Rapid PROTAC discovery platform: nanomole scale array synthesis and direct screening of reaction mixtures to facilitate the expedited discovery and follow-up of PROTAC hits

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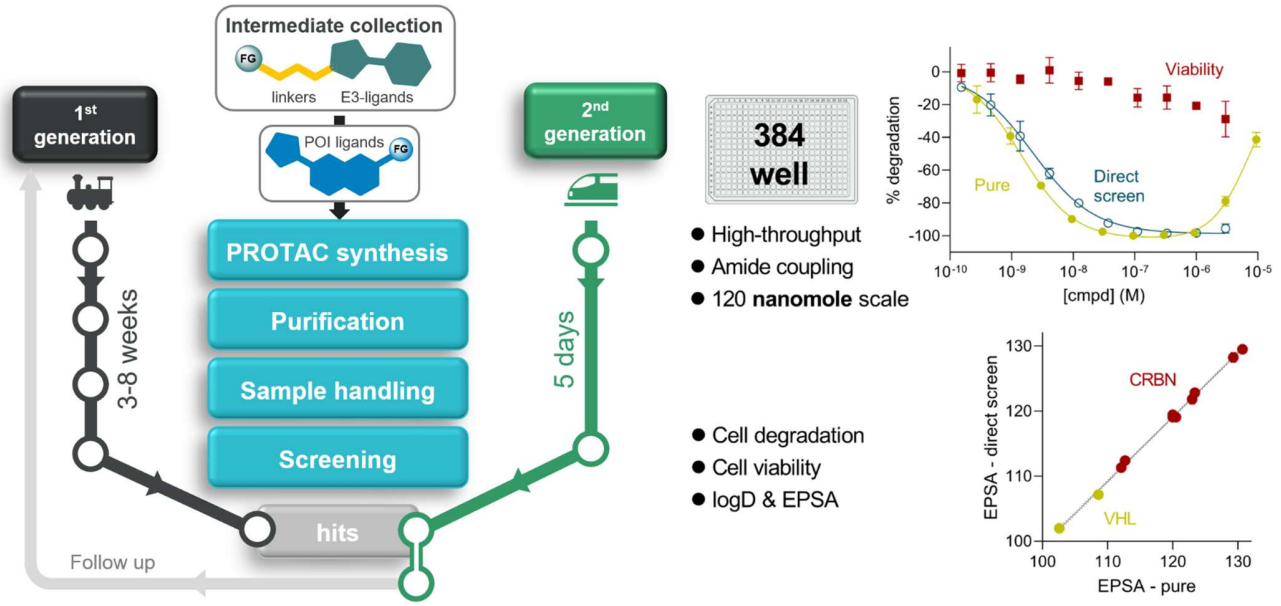


ABSTRACT: Precise linker length, shape and linker attachment point are all integral components to designing efficacious PROTACs. Due to the increased synthetic complexity of these heterobifunctional degraders and the difficulty of computational modelling to aid PROTAC design, the exploration of structure-activity-relationship (SAR) remains mostly empirical, which requires a significant time and resource investment. To facilitate rapid hit finding we developed capabilities for PROTAC parallel synthesis and purification by harnessing an array of pre-formed E3-ligand-linker intermediates. In the next iteration of this approach, we developed a rapid, nanomole-scale PROTAC synthesis methodology using amide coupling that enables direct screening of non-purified reaction mixtures in cell-based degradation assays, as well as logD and EPSA measurements. This approach greatly expands and accelerates PROTAC SAR exploration (5 days instead of several weeks) while using nanomole amounts of reagents. Lastly, it avoids laborious and solvent-demanding purification of the reaction mixtures, thus making it an economical and more sustainable methodology for PROTAC hit finding.

Proteolysis Targeting Chimeras (PROTACs) are a burgeoning therapeutic modality that has garnered significant interest across academia as well as the pharmaceutical and the biotechnology industries. Following the first disclosures of the PROTAC concept in the early 2000s,[1,2] numerous companies as well as academic groups have developed this modality and recently progressed PROTACs into clinical trials for a range of indications.[3-5] A PROTAC molecule consists of three components: an E3 ligase ligand (E3-L), a protein of interest ligand (POI-L) and a linker joining these two components. This modality utilises the ubiquitin proteasome degradation pathway where the E3 ligase complex and the POI are brought together

in a ternary complex with the PROTAC.[6] The close proximity of the two proteins allows the transfer of ubiquitin(s) from the E3 ligase complex to the POI, thus marking it for proteasomal degradation. In contrast to monovalent small molecule drug discovery, there are several levels of increased complexity when designing these larger (> 700 Da) and structurally complex modality that tend to lie beyond the realm of the rule of five (bRo5).[7,8] In addition to the POI-L optimisation process, successful PROTAC campaigns require exploration of multiple variables including: the type of E3 ligase[9] and E3-L pharmacophore, the linker attachment point to both the POI-L and the E3-L (exit vectors), as well as the linker-length, -morphology, and -polarity. Furthermore, due to the limited structural data on ternary complexes[10,11] and the challenges involved in computational approaches,[12] PROTAC hit finding and structure-activity relationship (SAR) exploration within this modality remains mainly empirical. Furthermore, the larger size of these molecules coupled with their intricate architectures demands increased synthetic efforts[13] further adding to the significant time and resource investments required to explore the aforementioned variables and their effects on efficacy and structure property relationships (SPR). To address these challenges several library approaches have been reported, featuring cycloaddition click reactions,[14] active esters,[15] Ugi reaction,[16] Staudinger ligation[17] and solid phase synthesis.[18] However, these methods often require extra step(s) to install a reactive handle in a starting material or introduce functionalities in the linker with specific properties e.g. aromaticity, higher MW, HBD and HBA count. If these properties are undesired, these linkers are expected to require another round of optimisation to help the overall PROTAC reach more medicinal chemistry friendly property space.[8] In addition, the reported methods have been showcased on conventional milli- to micromolar scale, and all disclosed PROTACs have been purified prior to testing for degradation of the POI.

At AstraZeneca, the exploration of a broad PROTAC chemical space has been realised by utilising a collection of designed E3-Ls connected to linkers for the automation-supported, library synthesis of PROTACs. These advanced building blocks in our collection are named herein as 'E3-L-linker intermediates'. They contain a reactive chemical handle (e.g. amine) at the terminus of the linker, amenable to robust and diverse chemistry transformations, enabling conjugation to the POI-L and thus rapid access to PROTACs (Figure 1A). In addition, the conjugation reactions bringing the E3-L-linker intermediates and the POI-L together, allow for the modulation of ion class, linker polarity, steric bulk and sp^2/sp^3 character of the overall PROTAC. This aspect provides additional opportunities for structural and physicochemical property variation.

