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Review article

A beginner's guide to current synthetic linker strategies towards VHL-recruiting PROTACs

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ABSTRACT

Over the last two decades, proteolysis targeting chimeras (PROTACs) have been revolutionary in drug development rendering targeted protein degradation (TPD) as an emerging therapeutic modality. These heterobifunctional molecules are comprised of three units: a ligand for the protein of interest (POI), a ligand for an E3 ubiquitin ligase, and a linker that tethers the two motifs together. Von Hippel-Lindau (VHL) is one of the most widely employed E3 ligases in PROTACs development due to its prevalent expression across tissue types and well-characterised ligands. Linker composition and length has proven to play an important role in determining the physicochemical properties and spatial orientation of the POI-PROTAC-E3 ternary complex, thus influencing the bioactivity of degraders. Numerous articles and reports have been published showcasing the medicinal chemistry aspects of the linker design, but few have focused on the chemistry around tethering linkers to E3 ligase ligands. In this review, we focus on the current synthetic linker strategies employed in the assembly of VHL-recruiting PROTACs. We aim to cover a range of fundamental chemistries used to incorporate linkers of varying length, composition and functionality.

1. Introduction

In recent years, the field of drug discovery and development has taken a turn from targeted protein inhibition to targeted protein degradation, using novel tool compounds, namely PROTACs (PROteolysis TArgeting Chimaeras) first reported by Sakamoto *et al.* in 2001.[1]

These heterobifunctional molecules are composed of three elements: a warhead that binds to the protein to be degraded (*i.e.* the protein of interest or POI), a linker that can be made from a variety of chemical moieties and an E3 ligase ligand. The mode-of-action of PROTACs is based on the hijacking of the Ubiquitin-proteasome system (UPS), one of the intracellular pathways used to clear proteins, which induces protein degradation *via* the recruitment of various E3 ligase enzymes (Fig. 1).[2,3] Only a judicious combination of anchor and warhead, connected by a suitable linker, leads to a productive ternary complex and efficient POI degradation.[4,5] With 28 PROTACs currently in clinical trials, protein degradation technologies have enabled the degradation of previously "undruggable" targets for the treatment of various conditions, including cancer [6,7] and non-oncogenic diseases.[8,9]

However, due to their high MWs and poor physicochemical properties, most PROTACs have suffered from poor cellular uptake and unfavourable PK/PD profiles.[10] Other than modifications of the POI ligand and E3-ligase for which many articles and reviews have been published, an obvious route for optimisation, is the linker modulation.[11] The distance between the POI and the Ub/E2, their relative orientation and the presentation and accessibility of suitably reactive POI lysine residues to the E2, are important parameters which ultimately depend on the linker unit. However, the relationship between the spatial distribution of lysine residues at the POI surface, the architecture and connectivity of the poly-Ub chains, and the overall efficiency of degradation are still poorly understood.[12,13]

The right combination of length, hydrophilicity and rigidity of ligand-connecting linkers form a basis for successful design of productive PROTACs. One commonly used approach is to generate a library of PROTAC incorporating linear unsaturated aliphatic linkers of various length until suitable spatial orientation between the target POI and the E3 ubiquitin ligase can be identified. Various length of flexible linkers and their precursors (either PEG or alkyl chains) are commercially accessible and have been found to produce potent degraders of diverse POI in given cell lines, ranging from a couple of atoms up to 29

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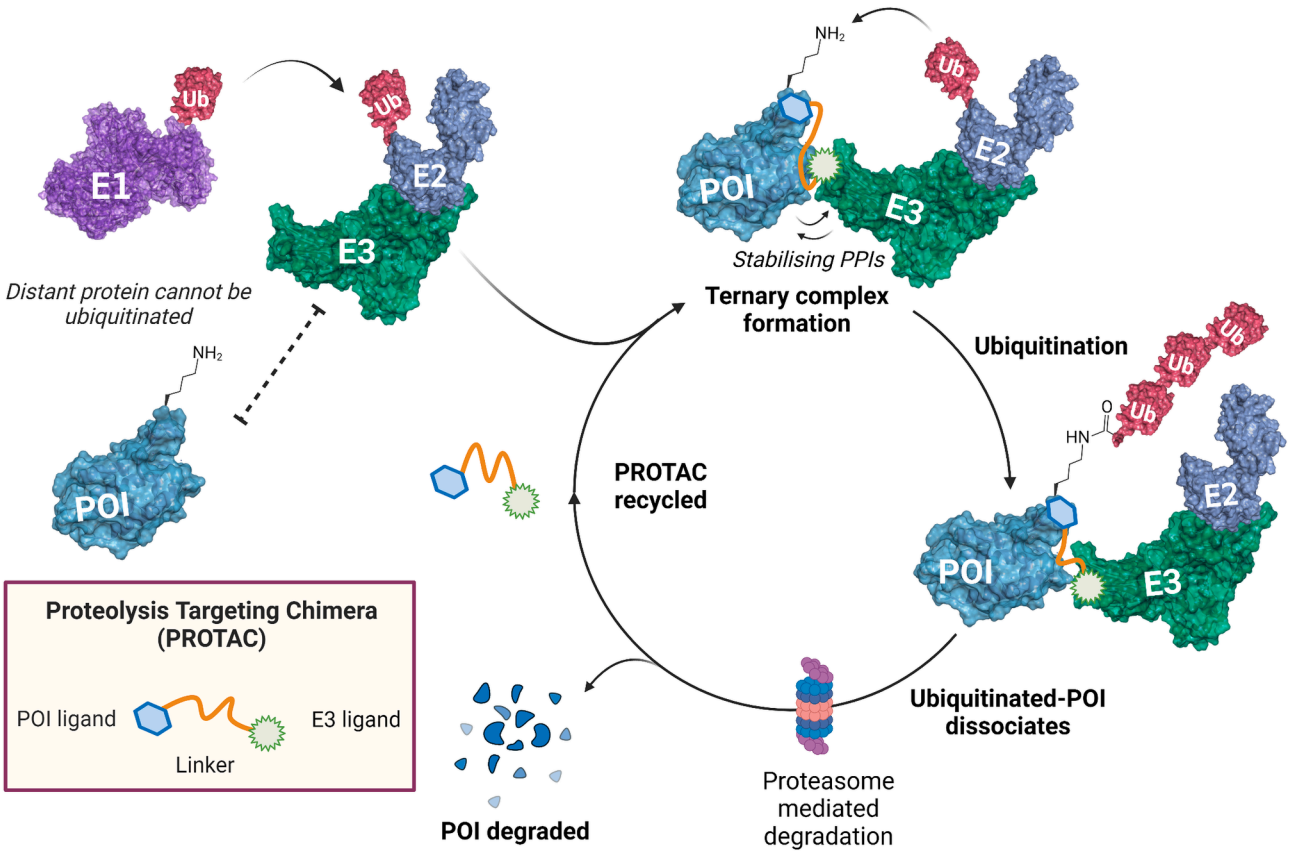


Fig. 1. Targeted protein degradation catalysed by a PROTAC (created with BioRender.com).

atoms.[9,13,14] In most cases, the longer aliphatic linkers have provided significant contribution to protein–ligand interactions within the ternary complex and stabilised its orientation by cooperative binding. In other cases, the energy gained in the ternary complex from new PPIs was offset by the entropic cost of reduced PROTAC flexibility.[14] Increasingly, the flexible, linear alkyl- and PEG linkers are being replaced by more rigid (e.g. alkyne) and cyclic scaffolds (e.g. piperazine, piperidine, triazole), providing opportunities for modulation of the physiochemical/PK properties of the PROTAC degraders (hydrophilicity/hydrophobicity, metabolic stability, bioavailability, cell membrane permeability) and of course, their biological activity. More recently, innovative PROTAC linker technologies have been developed, including photo-switchable linkers[15] and macrocyclisation.[16]

Although there are over 600 identified E3 ligases in the human genome, only a limited number have known ligands and of those, only a handful of them are employed in targeted protein degradation. Two E3 ligases in particular, Cereblon (CRBN) and Von Hippel-Lindau (VHL), are dominant within the field. While there are several reviews which report on the synthetic approaches surrounding CRBN ligands,[17,18] existing reviews of VHL-based PROTACs are focused on the synthesis of the E3 ligands themselves as well as the medicinal chemistry and SAR studies around the linkers, often termed as "linkerology".[13,14,19-21] This review focuses on the current synthetic methodologies that have been developed for the incorporation of linkers in VHL-targeting PROTACs.

2. General approaches to install linkers in VHL based PROTACs

The VHL protein is part of a multi-subunit of an E3 ubiquitin ligase, CUL2-RBX1-ElonginB-ElonginC-VHL, known as Cullin RING ligase complex ($CRL2^{VHL}$). VHL is comprised of two domains with specific binding sites to recruit primarily hypoxia inducible factor 1 (HIF-1A) for polyubiquitination and subsequent proteasomal degradation.[22] To date, VHL has been extensively employed as an E3 ligase protein to develop VHL-based PROTACs, degrading over 20 target proteins.[23] Following the discovery of the first small molecule VHL inhibitor VH032 (**1**) (K_d = 185 nM), a second generation of inhibitors was developed around the modification of VH032 LHS acetamide group.[24] Replacing the methyl group of the VH032 (**1**) with a fluorocyclopropyl group, generated a 4-fold more potent VHL inhibitor, VH101 (**2**) (K_d = 44 nM). Similarly, replacing the fluoro group with a cyano, led to the discovery of another inhibitor, VH298 (**3**), with a higher affinity than VH032 (K_d = 90 nM).[25] Based on these well-established VHL inhibitors, a series of small-molecule VHL ligands have been developed with exit vectors suitable for linker attachment to produce VHL-recruiting PROTACs (Fig. 2, A). Several representative examples of reported VHL-recruiting PROTACs are depicted in Fig. 2, **B**.

