Rational Design of CDK12/13 and BRD4 Molecular Glue Degraders

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ABSTRACT: Targeted protein degradation (TPD) is an emerging therapeutic approach for the selective elimination of disease-related proteins. While molecular glue degraders exhibit drug-like properties, their discovery has traditionally been serendipitous and often requires post-hoc rationalization. In this study, we demonstrate the rational design of molecular glue degraders using gluing moieties. By appending a chemical gluing moiety to several small molecule inhibitors, we successfully transformed them into degraders, obviating the need for a specific E3 ubiquitin ligase recruiter. Specifically, we found that incorporating a hydrophobic gluing moiety into a cyclin-dependent kinase 12 and 13 (CDK12/13) dual inhibitor enabled the recruitment of DNA damage-binding protein 1 (DDB1), thereby transforming a high-molecular-weight bivalent CDK12 degrader into a potent monovalent CDK12/13 molecular glue degrader. We also showcase that attaching a cysteine-reactive warhead to a BRD4 inhibitor converts it into a degrader by recruiting the DDB1 and CUL4 associated factor 16 (DCAF16) E3 ligase.

Molecular glues are small molecules that facilitate the dimerization of two proteins, forming a ternary complex through proximity-driven mechanisms.[1] By chemically recruiting specific proteins, these molecules provide precise control over various biological processes, including signal transduction, transcription, protein localization, stabilization, and degradation.[2] Among these, molecular glue degraders induce the proximity between E3 ubiquitin ligases and target proteins, resulting in the ubiquitination and degradation of the target proteins.[3] Unlike traditional drug discovery through occupancy-driven pharmacology, which mainly focuses on inhibiting the enzymatic activity of proteins, molecular glues exhibit unique event-driven pharmacology, including the ability to eliminate all functions of target proteins and maintain target depletion even at substoichiometric drug concentrations.

In the field of TPD, proteolysis-targeting chimeras (PROTACs) represent a promising strategy for the modular design of protein degraders.[4] These bivalent molecules consist of ligands for both the target protein and an E3 ubiquitin ligase, connected by a suitable linker. In contrast, the discovery of molecular glue degraders, advantageous because of their drug-like properties, is often serendipitous and their design remains challenging. Generally, two approaches are employed in the discovery of molecular glue degraders. The first approach involves chemical derivatizations of the few known E3 ubiquitin ligase ligands, enabling the recruitment of various neo-substrates by leveraging new protein-protein interactions.[5] Representative examples include the development of cereblon (CRBN) molecular glue degraders capable of degrading otherwise undruggable targets, such as transcription factor Ikaros family zinc finger proteins 1 and 3 (IKZF1/3),[6] G_1 to S phase transition protein 1 (GSPT1),[7] Helios (IKZF2),[8] widely-interspaced zinc finger-containing protein (WIZ)[9] and others. In the second approach, degraders are discovered serendipitously, *e.g.*, CR8 and BI-3802, in medicinal chemistry campaigns focused on small molecule inhibitors of the target of interest or related proteins.[10-12] Follow-up mechanistic studies reveal that these compounds contain gluing moieties that recruit components of the ubiquitin-proteasome system (UPS), initiating ubiquitination and subsequent degradation.

Herein, we investigated the prospective incorporation of gluing moieties in the design of molecular glue degraders. Minor modifications to parental inhibitors by adding gluing moieties resulted in a gain-of-function transition, effectively converting them into protein degraders. We demonstrated that addition of a hydrophobic gluing moiety to a CDK12/13 binder can recruit DDB1, transforming a high-molecular-weight CDK12 PROTAC into a potent, monovalent CDK12/13 molecular glue degrader. Additionally, we showed that appending a cysteine-reactive warhead to a BRD4 inhibitor enabled its conversion into a degrader by recruiting the DCAF16 E3 ligase. Overall, we envision that the derivatization of known inhibitors or binders of the target represents a promising approach for the discovery of molecular glue degraders.