Our internal collection consists of over 300 E3-L-linker intermediates with coverage of multiple E3 ligases, including the widely studied CRBN and VHL. The inclusion of E3-L-linker intermediates to additional E3 ligases, facilitates a more exploratory approach to broaden our understanding of the hijackable proteasomal degradation machinery and enhances the variety of chemical equity when exploring the PROTAC modality on a programme. In addition, within each E3 ligase class there are often several E3-L chemotypes offering diversity and/or off-target selectivity. This can be exemplified within the CRBN chemical space, which is populated with intermediates based on, amongst others, pomalidomide- and lenalidomide-type ligands. Furthermore, several exit vectors from E3-Ls are represented which have proven to be beneficial for degradation potency and selectivity. To quickly reach molecules with desirable ADME/DMPK properties, there is a focus on enriching the collection with E3-L-linker intermediates that possess attractive parameters with respect to low MW, HBD count and exposed polar surface area (EPSA) and within a certain lipophilicity space.[7,8]

The E3-L-linker intermediates available within our collection can be grouped into clusters based on their structures, to aid with the selection process for custom designed hit-finding arrays (Figure 1B).

Cluster 1: PEG-based linkers of systematic linker length variation with coverage of multiple E3-Ls and attachment points. The intermediates in this cluster are useful for early hit-finding but offer limited ADME/DMPK advantage.

Cluster 2: Linkers with preferential oral and inhaled ADME/DMPK properties (e.g. rigid secondary amines) covering select E3 ligase ligands and attachment points. The intermediates in this cluster are ideally suited for the pursuit of lead-like PROTAC arrays.

Cluster 3: Bespoke PROTAC intermediate singletons with optimised properties that have demonstrated advantages on specific programmes (internal and/or external) with respect to either selectivity and/or ADME/DMPK.[19]

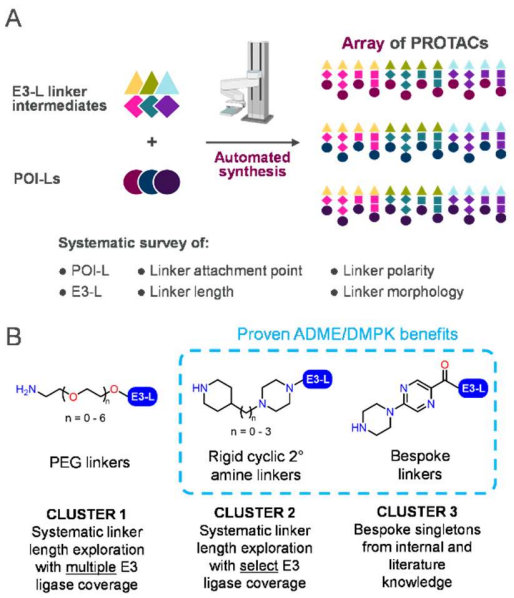


Figure 1. Use of E3-L-linker intermediates in PROTAC hit discovery: A. Parallel synthesis of PROTACs using E3-L-linker intermediates; B. Clustering of the E3-L-linker intermediates within the collection.

The arsenal of E3-L-linker intermediates contained within the collection is continually being enriched, with design inputs inspired by precedent from internal drug discovery programs as well as from the public domain.

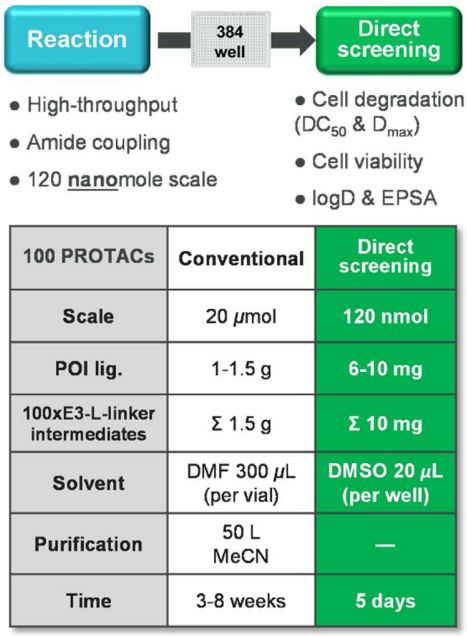
In our 1st generation approach to PROTAC hit finding, a POI-L, bearing a suitable functional handle, is coupled with an array of E3-L-linker intermediates at a standardised 20 μmol scale using an automated, multiple parallel based approach.[20] Following LC-MS analysis of the reaction mixtures, the successful reactions are progressed through to semi-automated, plate-based, reverse phase HPLC-MS purification to afford the desired purified PROTACs for further evaluation and profiling. The

platform provides a sufficient amount of material to allow for the further profiling of the PROTACs beyond the degradation assessment. This can include, but is not limited to, experiments evaluating: ADME/DMPK, safety/toxicity and off-target selectivity of the PROTAC. The approach allows for thorough profiling of PROTAC hits, thus further facilitating lead optimisation campaigns at an early stage of the investigation.

In the compound journey involving synthesis, purification, sample handling and screening, purification is a major bottleneck for speed and it hampers the possibility of miniaturization of the reaction scale. However, for primary project readouts like HiBiT or alphaLISA cell-based degradation assays,[21,22] only a few μL of 1 mM stock solution is needed, which is several thousand-fold less material than the conventional reaction scale (20 μmol). In addition, synthesis and testing of 100 purified PROTACs takes several weeks and approximately requires 1.0-1.5 g of POI-L, 1.5 g of E3-L-linker intermediates (combined mass) plus 50 L of acetonitrile for HPLC purification (Figure 2 - conventional). Therefore, this approach requires a significant time investment but also a significant cost expenditure. Furthermore, it is reliant on the time-consuming and costly scale-up of both the POI-L and the aforementioned arsenal of custom-made E3-L-linker intermediates for use in multiple programmes. While it is necessary to have purified samples in the later stages of a drug discovery project, in early hit finding, where broad chemical space needs to be explored, the whole process generates a vast amount of unnecessary chemical waste. This is potentially excessive, as often only a handful of synthesized PROTACs demonstrate degradation and are thus progressed further. In addition, due to the bifunctional and target-specific nature of PROTACs, it is less appealing to add them to HTS collections[23] for application in future screens, in contrast to monovalent small molecules.

For small molecules –that are not PROTACs– binary binding potency is of primary interest and several of the aforementioned issues could be tackled by miniaturised synthesis avoiding purification and applying direct screening of non-purified reaction mixtures. This can be achieved by means of off-rate screening,[24] sulfur (VI) fluoride exchange (SuFEx)[25] and 'direct-to-biology[26]' approaches. While many methods exist to quantify inhibitory potency, the primary readout for PROTACs comes solely from the cell-based degradation assays,[22] which is a more complex system and potentially more sensitive to non-purified mixtures. With respect to the screening of non-purified PROTAC reactions Tang *et. al.* reported the use of acyl hydrazones[27] or dialdehyde condensation[28] at 1 μmol scale in DMSO, both of which are high yielding reactions and give only water as a side product. In a related method, Hendrick *et.al.* reported a multistep approach to PROTACs at 5 μmol scale, using diamide linkers.[29] The approach involved resin filtrations and screening of these semi-purified mixtures, which provides rapid access to a set of various linkers. However, these methods above may require extra step(s) to install a reactive handle in a starting material, or introduce functionalities in the linker with specific properties e.g. aromaticity, higher MW, PSA, HBD and HBA count. If oral bioavailability is the end goal, many more iterations of optimisation will likely be necessary using these methodologies.[30,31] Most recently, Tang *et. al.* expanded the acyl hydrazone condensation approach to molecular glues.[32]

To shorten the time taken for PROTAC hit finding and transition from hit to lead, while reducing costs and lowering the environmental footprint, the synthesis and direct screening of non-purified mixtures would be beneficial. This ideally should be conducted on a miniaturised scale in a high-throughput fashion, using a POI-L with simple functional handles and a collection of E3-L-linker intermediates having a good representation of chemically diverse space with desirable ADME/DMPK properties.[8] In addition, the reaction components should be compatible with high-throughput cell-based degradation assays such as HiBiT or AlphaLISA.[22] Finally, DMSO would be the preferred solvent which would allow direct reaction dilution with more DMSO and subsequent screening, avoiding laborious solvent evaporation and reconstitution.