When synthesising VHL-targeting PROTACs, the type of chemistry to install linkers (flexible or rigid) is predetermined by the functionality of both, the VHL ligand and POI ligand. Conventionally, there are three

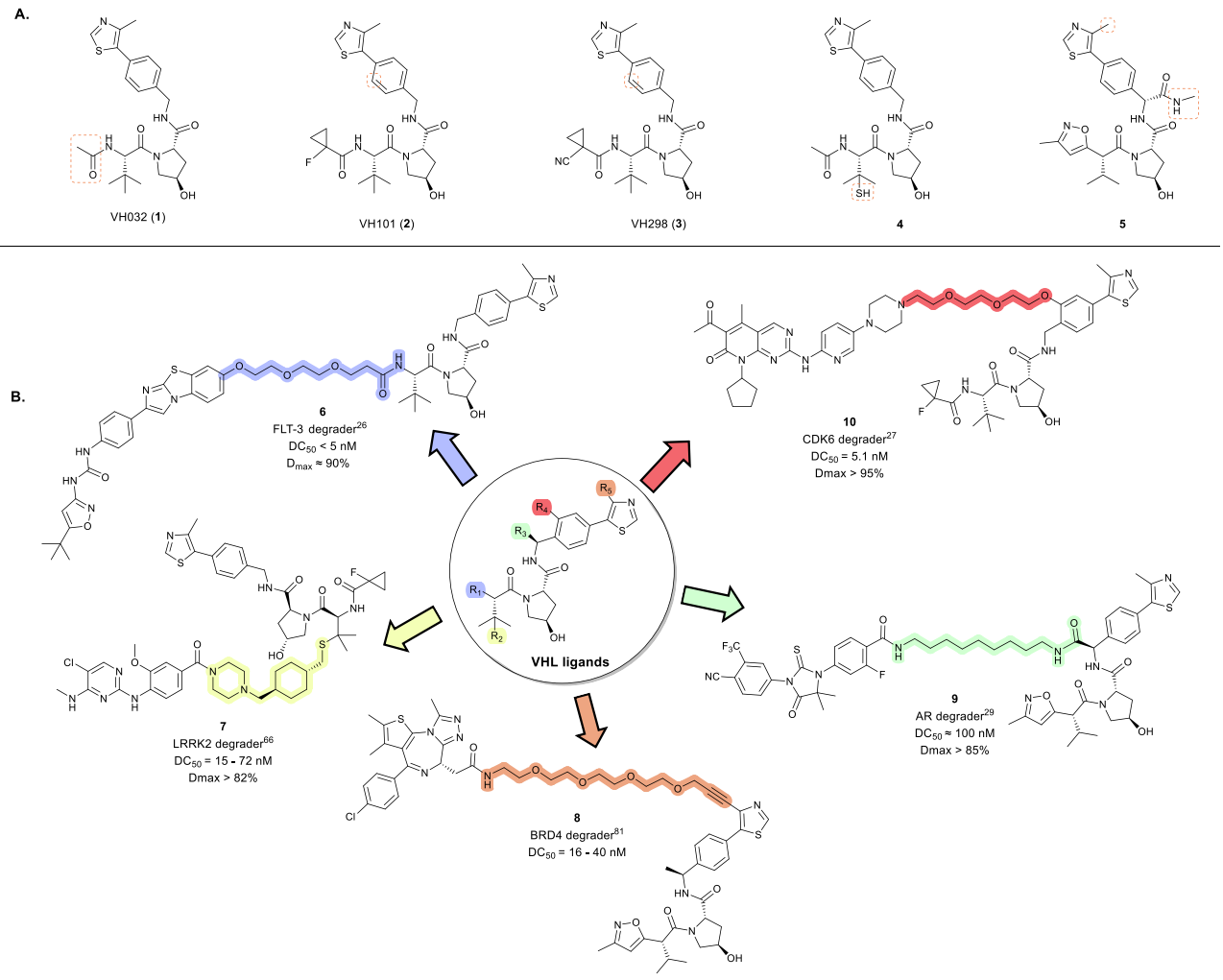


Fig. 2. A) Representative examples of VHL ligands recruited for PROTACs development, with their respective exit vectors highlighted in dashed orange boxes; B) Reported VHL ligand exit vectors and their utilisation in VHL-targeting PROTAC molecules.[26-29]

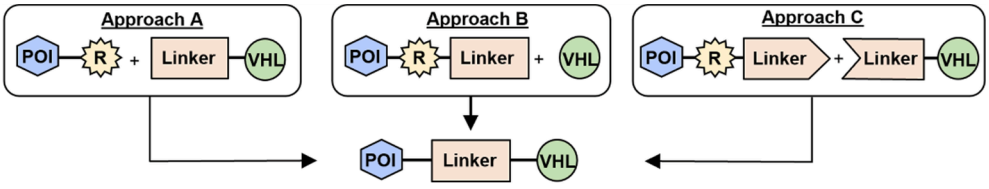
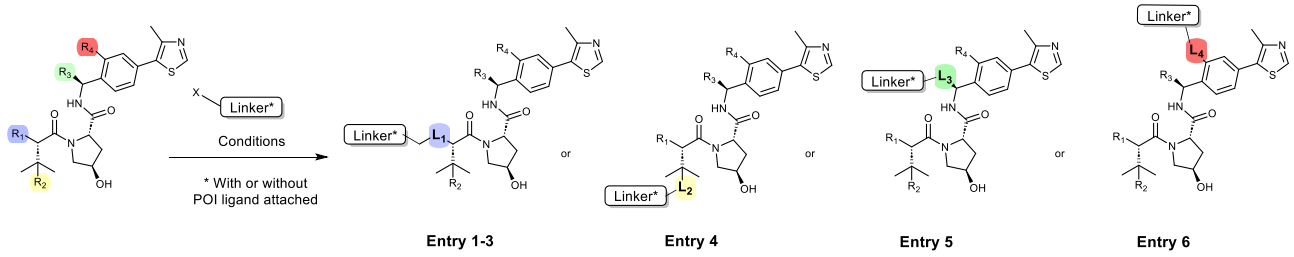


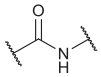
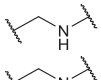
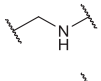
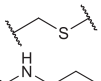
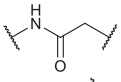
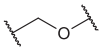
Fig. 3. A schematic representation of assembling VHL based PROTACs.

approaches that are implemented in assembling VHL-based PROTACs: a) coupling first the linker to the VHL ligand before attaching it to the warhead (**Approach A**); b) tethering the linker on the warhead first, followed by coupling to the VHL ligand (**Approach B**); c) installing two smaller linker fragments on both the POI and E3 ligands and connecting the two halves (**Approach C**). In all approaches, the linkage point (R) of the warhead is often modified to facilitate linker conjugation (Fig. 3). The choice of the approach is often based on two factors: 1) the availability and cost of building blocks, 2) the number of steps that are required to synthesise the warhead and linkers.

To date, more than 1500 different linkers have been reported in PROTACs design (data taken from PROTAC-DB 2.0; https://cadd.zju.edu.cn/protacdb/, as of the April 22, 2023). We used the PROTAC-DB to identify the types of linkers (flexible, rigid etc.), VHL ligands and attachment points that were used to assemble VHL-targeting PROTACs in existing literature. Depending on choice of the VHL ligand and its

Table 1
Commonly used synthetic methodologies for the attachment of linkers to VHL ligands. Linkers may already be attached to a POI ligand (Approach B) or may contain a handle for further functionalisation once it is attached to the VHL ligand (Approach A or C).



Entry	Linker Functionality (X =)	R_1	R_2	R_3	R_4	Typical Conditions	Linker Connectivity (L)	Refs
1	CO_2H	NH_2	Me	H, Me	H	HATU, DIPEA, DMF, r.t. *or* EDC·HCl, NMM, HOAt, DMSO, r.t.		40-53
2	CHO	NH_2	Me	H, Me	H	AcOH, MeOH *then* $NaBH_3CN$ *or* $NaBh(OAc)_3$, r.t.		54-60
3	Cl, Br, I, OTs, OMs	NH_2	Me	H, Me	H	KI, DIPEA, DMSO, Δ *or* KI, K_2CO_3, MeCN or DMF, Δ		61-65
4	Br, OTs, OMs	Amide	SH	H	H	DBU, DMF 0 °C to r.t.		27,66,67
5	NH_2	Amide, Oxazole	H, Me	CH_2CO_2H	H	EDC·HCl, NMM, HOAt, DMSO, r.t. *or* HATU, DIPEA, DMF, r.t.		29,68-71
6	Cl, Br, I, OTs, OMs	Amide	Me	H	OH	Cs_2CO_3 / K_2CO_3 in DMF (±KI), r.t. to 100 °C		65,72-79

respective exit vector, linkers with complementary functionalities are chosen to make the chemistry feasible. Representative examples of the chemistries that are implemented in the assembly of VHL PROTACs containing flexible and rigid linkers, will be further discussed in the following two sections, respectively.