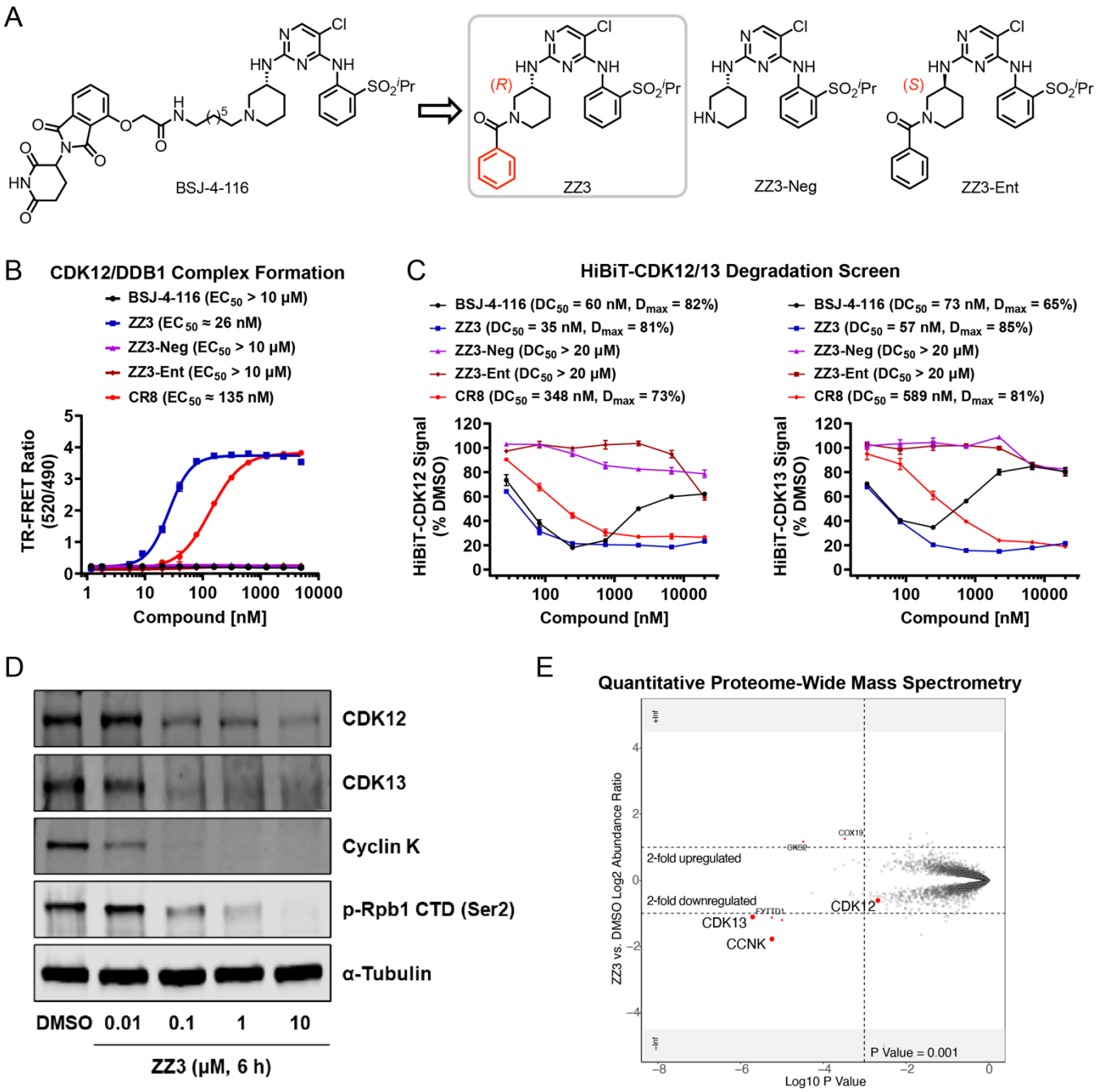


Figure 1. Introducing a hydrophobic gluing moiety yields a CDK12 molecular glue degrader. (A) Chemical structures of the parental CDK12 PROTAC (BSJ-4-116), its derivative (ZZ3) featuring a hydrophobic gluing moiety conferring degrader activity, a negative control of ZZ3 (ZZ3-Neg) without a gluing moiety, and an enantiomer of ZZ3 (ZZ3-Ent) that negates CDK12/13 binding. (B) TR-FRET assay quantifying the propensity of the indicated compounds to induce the CDK12/Cyclin K-DDB1 ternary complex formation. (C) HiBiT-CDK12 and HiBiT-CDK13 assay results for HEK293T cells treated with the indicated compounds for 6 h. (D) Western blots showing CDK12, CDK13, and Cyclin K degradation in Jurkat cells treated with the indicated concentrations of ZZ3 for 6 h. (E) Quantitative proteome-wide mass spectrometry in Jurkat cells after 3 h treatment with 100 nM of ZZ3.