100 PROTACs	Conventional	Direct screening
Scale	20 μmol	120 nmol
POI lig.	1-1.5 g	6-10 mg
100xE3-L-linker intermediates	Σ 1.5 g	Σ 10 mg
Solvent	DMF 300 μL (per vial)	DMSO 20 μL (per well)
Purification	50 L MeCN	—
Time	3-8 weeks	5 days

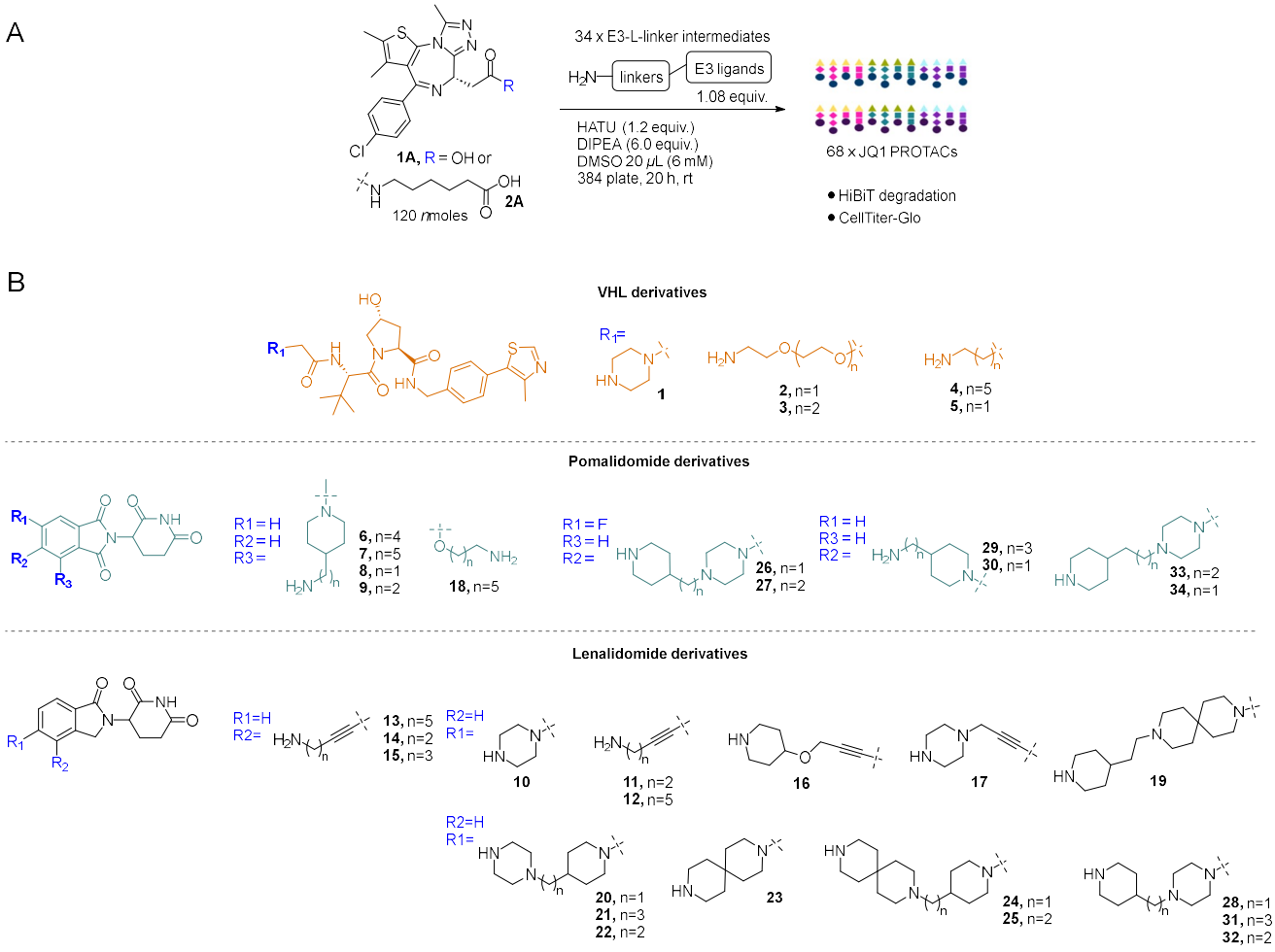
Figure 2. Comparison of 1st generation (purified) and 2nd generation (non-purified) workflows for a theoretical library of 100 PROTACs.

Having the desired requirements in mind, we additionally developed a rapid, nanomole scale PROTAC synthesis methodology using the E3-L linker intermediates employed in the 1st generation library synthesis. This approach allows for the direct screening of reaction mixtures using cell-based degradation and viability assays, as well as logD and EPSA measurements. We demonstrated the application of this integrated synthesis and screening platform on the widely benchmarked BRD4 system using the reported JQ1 ligand.[33] The approach greatly accelerates the workflow (5 days instead of several weeks), it expands the PROTAC chemical space exploration by facilitating the synthesis of hundreds of PROTACs using nanomole amounts of reagent and lastly it avoids the laborious and solvent-demanding purification of the library (Figure 2 – direct screening). Most importantly, direct screening does not compromise the data quality, which is suitable for the identification of actives and prioritisation of hits for re-synthesis on scale using our 1st generation approach. To our knowledge, this is the smallest scale (120 nanomoles in 20 μL of DMSO) of PROTAC library synthesis reported to date, which is approximately 170 times smaller than 20 μmol, 1st generation approach. We have chosen amide coupling as a model system which is a workhorse reaction for multi parallel synthesis (MPS), beyond PROTACs, due to its high yielding nature, functional group tolerance and efficiency on a small scale. [34] It was established that HATU and

DIPEA provided very good conversions while not interfering with the assay readout (HiBiT and CytoTox-Glo™).[35] This approach allows for a much broader chemical space exploration as only nanomole amounts of starting materials are required per reaction. Additionally, the scale allows for more POI-L–prelinker/chemotype combinations to be investigated using our set of custom-made and precious E3-L-linker intermediates, (Figure 2 – direct screening). Furthermore, the miniaturization of the array synthesis means we can more rapidly and opportunistically expand our arsenal of E3-L linker intermediates by incorporating frugal amounts of advanced intermediates from our internal PROTAC programs.