3. Flexible linkers in VHL PROTACs

Flexible linkers, often consisting of linear chains of alkyl and polyethylene glycol (PEG) units, remain the most commonly reported linker type within PROTAC literature. The high number of rotatable bonds enables PROTACs with flexible linkers a greater degree of freedom and can therefore maximise the number of low-energy conformations that exist in solution. Akin to casting a wide net, by allowing more solution-state conformations of the PROTAC to be accessible, it maximises the likelihood of forming a ternary complex with the POI and E3 ligase.

Because of the increased hit-rate of forming a ternary complex and thus achieving POI degradation, flexible linkers are often the first choice of linker upon the inception of a new PROTAC discovery campaign. As a result, alkyl and PEG linkers of various lengths with different functionalities are now widely available from commercial suppliers, albeit sometimes at great expense. The ability to purchase libraries of pre-functionalised linkers of various lengths has greatly increased the discovery rate of new PROTACs in the literature in recent years.

The conformational freedom conferred by flexible linkers also provide the ability for PROTACs to fold onto themselves in polar media (such as in plasma or in cytosol), reducing the effective polar surface area of the compound and often masking some of the hydrogen bond donors and acceptors by the formation of intramolecular hydrogen bonds (IMHB).[30-32] This hydrophobic collapse, often referred to as chameleonicity due the ability to fold/unfold in different media, is thought to provide improved permeability across hydrophobic membranes. The experimentally determined ADME properties therefore may differ significantly from those calculated *in silico*; in particular, it is strongly encouraged to use experimentally determined polar surface area (EPSA) rather than reliance on TPSA.

Although flexible linkers dominate the PROTAC literature, they are less favoured in the clinical landscape. Of the PROTACs in clinical trials which so far have disclosed structures only one contains a flexible linker. Furthermore, the only VHL-recruiting PROTAC that is currently in clinical trials, DT2216[33], utilises a flexible linker.

In addition to their increasing commercial availability, the versatility of chemistry amenable to PEG and alkyl linkers also make them the linker of choice for PROTAC synthesis as well as in other fields such as antibody-drug conjugates[34–36] and bioconjugation[37-39].

The assembly method of VHL-targeted PROTACs is often determined by the choice of POI ligand and VHL ligand such that the functional groups of the linkers are chosen to be compatible with the prior choice of ligands. While the chemistry used to attach linkers to the POI ligand are often specific to each series of POI ligand, the methodologies used to attach linkers or POI-linker conjugates to VHL ligands are well-established and are generally well tolerated across a wide range of linkers and POIs. In our experience, the chemistry used to incorporate flexible linkers into PROTACs is robust and often performs consistently

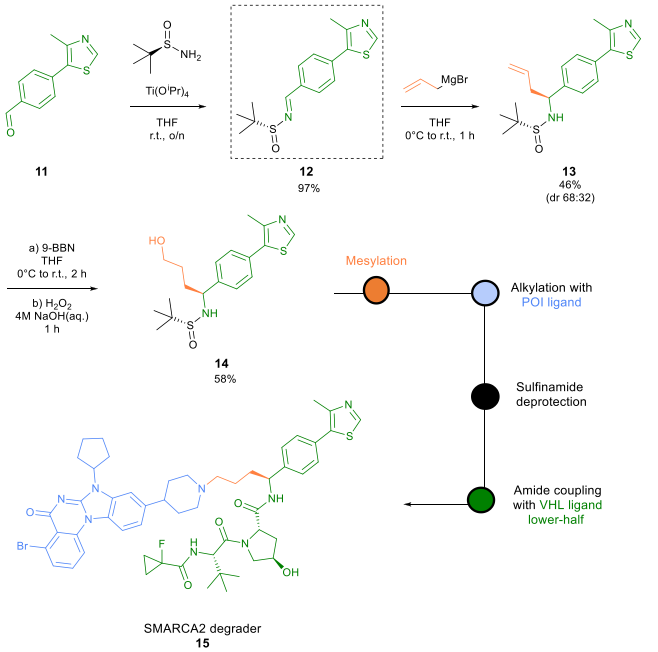


Fig. 4. Reported synthesis of SMARCA2 degrader (**15**) bearing carbon-tethered benzylic linkers by Kofink et al.[80.]

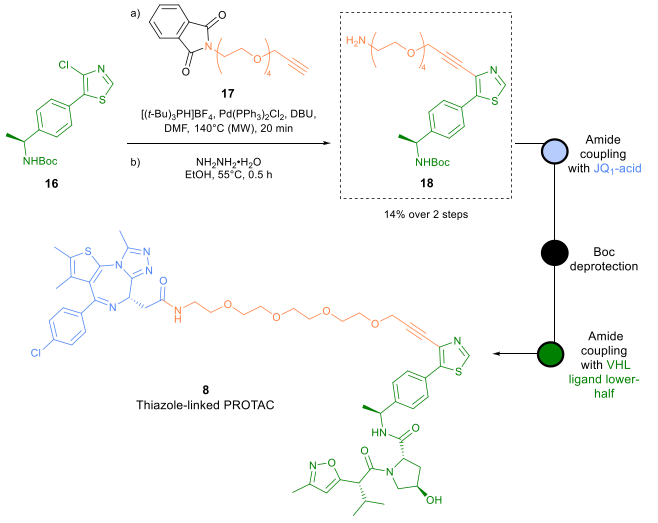


Fig. 5. Reported synthetic strategy for the development of thiazole-linked VHL PROTAC (**8**) by Krieger et al.[82.]

irrespective of the linker length or type, which enables general methods to be utilised in the construction of PROTAC libraries. Therefore, linkers of similar functionality are often attached to VHL ligands using established conditions, irrespective of which approach is taken (Fig. 3). Typical conditions for the attachment of linkers to VHL ligands are outlined in Table 1.

The first and most common method of linker attachment is through the terminal amino group of the VHL ligands ($R_1 = NH_2$), commonly referred to as "VH032-like" PROTACs. Amide coupling methodologies are widely used to create amide linkages to the linker (Table 1, Entry **1**)[40-53] but more recently, the use of reductive amination[54-60] and alkylation[61-65] methodologies are also being utilised to create secondary amine linkages from the same primary amine vector (Table 1, Entries **2** and **3**, respectively). There are now a diverse range of reported conditions for the coupling of amino VHL ligands with carboxylic acids, but the use of HATU and DIPEA remain the most dominant, followed by EDC

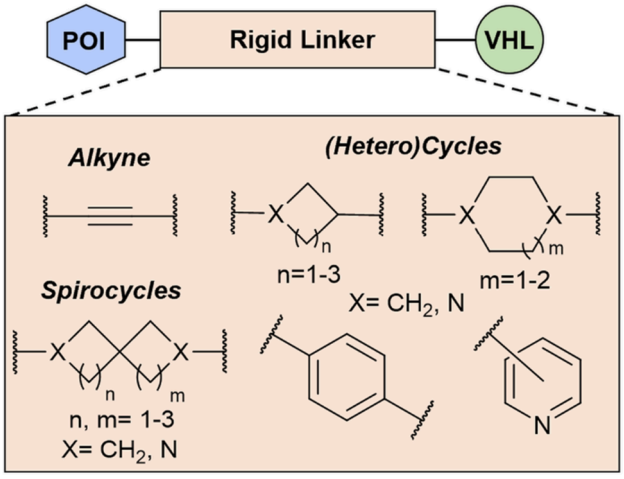


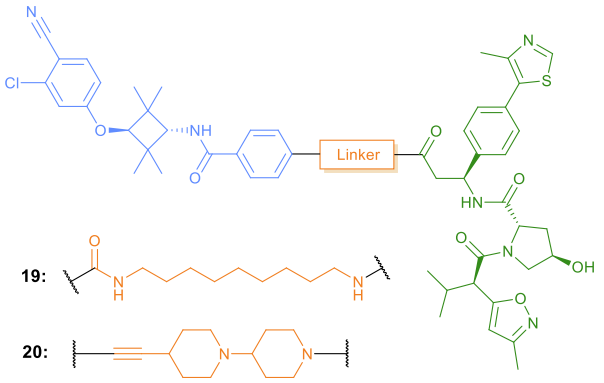
Fig. 6. Key rigid motifs used in PROTACs linker design.

and *N*-methylmorpholine (NMM). It is likely that the broad substrate tolerance for these peptide coupling reagents is the primary consideration for their use in PROTAC library assemblies, however conditions that are optimised specifically for each PROTAC are likely to be required as they head towards clinical development and process routes are developed.

Less than 1% of reported VHL PROTACs utilise the R_2 thioether exit vector (Table 1, Entry **4**) and those that are reported have so far been localised to the efforts of the Ciulli research group.[27,66,67] Initially, the R_2 thioether vector was utilised after the observation that in the BRD4^{BD2}-MZ1-VCB ternary complex, the *tert*-butyl group of MZ1 was pointing towards BRD4 at a short distance of roughly 5 Å away. Incorporation of penicillamine in lieu of *tert*-butyl leucine in the synthesis of the VHL ligand enabled a thiol handle to be installed which could then be exploited for linker attachment using a linker with a good leaving group and DBU as a base. The development of AT1 to utilise this new vector *via* a thioether linkage led to improved cellular potency and selectivity towards BRD4^{BD2}.