Introducing a hydrophobic gluing moiety into a CDK12/13 inhibitor triggered the gain-of-function transition into a potent CDK12/13 and cyclin K degrader

CDK12 and its highly homologous counterpart CDK13 are activated by binding to cyclin K, which regulates the transcription of DNA-damage response genes. Therefore, CDK12 and CDK13 are potential therapeutic targets for cancer treatment.[13, 14] Various orthogonal pharmacologic strategies have been developed, including reversible,[14, 15] covalent inhibitors[16] and PROTACs.[17] Among these strategies, the high molecular weight of PROTACs poses significant challenges for achieving suitable pharmacokinetic (PK) properties; therefore, developing a CDK12/13 degrader with drug-like properties is highly desirable.

Recently, various CDK inhibitors have been identified as cyclin K molecular glue degraders.[10, 18, 19] These molecular glues initially bind to the CDK12-cyclin K complex and subsequently recruit DDB1, a core component of CUL4-based E3 ubiquitin ligase complexes, triggering ubiquitination and degradation. Structural and biochemical mechanistic studies reveal that the π–cation interaction between the hydrophobic aromatic gluing moiety of CDK12 inhibitors and Arg928 of DDB1 is essential for the formation of the CDK12-DDB1 ternary complex.[18] Inspired by this, we prepared a series of analogs by incorporating aromatic groups into a solvent-exposed region of the parental CDK12/13 inhibitor from BSJ-4-116, aiming to induce interaction with DDB1 (Figure 1A).

We quantitatively measured the *in vitro* complex formation between CDK12 and DDB1 using a time-resolved fluorescence resonance energy transfer (TR-FRET) assay (Figure 1B).[18] CR8, a well-characterized cyclin K molecular glue, exhibited an EC_{50} value of 135 nM, consistent with previously reported potency.[10] Among the compounds in our library, ZZ3, which contains a phenyl moiety, induced robust complex formation with an EC_{50} value of 26 nM. In contrast, the parental CDK12 binder ZZ3-Neg, lacking the phenyl gluing moiety, failed to recruit DDB1 to CDK12 (EC_{50} > 10 μM). The enantiomer of ZZ3 (ZZ3-Ent), which lacks the binding affinity towards CDK12, completely lost its activity, indicating the necessity of binding to CDK12.

To investigate if the robust ternary complex formation of the hit compound results in cellular activities, we quantitatively measured endogenous protein levels in HEK293T cells using HiBiT-CDK12 and HiBiT-CDK13 assays, which employ CRISPR/Cas9-mediated fusion of an 11-amino acid peptide tag to the proteins of interest. The parental CDK12 PROTAC, BSJ-4-116, exhibited DC_{50} values of 60 nM and 73 nM for CDK12 and CDK13, respectively. The hit compound ZZ3 demonstrated potent degradation of CDK12 and CDK13, with DC_{50} values of 35 nM and 57 nM, respectively (Figure 1C). In contrast, the enantiomer ZZ3-Ent and the parental inhibitor ZZ3-Neg completely lost the ability to degrade CDK12 and CDK13 (Figure 1C). ZZ3 also induced degradation of cyclin K, consistent with previous reported cyclin K molecular glues (Figure 1D). By Western blotting we further confirmed that ZZ3 induced dose-dependent degradation of CDK12, CDK13, and cyclin K, as well as inhibition of RNA polymerase II phosphorylation (Figure 1D). ZZ3 induced time-dependent degradation of CDK12 and cyclin K, achieving complete depletion of cyclin K with a concentration of 1 μM for 0.5 h (Figure S1A). To assess the selectivity of ZZ3, we conducted quantitative proteome-wide mass spectrometry in Jurkat cells treated with a concentration of 100 nM for 3 h, aiming to capture the most immediate changes in protein levels. We observed selective degradation of CDK12, CDK13, and cyclin K (Figure 1E).