In the library setting we have used two POI-L acids: unmodified JQ1-acid (**1A**, Scheme 1) and a JQ1 equipped with an aliphatic prelinker capped with an acid (**2A**, Scheme 1). These two building blocks were reacted with 34 E3-L-linker-amine intermediates (Scheme 1). These were varied with respect to which E3 ligase protein they recruit (CRBN or VHL), type of E3-L chemotype (lenalidomide and pomalidomide), linker attachment point, length, rigidity, and amine class (primary or secondary). In this set, around half of the intermediates have desirable properties like short length, rigidity and a presence of a basic nitrogen that have been featured in orally bioavailable PROTACs, several of which are in clinical trials (e.g., linker **26**).[30] We have also included the linker from the known BRD4 degrader **MZ1**[36] (Scheme 1B, linker **3**).

Scheme 1. High-throughput synthesis of PROTACs using E3-L-linker intermediates; A. Nanomole scale, amide coupling reaction conditions; B. E3-L-linker intermediates used in this study.



The reactions were performed in an Echo® qualified 384 well plate, using HATU as the amide coupling agent and DIPEA as the base in 20 μL of DMSO (6 mM concentration). Reaction components were made as stock solutions in DMSO and added to the plate using a multi-channel pipette, followed by stirring for 20 h. Gratifyingly, all reactions showed conversions to the expected products, whereby most of the entries had high conversions (AUC - median 84%, average 79%) judged by the product area on the UV LC-MS trace (Figure 3A). Next, the reaction wells were diluted with DMSO to 1 mM and the solutions screened in parallel in a HiBiT degradation and cell viability (CytoTox-Glo™) assay. For the purposes of assay dilution and potency calculation, all reactions – irrespective of the conversion determined by UV LC-MS – were assumed quantitative.

Pleasingly, a number of PROTACs exhibited high potency in the HiBiT degradation assay (Figure 3B), with a few PROTACs in the picomolar range: those employing linkers **20** and **21**. Using linkers **14** and **15** showed no degradation when coupled with JQ1-acid **1A**, but the potency could be restored when JQ1-prelinker acid **2A** was used. Known JQ1 degrader **MZ1** (linker 3) showed lower than previously reported degradation,[33] but it is worth noting that a low (25%) conversion of this PROTAC was observed. In addition, none of the reactions showed

cytotoxicity, which would undoubtedly interfere with the assay readout. Lastly, the whole process from synthesis to biological profiling was performed in under 5 days. Detailed values of degradation potencies (pDC_{50} and D_{max}) including the degradation curves can be found in the SI.

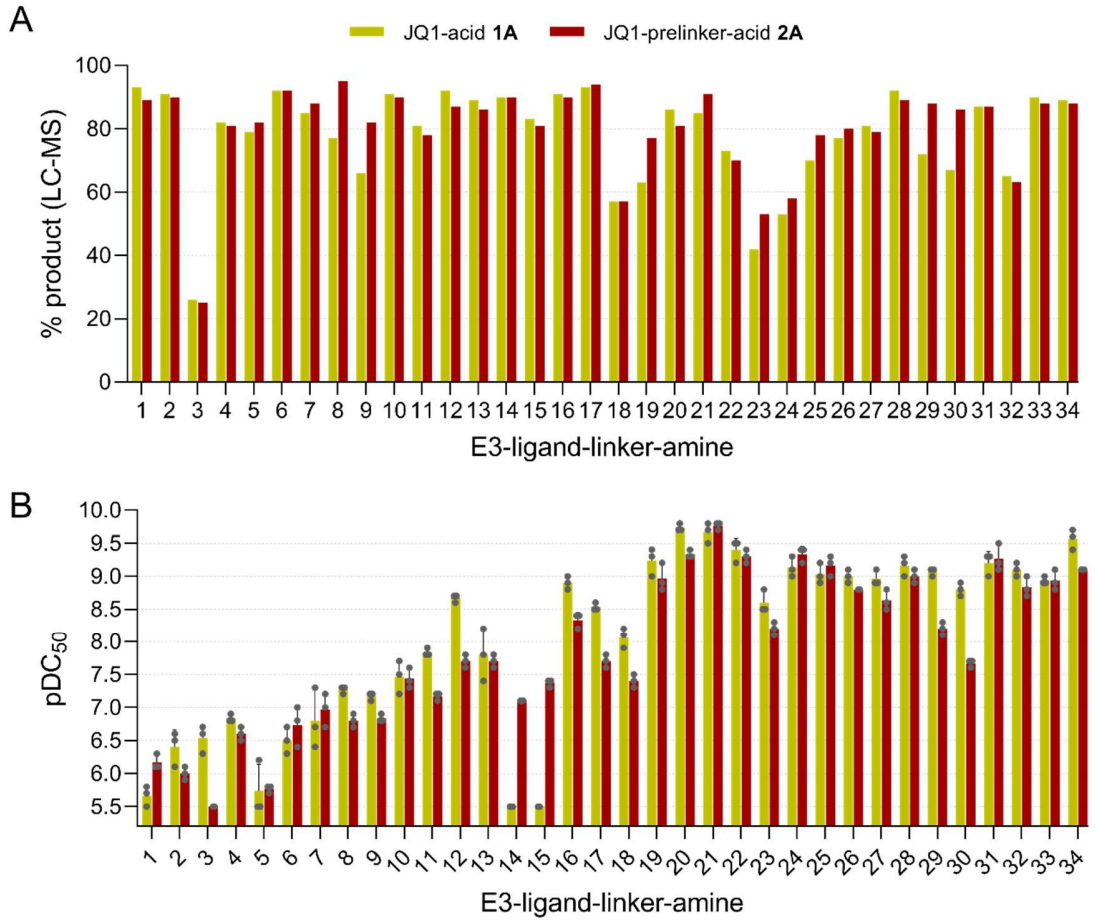
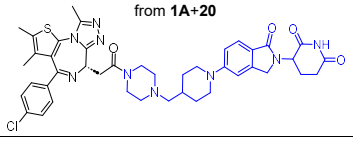
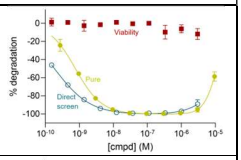
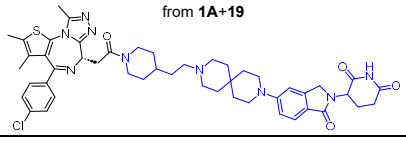
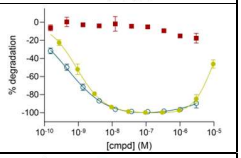
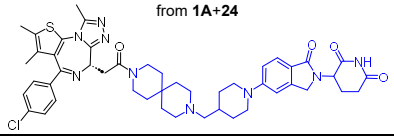
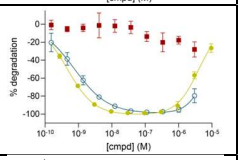
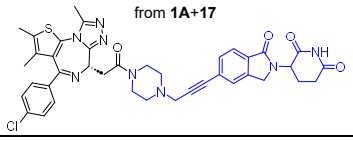
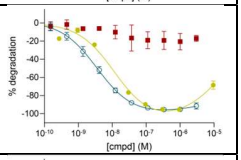
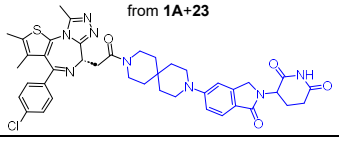
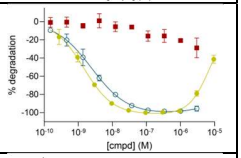
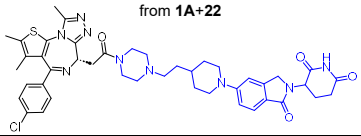
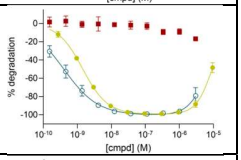
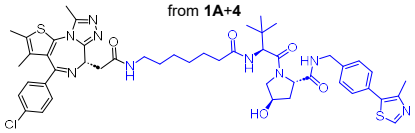
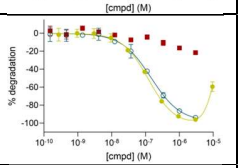
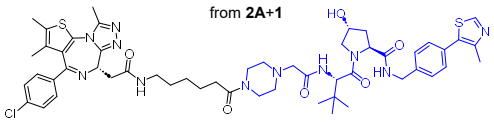
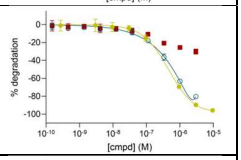
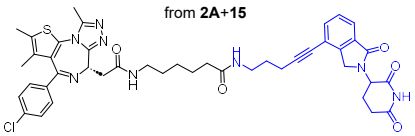
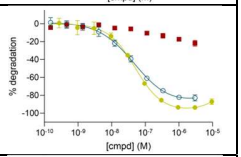
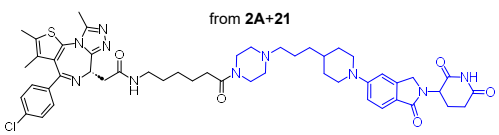
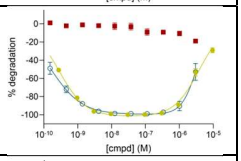
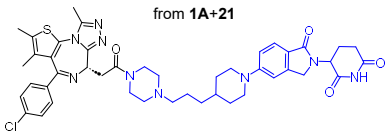
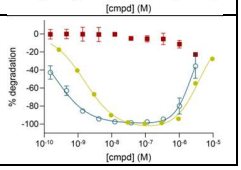