Another privileged VHL modality which is growing in popularity is the use of linkers attached to the benzylic R_3 position (Table 1, Entry **5**).[29,68–71] Here, the linker is installed to a modified VHL ligand which contains a benzylic CH_2CO_2H handle that is amenable to amide coupling methodologies. So far, the use of the benzylic exit vector has been limited in part due to the need to synthesise bespoke VHL ligands in which the CH_2CO_2H handle is incorporated early in the ligand synthesis. Once synthesised however, the methodologies used to install linkers to this benzylic handle can be high-yielding across a broad range of substrates. This is exemplified in work by Yu *et al.* in which a library of benzylic-tethered EGFR-VHL PROTACs were synthesised using EDC and NMM to afford an average isolated yield of 70% across a range of 36 different flexible PEG and alkyl linkers.[68] In addition to amide-linkages to the benzylic position of the VHL ligands, PROTACs containing benzylic ether- and carbon-linkages have also been reported. This is exemplified by work by Kofink *et al.* in which SMARCA2 selective degraders, such as **15**, were developed by the use of these novel benzylic linkage types (Fig. 4).[80] As substitution at only one of the benzylic sites is tolerated, the asymmetric synthesis of novel VHL ligands was required by means of formation of a chiral *N*-sulfinyl imine (**12**) followed by allylation and subsequent hydroboration to create a hydroxyl handle which could be then used for POI ligand installation. Attempts to make this method higher-throughput with respect to different linkers, addition of a vinyl group to the chiral *N*-sulfinyl imine (**12**) followed by reductive ozonolysis led to the creation of CH_2OH handle at the benzylic position, which could then be used to attach linkers in a more modular fashion.

Linkers can also be attached to the VHL ligand through the



Cpd	%AR degradation in LNCAP cells (μM)		
	0.01	0.1	1
19	43	91	96
20	76	98	99

Fig. 7. Representative example of developing AR degrader through linker modification.[29.]

incorporation and subsequent functionalisation of a phenolic moiety the R_4 position (Table 1, Entry **6**). [65,72–79] In a similar fashion to the benzylic functionality discussed previously, the phenolic handle needs integrating into the VHL ligand synthesis from the beginning. Despite this limitation, the phenolic exit vector is the 2nd most commonly utilised attachment position in the synthesis of VHL-recruiting PROTACs, albeit far behind the dominant terminal amide vector. The phenolic vector was first reported in collaborative work by Crews and GSK which showcased the discovery of HaloPROTACs, VHL ligands modified with chloroalkyl chains that target HaloTag7 fusion proteins for degradation.[79] Functionalisation of the phenolic vector is relatively robust due to the acidity of the phenol, which can be deprotonated using carbonate bases, and the improved nucleophilicity of the phenolate anion which can be readily alkylated with alkyl halides and pseudohalides.

More recently, it has been reported that the terminal thiazole group of the VHL ligand can be utilised as an attachment point for PROTAC linkers. The solvent-exposed nature of the 4-methyl substituent of the thiazole ring was identified early in the VHL ligand SAR and derivatisation of this position to incorporate linkers had been postulated in the patent literature.[81] However, the first reported example of a thiazole-linked VHL PROTAC was recently reported by Krieger and colleagues at Merck (Fig. 5).[82] Interestingly, the chlorothiazole handle was functionalised by a copper-free Sonogashira coupling early in the synthesis before the VHL ligand was fully formed.

While flexible linkers are by far the most commonly employed linkers in VHL-targeted PROTACs, many optimised PROTACs make use of more rigidified and functionalised linkers rather than simple linear chains. This is in part due to the improved potency that can be achieved by rigid linkers that bias the PROTACs solution-phase conformations towards the bioactive conformation found in the ternary complex ("preorganisation"). The linker is not just a passive bystander in the formation of the ternary complexes and linker-protein interactions can also play a vital role in the stability of the POI-PROTAC-E3 ternary complex. Thus, the use of more specialised linkers may exploit interactions with surface residues of the POI and E3 proteins better than simple PEG or alkyl chains. The change of linker type in the development cycle of PROTACs is also likely to be due to the undesirable physicochemical properties that flexible linkers can instil on the overall PROTAC molecule. The metabolic susceptibility of PEG chains is a particular concern as PROTACs head towards *in vivo* studies, whereas metabolic susceptibility may not have been a limiting factor during *in vitro* studies.[21] On the other hand, the use of linear alkyl chains can lead to poor aqueous solubility, particularly in VHL PROTACs where the E3 ligands are typically more lipophilic than their CRBN counterparts.[21] Both metabolic vulnerability and poor aqueous solubility are primary causes of the typically poor bioavailability of PROTACs during clinical development.

4. Rigid linkers in VHL PROTACs

Rigid linkers are incorporated in PROTAC design as an alternative linker strategy to modulate the degraders' physicochemical properties and optimise their degradation potency. Once the optimal linker length has been identified from biological evaluation or computational prediction of linear flexible linkers, rigidified linkers are next introduced with similar linker lengths.[14] Common rigid motifs that are incorporated in PROTAC linker design are (hetero)cycles, alkynes and spirocycles and these motifs account for 37%, 3.4% and 1.1% of total reported PROTACs, respectively (Fig. 6). Around 3.5% of PROTACs in the PROTAC-DB database contained both (hetero)cyclic and alkyne motifs. Incorporation of rigid linkers can be beneficial to enhance aqueous solubility, cell permeability and to improve the pharmacokinetic properties of the degrader.[13,14,19] As a result of these potential benefits, many of the PROTACs in clinical trials contain short and highly rigidified linkers.

Replacing linear chains with rigid scaffolds can reduce the number of rotatable bonds and orientate the whole conformation between the POI-E3 ligase ternary complex in a restricted manner, potentially leading to improved protein degradation. This is exemplified by Wang's work in which they developed and evaluated a series of AR degraders by performing an extensive optimisation around the linker. In particular, when replacing the flexible linker into a rigid linker this resulted in improving the solubility and the degradation potency to 76% at 0.01 μM (Fig. 7).[29] Interestingly, macrocyclic linkers have also been explored by the Ciulli

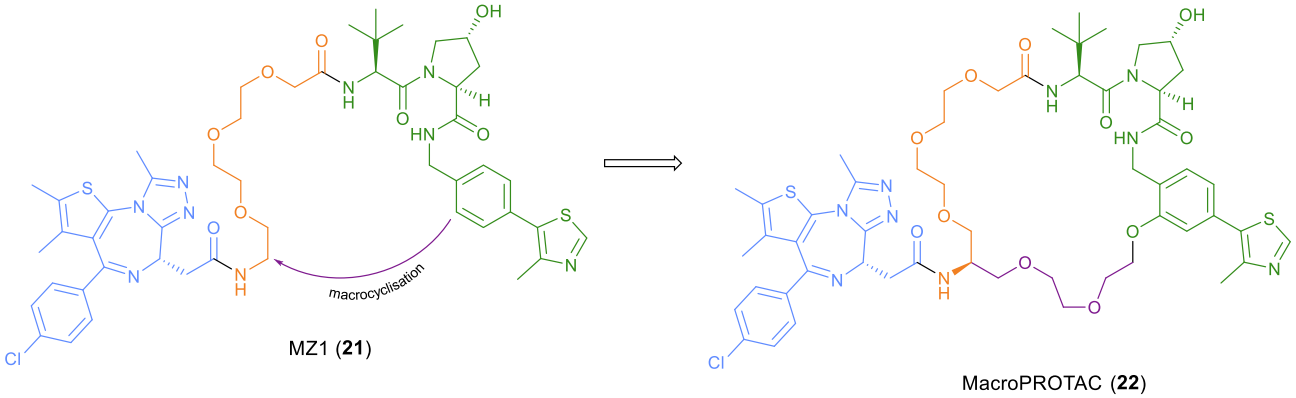
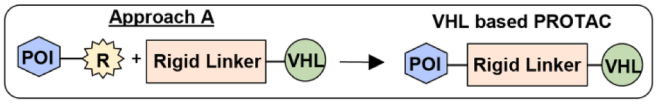
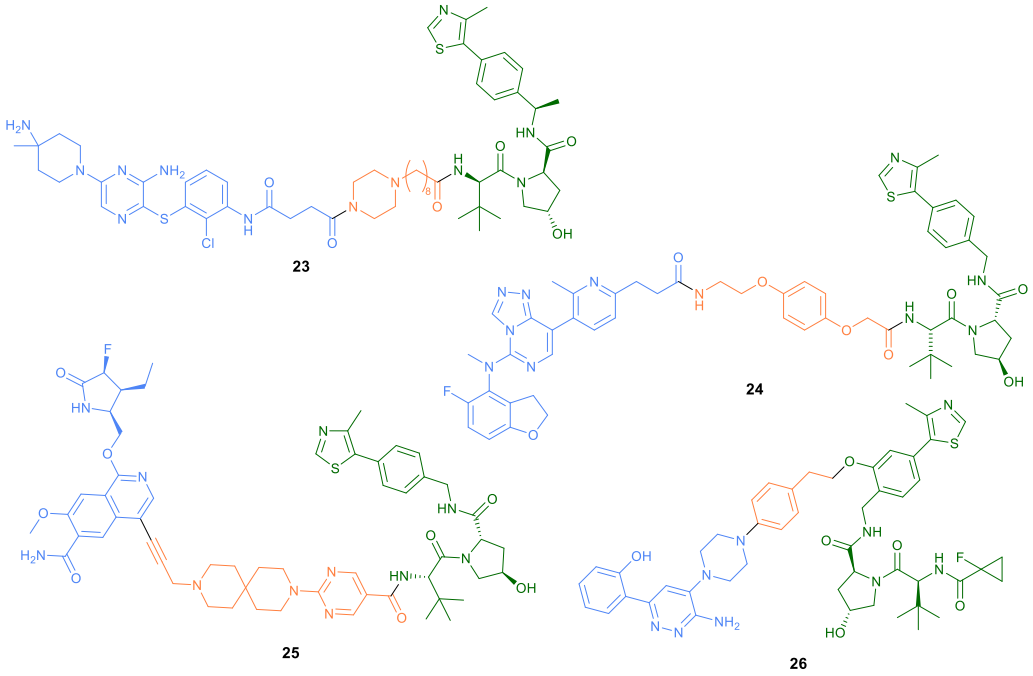


Fig. 8. Macrocyclisation of MZ1 (**21**) to lock the active conformation of the ternary complex $Brd4^{BD2}$-MZ1-VHL resulted in macroPROTAC (**22**).