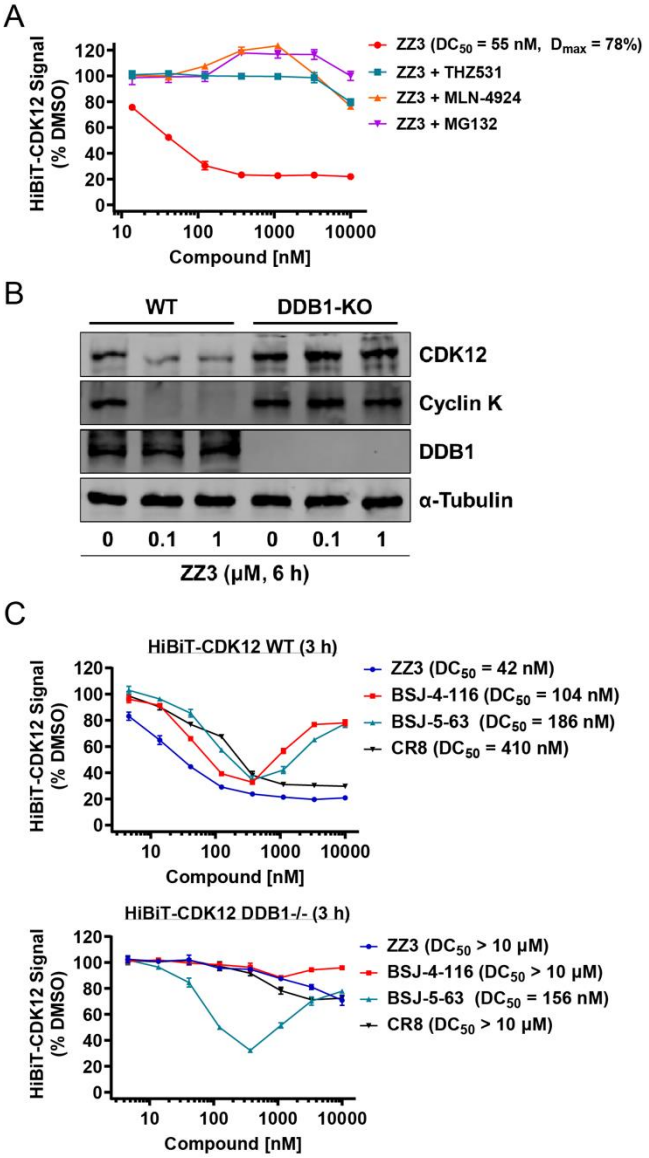


Figure 2. Mechanism of CDK12 and cyclin K degradation induced by ZZ3. (A) HiBiT-CDK12 assay results for HEK293T cells pretreated with the indicated inhibitors for 1 h, followed by treatment with ZZ3 for 6 h. (B) Western blots showing CDK12 and cyclin K degradation in WT or DDB1-KO HEK293T cells treated with the indicated concentrations of ZZ3 for 6 h. (C) HiBiT-CDK12 assay results for WT or DDB1-KO HEK293T cells treated with the indicated compounds for 3 h.

ZZ3-induced degradation of CDK12 and Cyclin K is DDB1-dependent

We next investigated the mechanism by which ZZ3 induces degradation. Pretreatment with MLN-4924, a NEDD8-activating enzyme inhibitor, and MG132, a proteasome inhibitor, rescued CDK12 degradation in the HiBiT-CDK12 degradation assay, indicating that ZZ3 promotes CDK12 degradation via the UPS (Figure 2A). Co-treatment with the covalent CDK12/13 inhibitor THZ531 also rescued CDK12 degradation, confirming the necessity of CDK12 binding (Figure 2A). We further validated that ZZ3-induced cyclin K degradation occurs

via the UPS through co-treatment with neddylation or proteasome inhibitors experiments confirmed by Western blotting (Figure S1B).

The knockout (KO) of DDB1 abrogated ZZ3's CDK12 and cyclin K degrading potency, suggesting that DDB1 is a critical component of the E3 ubiquitin ligase responsible for degradation (Figure 2B). To quantitatively measure endogenous CDK12 protein levels, we genetically knocked out DDB1 in HiBiT-CDK12 HEK293T cells. In the HiBiT assay using DDB1-KO cells, both CDK12 molecular glue degraders, ZZ3 and CR8, failed to induce CDK12 degradation, further supporting DDB1 as the ubiquitination factor responsible for driving degradation (Figure 2C). Additionally, degradation of CDK12 induced by the CRBN-based PROTAC, BSJ-4-116, was rescued in DDB1-KO cells, as DDB1 is part of the E3 ubiquitin ligase complex CUL4-RBX1-DDB1-CRBN (Figure 2C). In contrast, the VHL-based CDK12 PROTAC, BSJ-5-63, maintained the degradation of CDK12 in DDB1-KO cells, as the VHL E3 ligase function does not rely on DDB1 (Figure 2C).[20]