Figure 3. Overview of the PROTAC library using the JQ1 acid 1A (yellow bars) and the JQ1-prelinker-acid 2A (red bars): A. Product conversions measured by LC-MS; B. Degradation potencies (pDC_{50}) measured using a HiBiT assay.

In the next stage, we selected 11 PROTACs (**1P-11P**) to enter the methodology of our 1st generation approach. These were synthesized on a 14-20 μmol scale, purified by reverse phase HPLC and screened again in the HiBiT assay. These 11 compounds were selected to have a spread in observed potencies as well as differentiation in E3-L type and presence or lack of prelinker on the JQ1 ligand. We observed a very good correlation between pDC_{50} values for purified compounds with those obtained in the direct screening format. Slightly smaller D_{max} values were observed for the latter approach, which is in line with observations from Hendrick *et.al.*[29] Gratifyingly, even for non-purified mixtures with moderate conversions, in the range of 40-60% by UV LC-MS (PROTACs **2P**, **4P**, **5P**), the measured potencies were comparable (Table 1).

Our attention then turned to investigate how a varying reaction conversion could affect the readout in the HiBiT degradation assay, including the potential interference from binary binding of the reaction components (POI-L-acid & E3-L-linker intermediate). We performed a competition experiment (Figure 4) using a known PROTAC **MZ1** and PROTAC **4P** that were scaled up and purified using the 1st generation approach. In this experiment, pure PROTAC solutions were mixed (without coupling agent or base) with a solution containing their respective reaction components (JQ1 acid **1A** and E3-L-linker amine in 1:1 ratio), in order to mimic partial conversions (from 10-100%) of the coupling reaction. The total concentration of POI-L and E3-L moieties from the reaction component plus concentration of these ligands joined in PROTAC are the same in all the samples (Figure 4A). For each of the PROTAC percentages the HiBiT assay was performed, assuming 100% concentration of PROTAC. The potencies from the HiBiT assay are plotted in Figure 4B together with the potencies corrected for the actual PROTAC concentration. The degradation curves for these experiments are shown in Figure 4C and 4D. The degradation follows the concentration-response correlation, judged by corrected concentration in close alignment with the pDC_{50} values for the pure compounds (dashed line Figure 4B) and no sharp degradation shutdown was seen. The competition experiment results for **MZ1** at 20% (pDC_{50}=6.7) and 30% (pDC_{50}=6.9) PROTAC ratio correlates with the value from the direct screen

Table 1. Comparison of degradation potency (pDC_{50} and Dmax) between the direct screening method and purified samples including cell viability curves for direct screening. E3-L-linker intermediate part colored in blue and acid in black.

No	PROTAC structure	Direct screening			Pure		Curves
		LC-MS%	pDC_{50}	D_{max}%	pDC_{50}	D_{max}%	
1P	 from **1A+20**	86	9.7	89	9.1	100	
2P	 from **1A+19**	63	9.2	90	9.0	100	
3P	 from **1A+24**	53	9.1	80	9.2	99	
4P	 from **1A+17**	93	8.5	91	8.0	96	
5P	 from **1A+23**	42	8.6	96	8.8	100	
6P	 from **1A+22**	73	9.4	79	8.8	99	
7P	 from **1A+4**	82	6.8	94	6.9	96	
8P	 from **2A+1**	89	6.2	80	6.3	101	
9P	 from **2A+15**	81	7.4	83	7.3	93	
10P	 from **2A+21**	91	9.8	99	9.3	100	
11P	 from **1A+21**	85	9.7	99	8.8	100	

library where only 25% (pDC_{50}=6.5) conversion of **MZ1** was observed (Figure 3, linker **3**). Based on these results, together with correlations in Table 1, the discrepancy between pure and direct screening, for most reactions containing above 50% of product by UV LC-MS falls within 0.5 units of pDC_{50}, without the adjustments for the actual concentration. This is rationalized to be accurate enough for the hit finding and selection strategy for follow-up purified arrays.

Interestingly for lower concentrations of PROTACs (10-40%) the degradation curve shape is still maintained (Figure 4C and 4D) and the expected potency of the pure PROTAC can be roughly recalculated from the % conversion (within 0.5 units of pDC_{50}). This concentration response suggests that there is little additional interference in PROTAC mediated degradation by the competing binary binding of the POI-L acid and the E3-L-linker intermediate. This could be attributed to several potential explanations, including catalytic mechanism of action, positive cooperativity[4,37] for the formation of the ternary complex, better cell permeability or stability of the PROTAC than its reaction components, or rapid equilibration of the POI binding event compared to the relatively long (5h) HiBiT incubation time (event-driven[4,37] vs occupancy-driven pharmacology). In addition, the degradation of BRD4 using the purified PROTACs **4P** and **11P** was confirmed by Western Blot (WB). Repeating the same experiments, with a prior incubation of thalidomide at 20 μM confirmed the CRBN-mediated PROTAC mode of action of the compounds (Details in the SI)