Table 2
Representative examples of VHL-recruiting PROTACs containing different rigid linkers synthesised using Approach A.





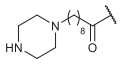
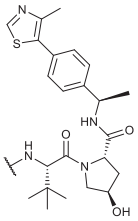
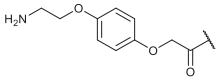
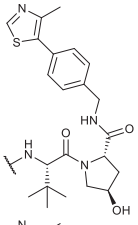
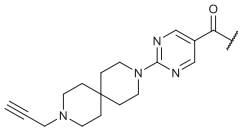
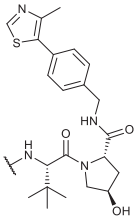
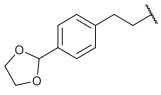
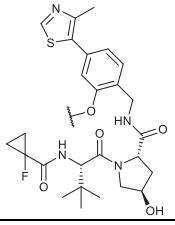
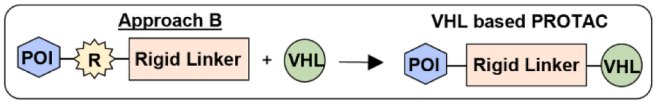
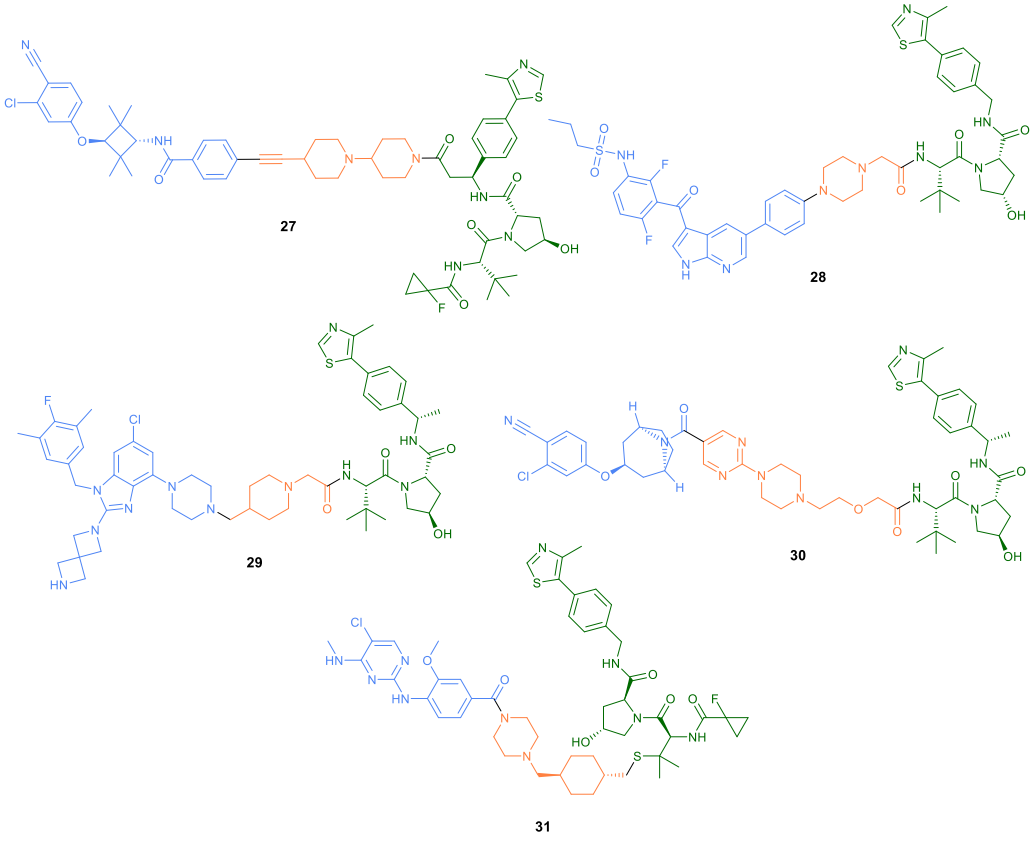
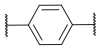
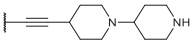
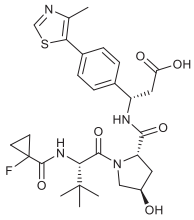
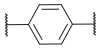
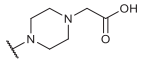
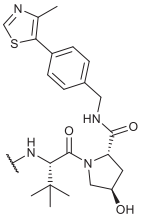
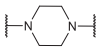
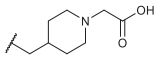
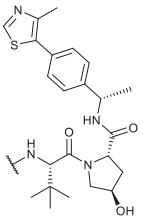
Cpd	R	Rigid Linker	VHL	Assembly Conditions	Ref
23				HATU, DIPEA, DMF	[85]
24				HATU, Et_3N, DCM	[42]
25				K_2CO_3, Xphos, $Pd_2(dba)_3$, DMA	[83]
26				i) 0.5 M HCl, THF, 50 °C, 1 h, ii) Et_3N, $NaBH(OAc)_3$, $MgSO_4$, DMF	[84]

Table 3
Representative examples of VHL-recruiting PROTACs containing different rigid linkers synthesised using Approach B.

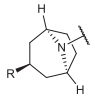
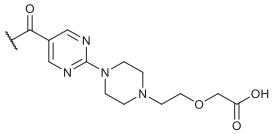
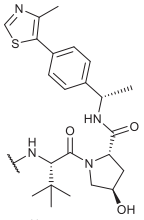
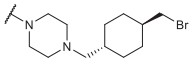
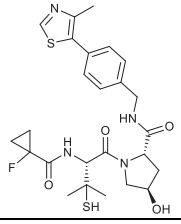




Cpd	R	Rigid Linker	VHL	Assembly Conditions	Ref
27				HATU, DIPEA, DMF	29
28				PyBOP, Et_3N, DMF	86
29				HATU, DIPEA, DMF	87

(*continued on next page*)

Table 3 (*continued*)

Cpd	R	Rigid Linker	VHL	Assembly Conditions	Ref
30				DIPEA, HATU, DCM	[88]
31				DBU, THF	[66]

group as alternative strategy to lock the conformation of the BET degrader, MZ1 (**21**), close to that of the active conformation. After analysis of the $BRD4^{BD2}$-MZ1-VHL ternary complex by x-ray crystallography, it was envisaged that a second linker could be placed between the phenyl ring of the VHL ligand and the first PEG linker of MZ1 (Fig. 8) to form the macrocyclic linker. Despite the 12-fold loss in binding affinity, macroPROTAC (**22**) exhibited similar cellular potency to MZ1.[16]

In the assembly of VHL-targeting PROTACs, the chemistry that is chosen to install rigid linkers is often determined by the functionality of both, the VHL ligand, with its respective exit vector, and the POI ligand. Based on reported examples, incorporation of rigidified linkers in the design of VHL-PROTACs can be achieved with either of the three approaches, as previously described in Fig. 3.

For the first approach, amide coupling conditions are commonly applied for PROTACs assembly by conjugating amino containing chains to the carboxylic acid bearing warhead (Table 2, VHL-recruiting PROTACs **23**, **24**). Another linking strategy that has been reported by Nunes *et al.* for the development of IRAK4 PROTACs, was the coupling of VHL ligand that was pre-attached to alkyne bearing linkers onto the warhead *via* cross coupling reaction, Sonogashira.[83] Following a small molecule docking study to predict the solvent-exposed region and after short optimisation around the linker design, a potent IRAK4 degrader (DC_{50} = 151 nM in PBMC cells) was discovered that contained a rigid, polar, spirocyclic pyrimidine linker (Table 2, VHL-recruiting PROTAC **25**). Reductive amination is also employed as an alternative synthetic strategy utilising carbonyl bearing linkers tethered to VHL ligands to assemble PROTACs. This is exemplified by Farnaby's work in which ACBI1, a potent degrader of BAF complex ATPases, was synthesised by coupling first the protected carbonyl, acetal containing linkers on the VHL ligand followed by an acidic deprotection and a subsequent reductive amination on the amine of the warhead (Table 2, VHL-recruiting PROTAC **26**).[84]

In regard to the second approach (Approach B), the final conditions for conjugation are often determined by the type of VHL ligand and its respective functionality that is employed. Apart from the common amide coupling conditions that are utilised (Table 3, VHL-recruiting PROTACs **27**–**30**), the Ciulli group recently discovered XL01126, a BBB-penetrant and potent degrader of LRRK2, that contains a thioether conjugated VHL ligand (VH101, **2**) connected to a rigid polar carbocycle ring.[66] For the synthesis of XL01126, the final linker attachment on the VHL ligand took place by using a linker with a good leaving group and DBU as a base (Table 3, VHL-recruiting PROTAC **31**).