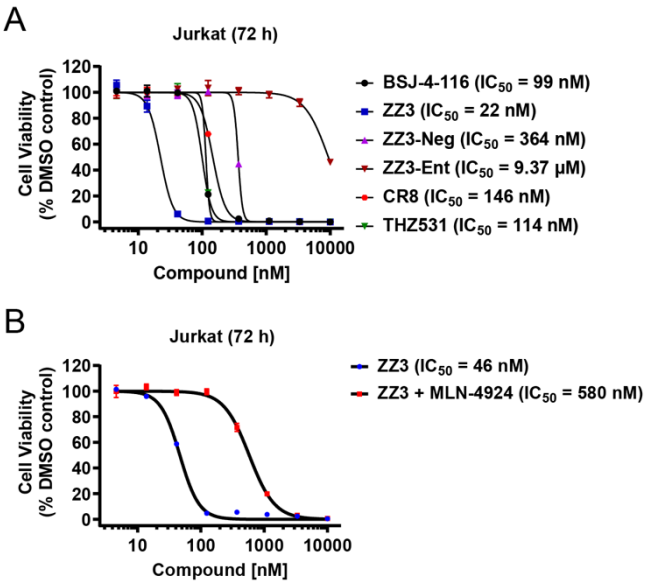
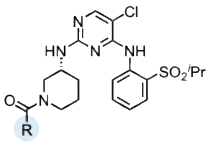


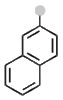
Figure 3. Antiproliferative activity of CDK12/13 and cyclin K degraders. (A) Cell viability results for Jurkat cells treated with the indicated compounds for 72 h. (B) Cell viability results for Jurkat cells pre-treated with MLN-4924 for 1 h, followed by treatment with ZZ3 for 72 h.

CDK12/13 and cyclin K degradation leads to potent antiproliferative activity

We found that the CDK12/13 molecular glue degrader ZZ3 exhibited more potent antiproliferative activity in Jurkat cells than the parental CDK12 PROTAC BSJ-4-116, the covalent CDK12/13 inhibitor THZ531, and the cyclin K molecular glue CR8 (Figure 3A). Importantly, ZZ3 demonstrates good *in vitro* kinase selectivity, with RSK1 and CHEK2 being the only two off-targets identified in a KINOMEscan evaluation of a panel of 468 human kinases (Figure S2). The reduced antiproliferative activity of the parental CDK12/13 inhibitor ZZ3-Neg indicated that the observed antiproliferative activity of ZZ3 is unlikely solely attributable to the inhibition of CDK12/13 (Figure 3A). To further confirm whether this potent antiproliferative effect was due to the degradation of CDK12/13 and cyclin K, we pretreated the cells with MLN-4924, a NEDD8-activating enzyme inhibitor, and assessed the changes in cell viability. As a result, the pretreatment with MLN-4924 reduced ZZ3-induced antiproliferative activity by more than 10-fold (Figure 3B).

Table 1. Structure-activity relationship (SAR) study of ZZ3's gluing moiety[a]



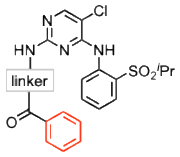
compound	gluing moiety R	HiBiT-CDK12 DC_{50} (μM)	TR-FRET CDK12/DDB1 EC_{50} (nM)
ZZ3		0.035	26
ZZ7-14-046		> 20	n.d.
ZZ7-16-063		5.13	55
ZZ7-16-064		3.37	39
ZZ7-16-065		14.03	49
ZZ7-16-066		> 20	56
ZZ7-16-067		> 20	68
ZZ7-16-068		> 20	56
ZZ7-16-069		> 20	n.d.
ZZ7-16-070		> 20	85
ZZ7-16-071		> 20	n.d.
ZZ7-16-072		> 20	n.d.