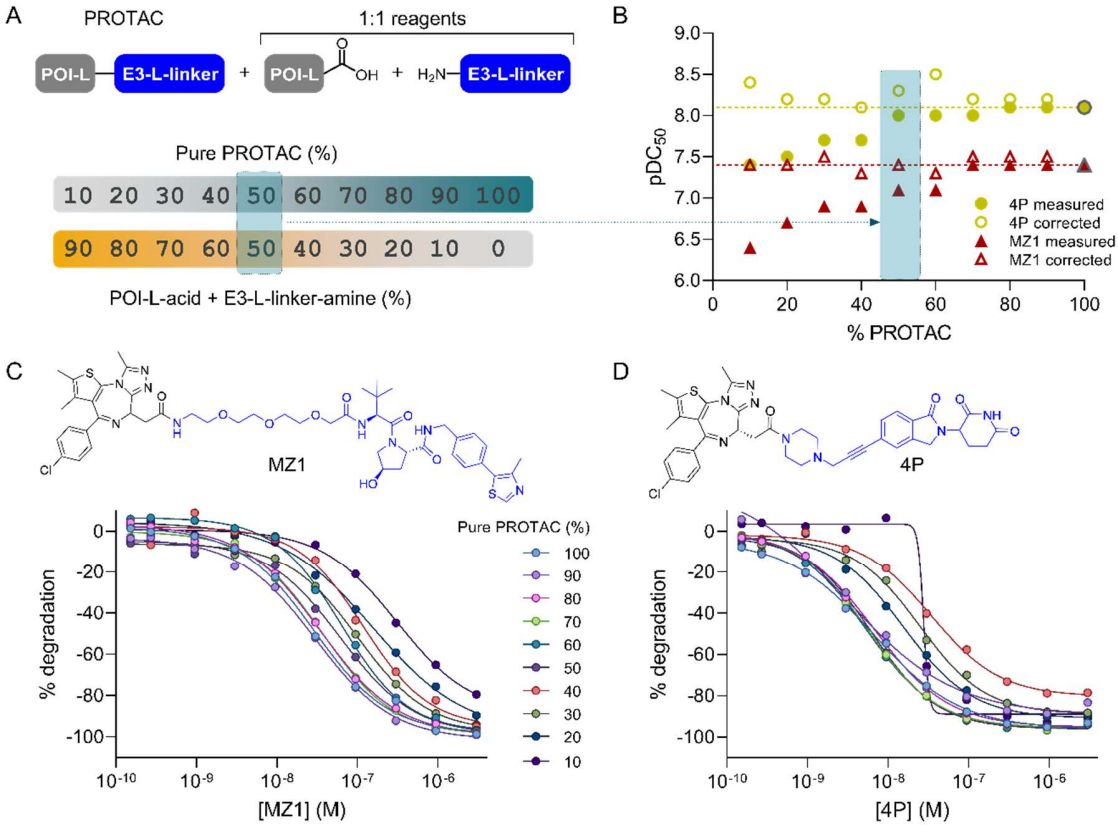


Figure 4. Illustration of doping experiments for PROTAC MZ1 and PROTAC 4P: A. Schematic of the experimental setup B; Measured pDC_{50} values for each condition – closed symbols represent measurements; open symbols are concentration corrected values; C and D. Concentration response curves for MZ1 (red) and 4P (yellow), respectively.

Optimisation of PROTAC properties requires broad assessment in a set of assays beyond degradation and functional cell-based assays. We therefore investigated the correlation between non-purified mixtures and the pure PROTACs in chromatographic logD and EPSA assays, which measure two of the important parameters to consider while designing for high permeability and oral bioavailability. The chromatographic logD (chrom logD) method has been shown to offer rapid determination, high accuracy, and broader range than the traditional shake flask method,[38] especially for bRo5 molecules. In addition, it is often challenging to measure permeability for larger MW compounds, such as peptides, and it was demonstrated that the EPSA[39] assay may act as a viable surrogate. This method employs supercritical fluid chromatography, that provides a low dielectric constant environment where molecular folding *via* intramolecular hydrogen bonding can take place, thereby greatly reducing the solvent-exposed polarity. Specifically, chrom logD and EPSA assays are promising for handling crude mixtures as they involve chromatographic separation of the reaction components.

In order to match the standardized assay concentration of 10 mM we remade the solutions for direct screening (**1P-11P**) on 200 nmole scale to reach the 10 mM concentration in DMSO (assuming 100% conversion; details in the SI). We then performed comparative testing for the selected cohort of 11

PROTACs and observed near to excellent correlation in the EPSA assay (Figure 5A). The two VHL PROTACs **7P** & **11P** (Figure 5A) had the lowest EPSA values despite the large number of *N* and *O* atoms, which is in line with the findings from Kihlberg *et. al.* about the ability of some PROTACs to fold in apolar solutions and shield the polar functionalities *via* intramolecular hydrogen bonding from the surrounding solvent molecules.[40,41] For PROTACs **1P**, **6P**, **11P** (Figure 5A) differing only by the length of $(CH_2)_{1\text{-}3}$ aliphatic spacers, which have negligible contribution to PSA, nearly identical values of EPSA (120 Å^2) were observed. For the logD assay we observed very good correlation for the majority of the tested examples (Figure 5B). PROTACs with a logD<3.6 all showed concordance within 0.1 units of logD. Detailed tabularised values for logD and EPSA measurements can be found in the SI. These two parameters can provide valuable information, in addition to the degradation potency, to potentially aid ranking/selecting of compounds for scale-up synthesis and purification on a drug discovery program.

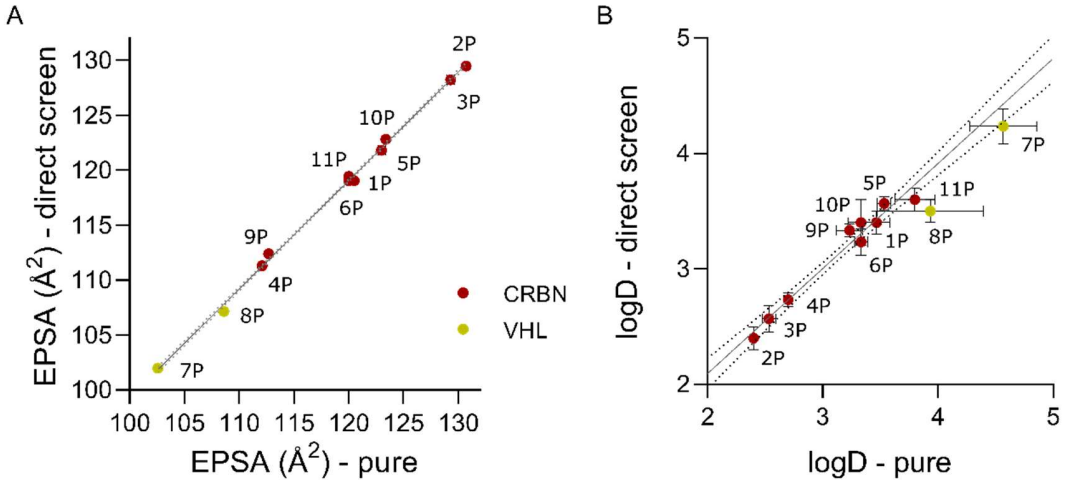


Figure 5. Measured physicochemical properties for purified and direct screening for VHL (yellow) and CRBN-targeted (red) PROTACs: A. EPSA. The solid line represents the best fitted straight line with a slope of 0.99 (0.80 - 1.0) and an intercept of 0.52 (-1.2 - 2.2) with r^2=0.996; B. Chromatographic logD. The best fitted straight line to CRBN-based PROTACs has a slope of 0.91 (0.71 - 0.89) and intercept of 0.28 (-0.064 - 0.61) with r^2=0.925. VHL-based PROTACs were associated with higher experimental variability and were not included in the regression. The dotted lines represent the 95% confidence interval (not visible in A).