For the final approach (Approach C), rigid heterocycle rings are firstly incorporated on the warhead followed by a final coupling to the pre-attached linkers on the VHL ligand. One representative example is the development of a potent BCL-xL/2 dual degrader, PPC8, in which the linker of the VHL ligand was tethered to the piperazine ring of the warhead using HATU, as the final coupling reagent (Table 4, VHL-recruiting PROTAC **32**).[89] Similar synthetic strategy was also described by Bollu *et al.* to synthesise IDO1 degraders (Table 4, VHL-recruiting PROTAC **33**).[65]

Despite the wide use of these approaches, developing PROTAC libraries with rigid linkers of varying lengths remains time-consuming. In particular, multiple synthetic steps and linkage point modifications are typically required, which when followed by challenging purifications, often leads to low isolated yields. Moreover, the commercial availability of preassembled rigid linker-VHL ligand conjugates is limited and expensive. It would be invaluable to have a methodology that can rapidly expand the library of PROTACs bearing rigid linkers of different lengths.

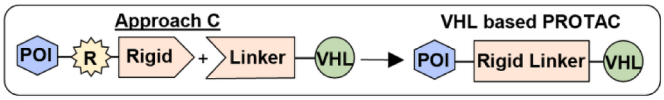
Interestingly, Bhela *et al.* recently reported a novel modular synthetic platform which is based on multicomponent reactions (MCR) to assemble a variety of protein degraders in a cost-effective manner. In their work, BRD4 degrading PROTACs were synthesised using Ugi and Passerini reactions, two of the most well-established MCR.[90-92] The VHL ligand-linker conjugates **36**/**37** were made in a two-step procedure. The first step involved the saponification and subsequent amide coupling of the isocyanide methyl esters **34**/**35** with the VHL ligand, VH032 amine. Finally, an Ugi reaction using JQ1 carboxylic acid **38**, piperazine **39**, formaldehyde **40** and isocyanide **36**/**37** afforded VHL-recruiting PROTACs bearing piperazine linkers **41**/**42**, respectively, in moderate yields (Fig. 9).

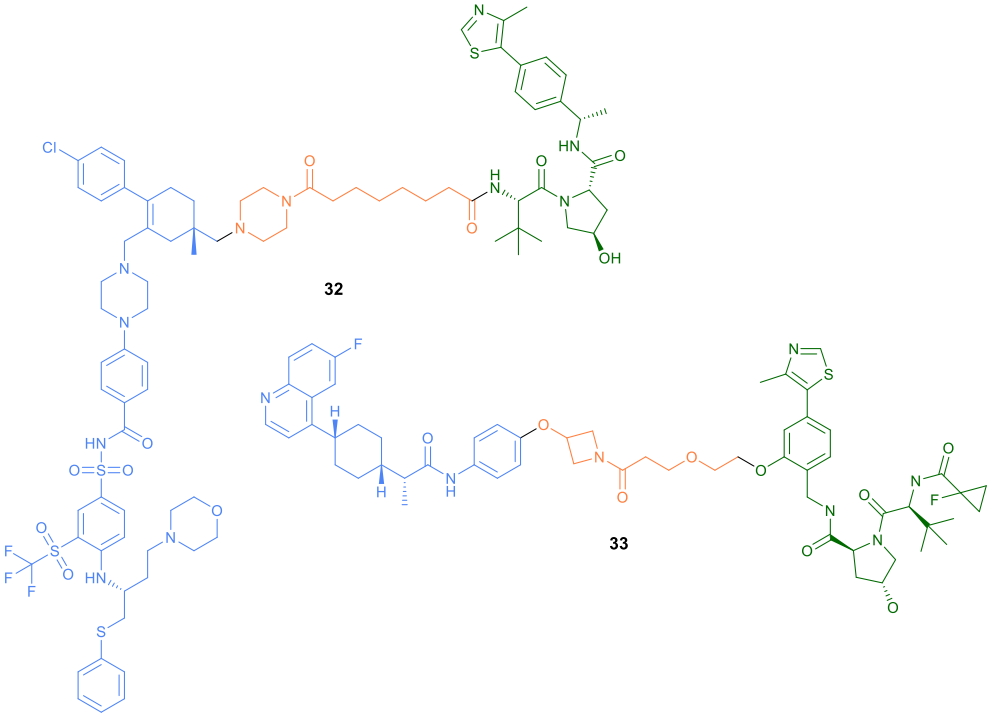
5. Clickable linkers in VHL PROTACs

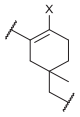
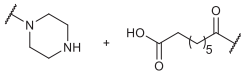
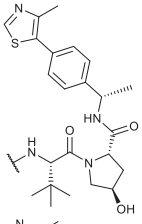
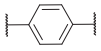
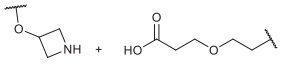
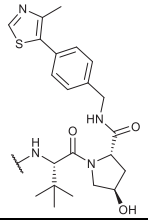
Click chemistry has been widely used as an expeditious approach in PROTACs research to facilitate the development of diverse PROTAC libraries.[13,93–100] Lebraud *et al.* pioneered the concept of clickable linkers (CLIPTACs) to overcome problems associated with poor solubility and cell permeability by "clicking" two low molecular weight PROTAC precursors intracellularly.[96] Since then, the click methodology has been conveyed as a platform to rapidly access a library of PROTACs of varying linker lengths.

The copper catalysed-Huigsen cycloaddition is one of the most common "click" platforms used to assemble triazole moieties. This reaction takes place between an azide and an alkyne under mild conditions to generate 1,4-disubstituted 1,2,3-triazoles often in high yields and high selectivity.[101] The triazole moiety has been commonly incorporated in linker strategies, partly due to the ease with which it can be installed, along with its chemical robustness to metabolism.[102] As

Table 4
Representative examples of synthesised VHL-recruiting PROTACs containing different rigid linkers using Approach C.





Cpd	R	Rigid Linker	VHL	Assembly Conditions	Ref
32				HATU, Et_3N, DCM	[89]
33				HATU, DIPEA, DMF	[65]

judicious bioisosteric replacements of amide bonds, triazoles have also been used to modulate the physicochemical properties of PROTACs, notably improve their aqueous solubility.[103] In addition, triazole rings can introduce rigidity to the linker chain, and depending on their position on the linker, they may exploit new intermolecular interactions that may improve the ternary complex stability, and ultimately, improve the target degradation efficiency.[104]

The assembly of the triazole-containing linkers often takes place as the final step of PROTAC synthesis by "clicking" both, the warhead ligand and the E3 ligase ligand together.[93,95,98–100,105] When employing the VHL ligand, commercially available VH032 amine and carboxylic acid-bearing linkers have commonly been conjugated *via* amide coupling conditions. The terminal end of these linkers can bear either: a good leaving group (such as halide or tosyl group), which can then be displaced by nucleophile NaN_3 (Fig. 10, **A**) or a terminal alkyne (Fig. 10, **B**). The anchor point of the warhead can judiciously be modulated proportionally to introduce the respective terminal alkyne- or azide-bearing linkers of various lengths. Final coupling of these two PROTAC precursors (warhead-linker and E3-linker) are performed by a Cu (I)-catalysed "click" cycloaddition.

Liu *et al.* recently reported the development of VHL based PROTACs to target transcriptional factors (TF PROTACs), using an alternative

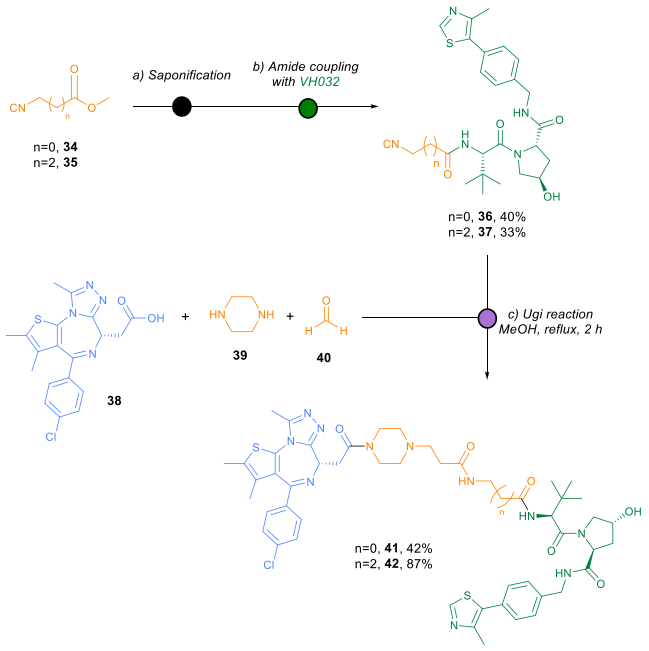


Fig. 9. A summary of the synthetic route for the development of VHL-based PROTACs targeting BRD4, developed by Bhela et al.[90.]

copper-free "click" reaction, known as strain-promoted azide-alkyne cycloaddition (SPAAC).[106] In general, the SPAAC reaction requires a strained-alkyne scaffold, bicyclooctyne (BCN), which will bioconjugate with azide-tagged molecules and form the triazole ring intracellularly.[107] The TF PROTACs are comprised of a DNA oligomer, which selectively binds to the TF of interest, a VHL ligand and a clickable linker that will be formed in the cell. To achieve TF PROTACs assembly, BCN (**43**) is firstly conjugated to pre-attached alkyl/PEG linkers of varying lengths to VHL ligand, whilst the azide moiety is incorporated on the 5' end of the DNA sequence (N3-ODN, **44**). Finally, the two parts are "clicked" *in vitro via* the SPAAC reaction forming the TF-PROTAC, and then subsequently is transferred into the cell to target TFs for proteasomal degradation (Fig. 11).