In conclusion, we have developed two complementary PROTAC synthesis approaches that utilise our internal collection of E3-L-linker intermediates enabling hit finding followed by extensive SAR and SPR exploration. The 1st generation approach provides robust and reliable PROTAC arrays, with ample amounts of purified compounds useful for further profiling. This approach is most beneficial after the initial hits have been established. On the other hand, the 2nd generation takes advantage of the versatility of the amide bond reaction, miniaturization and direct reaction screening of non-purified mixtures. This method greatly reduces cost, shortens timelines to 5 days, expands SAR exploration by sampling a wide breadth of PROTAC chemical space using nanomole amounts of reagents and avoids the laborious and solvent demanding purification process. As only a selection of hits will be synthesised and purified on scale using the 1st generation platform, this represents a more efficient and sustainable approach to PROTAC hit finding.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Protocols for direct screening experiments (HiBiT, logD, EPSA), detailed HiBiT degradation data, Western Blot protocol, synthesis protocols, NMR spectra & HPLC traces (PDF).

INFORMATION

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ABBREVIATIONS

SAR, structure−activity relationships; SPR, structure-property relationship; HBD, hydrogen bond donor; HBA, hydrogen bond acceptor; POI-L, protein of interest ligand; E3-L, E3 ligase ligand; PROTACs, proteolysis targeting chimeras; BRD4, bromodomain-4; CRBN, cereblon; VHL, Von Hippel–Lindau tumor suppressor; ADME, absorption, distribution, metabolism, and excretion; DMPK, drug metabolism and pharmacokinetics; PEG, polyethylene glycol; HTS, high-throughput screening; PSA, polar surface area

REFERENCES

(1) Kenten, J. H.; Roberts, S. F.; Lebowitz, M. S. Controlling Protein Levels in Eukaryotic Organisms Using Novel Compounds Comprising a Ubiquitination Recognition Element and a Protein Binding Element. WO2000047220A1, **2000**.

(2) Sakamoto, K. M.; Kim, K. B.; Kumagai, A.; Mercurio, F.; Crews, C. M.; Deshaies, R. J. Protacs: chimeric molecules that target proteins to the Skp1-Cullin-F box complex for ubiquitination and degradation. *Proc. Natl. Acad. Sci. U. S. A.* **2001**, 98, 8554−8559.

(3) Mullard, A. Targeted protein degraders crowd into the clinic. *Nat. Rev. Drug. Discov.* **2021**, 20 (4), 247-250.

(4) Bekes, M.; Langley, D. R.; Crews, C. M. PROTAC targeted protein degraders: the past is prologue. *Nat. Rev. Drug. Discov.* **2022**, 21 (3), 181-200.

(5) He, M.; Cao, C.; Ni, Z.; Liu, Y.; Song, P.; Hao, S.; He, Y.; Sun, X.; Rao, Y. PROTACs: great opportunities for academia and industry (an update from 2020 to 2021). *Signal. Transduct. Target. Ther.* **2022**, 7 (1), 181.

(6) Casement, R.; Bond, A.; Craigon, C.; Ciulli, A. Mechanistic and Structural Features of PROTAC Ternary Complexes. *Methods. Mol. Biol.* **2021**, 2365, 79-113.

(7) Doak, B. C.; Over, B.; Giordanetto, F.; Kihlberg, J. Oral druggable space beyond the rule of 5: insights from drugs and clinical candidates. *Chem. Biol.* 2014, 21 (9), 1115-1142.

(8) Edmondson, S. D.; Yang, B.; Fallan, C. Proteolysis targeting chimeras (PROTACs) in 'beyond rule-of-five' chemical space: Recent progress and future challenges. *Bioorg. Med. Chem. Lett.* **2019**, 29 (13), 1555-1564.

(9) Yang, Q.; Zhao, J.; Chen, D.; Wang, Y. E3 ubiquitin ligases: styles, structures and functions. *Mol. Biomed.* **2021**, 2 (1), 23.

(10) Gadd, M. S.; Testa, A.; Lucas, X.; Chan, K. H.; Chen, W.; Lamont, D. J.; Zengerle, M.; Ciulli, A. Structural basis of PROTAC cooperative recognition for selective protein degradation. *Nat. Chem. Biol.* **2017**, 13 (5), 514-521.

(11) Wijaya, A. J.; Farnaby, W.; Ciulli, A. Crystallization of VHL-based PROTAC-induced ternary complexes. *Methods. Enzymol.* **2023**, 681, 241-263.

(12) Ward, J. A.; Perez-Lopez, C.; Mayor-Ruiz, C. Biophysical and Computational Approaches to Study Ternary Complexes: A 'Cooperative Relationship' to Rationalize Targeted Protein Degradation. *Chembiochem.* **2023**, e202300163.

(13) Cao, C.; He, M.; Wang, L.; He, Y.; Rao, Y. Chemistries of bifunctional PROTAC degraders. *Chem. Soc. Rev.* **2022**, *51* (16), 7066-7114.

(14) Wurz, R. P.; Dellamaggiore, K.; Dou, H.; Javier, N.; Lo, M. C.; McCarter, J. D.; Mohl, D.; Sastri, C.; Lipford, J. R.; Cee, V. J. A "Click Chemistry Platform" for the Rapid Synthesis of Bispecific Molecules for Inducing Protein Degradation. *J. Med. Chem.* **2018**, *61* (2), 453-461.

(15) Brownsey, D. K.; Rowley, B. C.; Gorobets, E.; Gelfand, B. S.; Derksen, D. J. Rapid synthesis of pomalidomide-conjugates for the development of protein degrader libraries. *Chem. Sci.* **2021**, *12* (12), 4519-4525.

(16) Bhela, I. P.; Ranza, A.; Balestrero, F. C.; Serafini, M.; Aprile, S.; Di Martino, R. M. C.; Condorelli, F.; Pirali, T. A Versatile and Sustainable Multicomponent Platform for the Synthesis of Protein Degraders: Proof-of-Concept Application to BRD4-Degrading PROTACs. *J. Med. Chem.* **2022**, *65* (22), 15282-15299.

(17) Bemis, T. A.; La Clair, J. J.; Burkart, M. D. Traceless Staudinger ligation enabled parallel synthesis of proteolysis targeting chimera linker variants. *Chem. Commun. (Camb.)* **2021**, *57* (8), 1026-1029.

(18) Krajcovicova, S.; Jorda, R.; Hendrychova, D.; Krystof, V.; Soural, M. Solid-phase synthesis for thalidomide-based proteolysis-targeting chimeras (PROTAC). *Chem. Commun. (Camb.)* **2019**, *55* (7), 929-932.