To the best of our knowledge, "click" chemistry hasn't been applied on alternative VHL ligands other than VH032.

6. Photoswitchable linkers in VHL PROTACs

Photoswitchable PROTACs (PhotoPROTACs) is a novel class of degraders which combines the strategies of two emerging areas, photopharmacology and PROTACs. The concept of photoPROTACs was firstly introduced by Crews' group as a tool to control PROTACs activity in a spatiotemporal manner enabling reversible on/off switch of target protein degradation.[15] The first design of photoPROTAC was based on the well-established BET protein degrader, ARV-771, which is comprised of BRD4 ligand (JQ1 amine) and a VHL ligand (VH032 amine), connected *via* a 11 Å long PEG linker. Replacing the hydrophilic linker to an *ortho*-F_4-azobenzene linker **49** resulted in the development of the first photoPROTAC **50**.

The synthesis of the red-light switchable azobenzene linker **49** started with the conversion of aniline **45** to the corresponding diazonium salt **46** using $NOBF_4$ followed by ortholithiation of difluorobenzene **47** and subsequently, coupling with the aryldiazonium tetrafluoroborate salt **46**.[108] The TBS group of the resulted unsymmetrically substituted *ortho*-F_4-azobenzene **48**, was removed using TBAF. A final oxidation of the benzylic alcohol furnished the desired photoswitchable linker **49** (Fig. 12**, A**). For the assembly of photoPROTAC-1 **50**, the azobenzene linker was first coupled with the VHL ligand, followed by a subsequent hydrolysis of the *tert*-butyl group and a final amide coupling with JQ1 amine, affording both *cis*- and *trans*- isomers of photoPROTAC-1 (**50**).

Irradiation of photoPROTAC-1 at 415 nm and 530 nm LEDs resulted in the formation of 95% *trans*-photoPROTAC-1 (**51**) and 68% *cis*-photoPROTAC-1 (**52**), respectively. The distance of the azobenzene linker in both forms differed by 3 Å (11 Å in *cis*-form and 8 Å in *trans*-form) (Fig. 12**, B**). Interestingly, in Ramos cells and after 7 h incubation, the

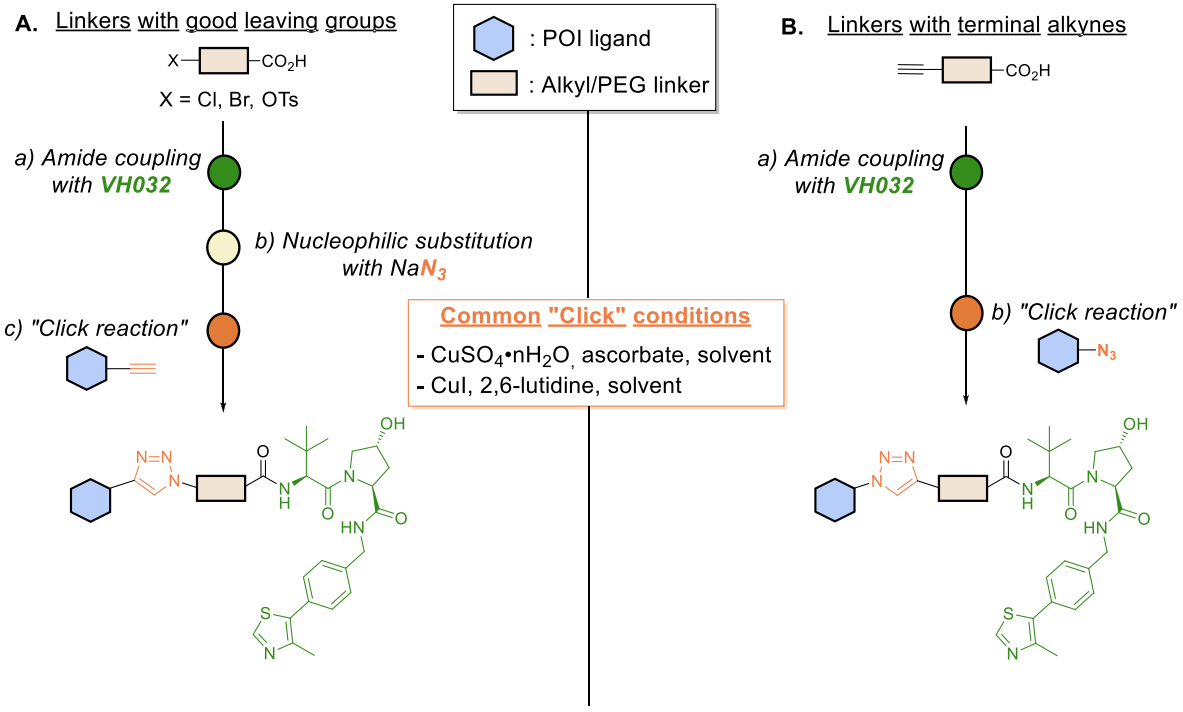


Fig. 10. Schematic representation of the assembly of VHL-based PROTACs *via* "click" chemistry using either: (**A**) linkers containing a good leaving group and (**B**) linkers containing a terminal alkyne.

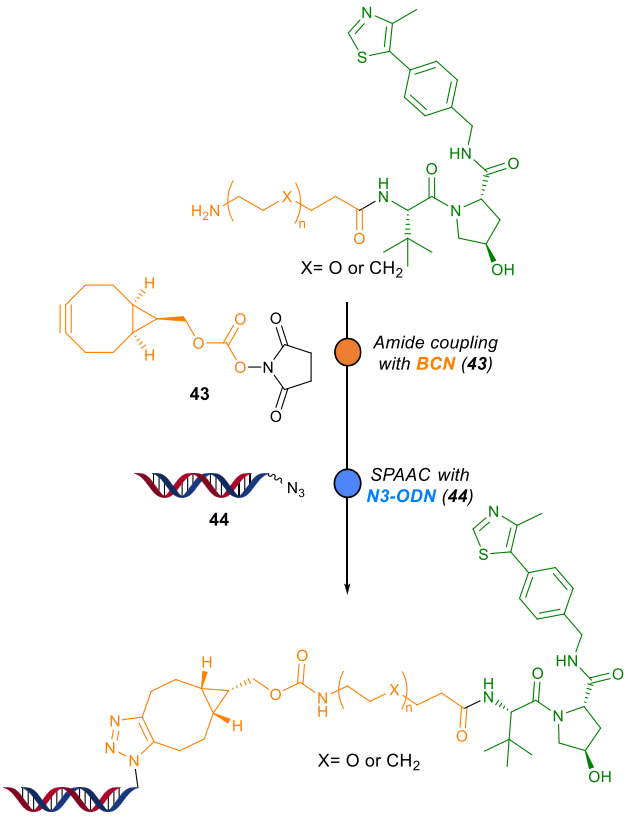


Fig. 11. A schematic representation on assembling TF PROTACs using SPAAC reaction recruiting VHL to ubiquitinate the targeted TFs, developed by Liu *et al.*

trans-photoPROTAC (**51**) induced BRD2 degradation which was sustained for a further 17 h, whereas the *cis*-form **52** did not show any significant effect on BRD2 levels.[15] Incorporating photoswitchable linkers in PROTACs design as a "remote control" of activating/deactivating degradation, has proven to be a feasible strategy to selectively target proteins and prevent from causing off-target effects.

7. Summary and future perspective

In the last decade, the TPD field has progressed beyond its 'academic curiosity' origins and is poised to bring great clinical benefit to patients in the coming decades. This new promising therapeutic modality has the potential to revolutionise modern medicine by overcoming some of the drawbacks of traditional occupancy-based drugs. In particular, TPD has the potential to target previously 'undruggable' areas of the human proteome and so may be able to provide treatments for many orphan diseases, however, most PROTACs currently in clinical trials target existing clinical indications, typically in oncology. The improved selectivity and potency conferred from proximity-driven pharmacology has led to the development of several best-in-class clinical candidates and time will tell whether these improved *in vitro* properties will transfer into beneficial outcomes *in vivo*. This should translate into safer and more effective drugs, with less off-target toxicity.

PROTACs are large and complex molecules that are challenging our preconceptions of what drugs look like and are establishing a frontier beyond the 'Lipinski's rule of five' (bRo5). PROTACs do however bring their own challenges in terms of design and development.

A lack of structure-guided design in a majority of projects means that iterative modulation of the linker region and exploitation of different E3 ligand exit vectors remains the most common strategy for PROTAC optimisation. The use of structural biology techniques, such as crystallography and cryo-EM, to image POI-PROTAC-E3 ternary complexes is still possible but can be limited to cases where highly cooperative ternary complexes are formed. This often requires an already optimised PROTAC and so defeats the objective of using structure-guided design as a lead optimisation strategy. When structural information about the ternary complex is obtained, however, it can be highly beneficial and numerous examples are now reported where structure-guided design has been effective in the development of potent and selective PROTACs.[16,27,80] The use of *in silico* predictive models, such as PRosettaC and DeLinker, are emergent tools for the PROTAC field and recent examples provide evidence that effective computational models are in fact possible.[109-112] Rapid development in this area in the coming decade is likely to be revolutionary in the ability to predict the 'PROTACability' of biological targets and in the rational design of effective PROTACs.

From a synthetic chemist's point of view, the high complexity of PROTACs poses challenges in both, design and synthesis. These large multi-component structures often don't appear in structural searches in chemical databases (e.g. Reaxys® and SciFinder®) and this coupled with the rate at which PROTACs are reported, make keeping on top of the latest synthetic approaches challenging. This is one of the key motivations behind this review. The recent developments of PROTAC-specific curated databases[113,114,115] and the emergence of the revered *CeTPD Journal Club*[116] provide accessible community-led tools to overcome this growing complexity.

VHL-targeting PROTACs remain the most reported PROTACs in the literature and widely-adopted synthetic methodologies are being established for classical transformations such as amide couplings, alkylations and reductive aminations. These methodologies are efficient at building libraries of VHL PROTACs with simple linkers, however, as the linkers and VHL ligands being utilised become more complex there is a growing diversity of chemistry being employed.

New synthetic trends that appear in the wider medicinal chemistry literature will also likely be applied to PROTAC synthesis in the future. One example of a methodology taken from chemical biology and applied to PROTAC synthesis is the use of traceless Staudinger ligations to assemble BRD4-CRBN PROTACs from azide and thioester-phosphine building blocks (Fig. 13, **A**).[116] This biorthogonal chemistry has yet to be reported in the synthesis of VHL-recruiting PROTACs but this methodology could provide great utility if applied. Work by Kodakek and Gui has demonstrated that PROTACs containing oxime-based linkers can be readily formed between precursors bearing aldehyde and hydroxylamine moieties (Fig. 13, **B**).[117,118] They highlighted the potential use of this methodology in "Split-PROTACs" where POI and E3 ligands could be connected in a modular, array-based format. The authors postulated that this could even enable the formation of PROTACs within a cellular environment, although, the oxime ligation was shown to be ineffective at the low intracellular concentrations of compounds. Photoredox chemistry has also been utilised to generate C—C bonds in the synthesis of CRBN-targeting PROTACs[119] but to our knowledge has yet to be applied to VHL-targeting PROTACs (Fig. 13, **C**). Overall, these innovative methods of synthesising PROTACs show great promise but are yet to be adopted by the wider field. We hope that as these methods are

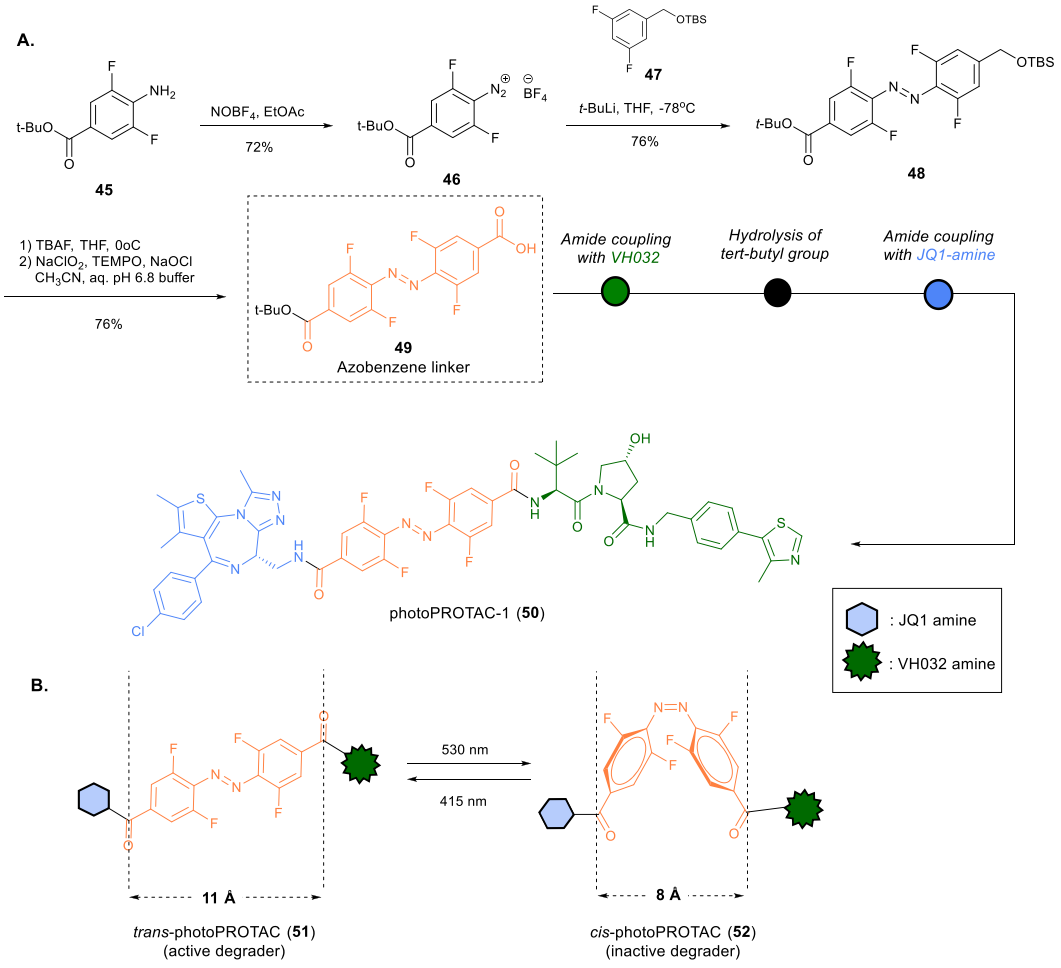


Fig. 12. A) Synthetic route for azobenzene linker 49 and assembly of photoPROTACs-1 (50) developed by Pfaff et al.[15]; B) Schematic representation of forming active *trans*-photoPROTAC (51) and inactive *cis*-photoPROTAC (52) after irradiating Ramos cells at 415 nm and 530 nm, respectively.

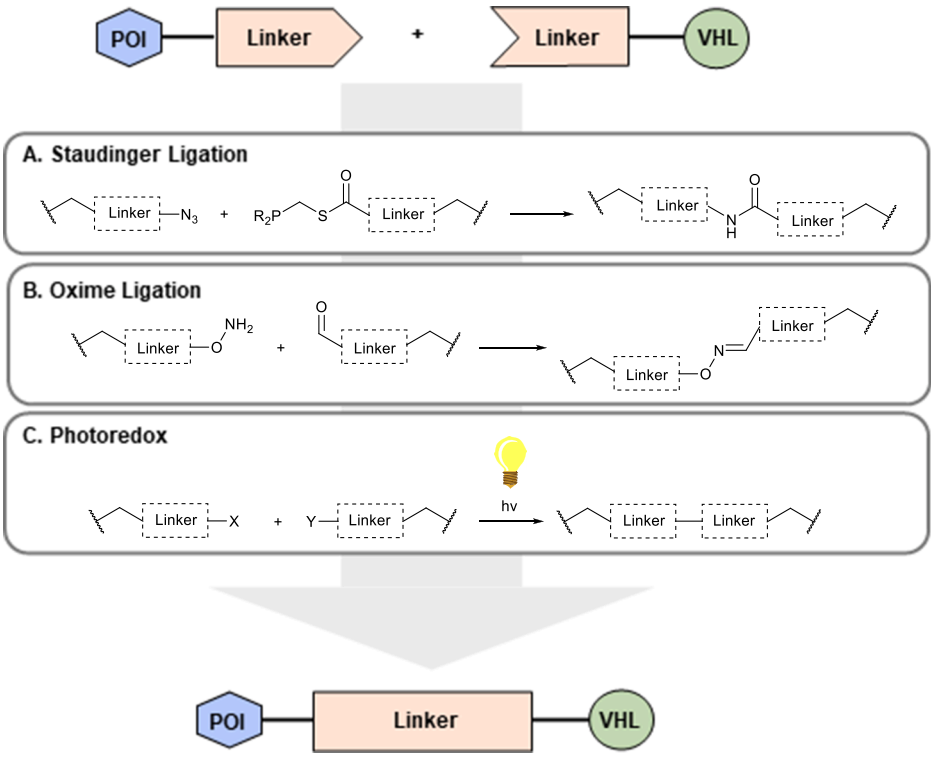


Fig. 13. Emergent methods for the assembly of VHL-recruiting PROTACs. Many of which have been applied to CRBN-based systems but are yet to be applied to VHL-recruiting PROTACs.

optimised that they are taken up by the synthetic chemists working within the PROTAC field.

In general, the modular nature of PROTACs makes them well suited for parallel synthesis, enabling rapid and efficient generation of PROTAC libraries, although purification of the resulting products can remain a challenge. The use of "Direct-to-Biology" (D2B) methods which Janssen and GSK have both pioneered circumvents the need to purify PROTACs and greatly accelerates the rate of library synthesis and analysis.[119,120] It is our understanding that most PROTAC drug discovery campaigns conducted within pharmaceutical and biotech companies are often initiated by the use of automated array-based methods to both synthesise and screen thousands of PROTACs in a high-throughput fashion. The lead optimisation phase of PROTACs, which often requires specific and non-trivial alterations in the PROTAC composition, is still likely to be carried out by traditional sequential synthesis by medicinal chemists.

In summary, PROTACs are poised to revolutionise drug discovery but the challenges in the design and synthesis of novel PROTACs threatens to slow the pace of their development. While improvements in structural biology and computational methods may improve our understanding of PROTAC design, frontiers in chemistry are being pushed to help drive their synthetic accessibility. The use of parallel-synthesis and automation aid in the synthesis of large PROTAC libraries but are expensive and often limited to an industrial setting. This guide acts as a starting point for understanding what synthetic methodologies are amenable in the synthesis of VHL-targeting PROTACs, but it is by no means exhaustive. We hope the reader finds great utility in this toolbox and that it motivates them to continue driving the development of the TPD field.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

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

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