(19) Miah, A. H.; Smith, I. E. D.; Rackham, M.; Mares, A.; Thawani, A. R.; Nagilla, R.; Haile, P. A.; Votta, B. J.; Gordon, L. J.; Watt, G.; et al. Optimization of a Series of RIPK2 PROTACs. *J. Med. Chem.* **2021**, *64* (17), 12978-13003.

(20) Flanagan, S. R.; Harrowven, D. C.; Bradley, M. A new benzannulation reaction and its application in the multiple parallel synthesis of arylnaphthalene lignans. *Tetrahedron* **2002**, *58* (30), 5989-6001.

(21) Daniels, D. L.; Riching, K. M.; Urh, M. Monitoring and deciphering protein degradation pathways inside cells. *Drug. Discov. Today Technol.* **2019**, *31*, 61-68.

(22) Simard, J. R.; Lee, L.; Vieux, E.; Improgo, R.; Tieu, T.; Phillips, A. J.; Fisher, S. L.; Pollock, R. M.; Park, E. High-Throughput Quantitative Assay Technologies for Accelerating the Discovery and Optimization of Targeted Protein Degradation Therapeutics. *SLAS Discov.* **2021**, *26* (4), 503-517.

(23) Diller, D. J.; Hobbs, D. W. Deriving knowledge through data mining high-throughput screening data. *J. Med. Chem.* **2004**, *47* (25), 6373-6383.

(24) Murray, J. B.; Roughley, S. D.; Matassova, N.; Brough, P. A. Off-rate screening (ORS) by surface plasmon resonance. An efficient method to kinetically sample hit to lead chemical space from unpurified reaction products. *J. Med. Chem.* **2014**, *57* (7), 2845-2850.

(25) Kitamura, S.; Zheng, Q.; Woehl, J. L.; Solania, A.; Chen, E.; Dillon, N.; Hull, M. V.; Kotaniguchi, M.; Cappiello, J. R.; Kitamura, S.; et al. Sulfur(VI) Fluoride Exchange (SuFEx)-Enabled High-Throughput Medicinal Chemistry. *J. Am. Chem. Soc.* **2020**, *142* (25), 10899-10904.
(26) Thomas, R. P.; Heap, R. E.; Zappacosta, F.; Grant, E. K.; Pogany, P.; Besley, S.; Fallon, D. J.; Hann, M. M.; House, D.; Tomkinson, N. C. O.; et al. A direct-to-biology high-throughput chemistry approach to reactive fragment screening. *Chem. Sci.* **2021**, *12* (36), 12098-12106.
(27) Roberts, B. L.; Ma, Z. X.; Gao, A.; Leisten, E. D.; Yin, D.; Xu, W.; Tang, W. Two-Stage Strategy for Development of Proteolysis Targeting Chimeras and its Application for Estrogen Receptor Degraders. *ACS Chem. Biol.* **2020**, *15* (6), 1487-1496.
(28) Guo, L.; Zhou, Y.; Nie, X.; Zhang, Z.; Zhang, Z.; Li, C.; Wang, T.; Tang, W. A platform for the rapid synthesis of proteolysis targeting chimeras (Rapid-TAC) under miniaturized conditions. *Eur. J. Med. Chem.* **2022**, *236*, 114317.
(29) Hendrick, C. E.; Jorgensen, J. R.; Chaudhry, C.; Strambeanu, II; Brazeau, J. F.; Schiffer, J.; Shi, Z.; Venable, J. D.; Wolkenberg, S. E. Direct-to-Biology Accelerates PROTAC Synthesis and the Evaluation of Linker Effects on Permeability and Degradation. *ACS Med. Chem. Lett.* **2022**, *13* (7), 1182-1190.
(30) Han, X.; Sun, Y. Strategies for the discovery of oral PROTAC degraders aimed at cancer therapy. *Cell Rep. Phys. Sci.* **2022**, 3 (10).
(31) Hornberger, K. R.; Araujo, E. M. V. Physicochemical Property Determinants of Oral Absorption for PROTAC Protein Degraders. *J. Med. Chem.* **2023**, *66* (12), 8281-8287.
(32) Li, J.; Li, C.; Zhang, Z.; Zhang, Z.; Wu, Z.; Liao, J.; Wang, Z.; McReynolds, M.; Xie, H.; Guo, L.; *et al.* A platform for the rapid synthesis of molecular glues (Rapid-Glue) under miniaturized conditions for direct biological screening. *Eur. J. Med. Chem.* **2023**, *258*. DOI: 10.1016/j.ejmech.2023.115567.
(33) Filippakopoulos, P.; Qi, J.; Picaud, S.; Shen, Y.; Smith, W. B.; Fedorov, O.; Morse, E. M.; Keates, T.; Hickman, T. T.; Felletar, I.; *et al*. Selective inhibition of BET bromodomains. *Nature* **2010**, *468* (7327), 1067-1073.
(34) Dombrowski, A. W.; Aguirre, A. L.; Shrestha, A.; Sarris, K. A.; Wang, Y. The Chosen Few: Parallel Library Reaction Methodologies for Drug Discovery. *J. Org. Chem.* **2022**, *87* (4), 1880-1897.
(35) Riching, K. M.; Mahan, S.; Corona, C. R.; McDougall, M.; Vasta, J. D.; Robers, M. B.; Urh, M.; Daniels, D. L. Quantitative Live-Cell Kinetic Degradation and Mechanistic Profiling of PROTAC Mode of Action. *ACS Chem. Biol.* **2018**, *13* (9), 2758-2770.
(36) Zengerle, M.; Chan, K. H.; Ciulli, A. Selective Small Molecule Induced Degradation of the BET Bromodomain Protein BRD4. *ACS Chem. Biol.* **2015**, *10* (8), 1770-1777.
(37) Cecchini, C.; Pannilunghi, S.; Tardy, S.; Scapozza, L. From Conception to Development: Investigating PROTACs Features for Improved Cell Permeability and Successful Protein Degradation. *Front. Chem.* **2021**, *9*, 672267.
(38) Ermondi, G.; Vallaro, M.; Goetz, G.; Shalaeva, M.; Caron, G. Experimental lipophilicity for beyond Rule of 5 compounds. *Future Drug Discovery* **2019**, *1* (1), 2631-3316.
(39) Goetz, G. H.; Philippe, L.; Shapiro, M. J. EPSA: A Novel Supercritical Fluid Chromatography Technique Enabling the Design of Permeable Cyclic Peptides. *ACS Med. Chem. Lett.* **2014**, *5* (10), 1167-1172.
(40) Atilaw, Y.; Poongavanam, V.; Svensson Nilsson, C.; Nguyen, D.; Giese, A.; Meibom, D.; Erdelyi, M.; Kihlberg, J. Solution Conformations Shed Light on PROTAC Cell Permeability. *ACS Med. Chem. Lett.* **2021**, *12* (1), 107-114.
(41) Poongavanam, V.; Atilaw, Y.; Siegel, S.; Giese, A.; Lehmann, L.; Meibom, D.; Erdelyi, M.; Kihlberg, J. Linker-Dependent Folding Rationalizes PROTAC Cell Permeability. *J. Med. Chem.* **2022**, *65* (19), 13029-13040.