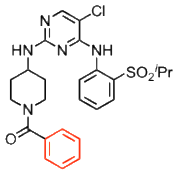
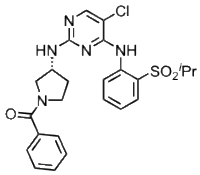
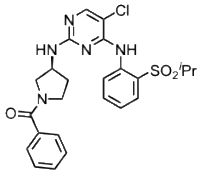
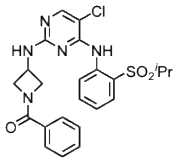
[a]DC_{50} and EC_{50} values are given as averages of two replicates. n.d., not detected.

Structure-activity relationship (SAR) study yields ZZ4 with comparable potency

Next, we synthesized several analogs of ZZ3 to study structure-activity relationships (SAR) by modifying the gluing moiety (Table 1). The use of an alkyl gluing moiety (ZZ7-14-046) failed to induce the formation of the ternary complex and the degradation of CDK12, highlighting the critical role of the phenyl group to facilitate key π–cation interaction. Introducing larger π-systems, such as naphthyl (ZZ7-16-063~064), significantly reduced the potency of CDK12 degradation, despite maintaining the ternary complex formation. Replacing the phenyl group with heterocycles, including pyridine (ZZ7-16-065~067), pyrimidine (ZZ7-16-068~070), and pyridazine (ZZ7-16-071~072), mostly abolished CDK12 degradation, indicating that a hydrogen bond acceptor is not required.

Table 2. Structure-activity relationship (SAR) study of ZZ3's linker[a]



compound	structure	HiBiT-CDK12 DC_{50} (nM)	TR-FRET CDK12/DDB1 EC_{50} (nM)
ZZ4		40	24
ZZ7-16-018		1,660	27
ZZ7-16-019		> 10,000	n.d.
ZZ7-16-020		> 10,000	85

[a]DC_{50} and EC_{50} values are given as averages of two replicates. n.d., not detected.

We next investigated the linker length and preferred orientation of the phenyl gluing moiety with respect to the pyrimidine hinge binding motif (Table 2). Both five-membered (ZZ7-16-018, 19) and four-membered (ZZ7-16-020) rings resulted in decreased CDK12 degradation potency. However, a compound with a 4-aminopiperidine linker, ZZ4, demonstrated comparable ternary complex formation (EC_{50} = 24 nM) and CDK12 degradation potency (DC_{50} = 40 nM). ZZ4 induced potent, UPS-dependent CDK12 and cyclin K degradation, with good degradation selectivity (Figure S3). Taken together, these results indicate the importance of both the hydrophobic aromatic gluing moiety and its orientation for the activity of CDK12 molecular glue degraders.

Introducing a covalent warhead into an inhibitor of BRD4 triggered the gain-of-function transition into a BRD4 degrader

We then investigated whether the introduction of a chemical glueing moiety could be broadly applied to the development of degraders of the bromodomain and extra-terminal domain (BET) family of protein BRD4. BRD4 has been investigated as a potential target for cancer and immune diseases.[21] Substantial efforts have been devoted to developing BET inhibitors,[22] and more recently, to transitioning to degraders.[23, 24] Several BRD4 molecular glue degraders have been reported, particularly those that recruit E3 ubiquitin ligases such as DDB1- and CUL4-associated factor 11 (DCAF11) and DDB1- and CUL4-associated factor 16 (DCAF16), indicating complementary protein-protein interactions between BRD4 and DCAF proteins.[12, 25, 26] Notably, our group and others have reported that tethering a cysteine-reactive covalent warhead to the well-studied BET inhibitor JQ1 generates BRD4 degraders that covalently label E3 ligases.[25-27]

To investigate whether covalent warheads could serve as gluing moieties to recruit E3 ligases for other BET inhibitors, we synthesized ZZ5 by attaching an acrylamide warhead into GSK778, a reported selective inhibitor of first bromodomain of BRD4 (Figure 4A).[28] We quantitatively measured endogenous BRD4 protein levels in Jurkat cells using a HiBiT-BRD4 assay.[25] The JQ1-based BRD4 PROTAC dBET6 exhibited DC_{50} values of 18 nM, consistent with previously reported potencies.[24] ZZ5 induced BRD4 degradation, with DC_{50} and D_{max} values of 3.26 μM and 54%, respectively (Figure 4B). ZZ5-Neg, which lacks the acrylic double bond, did not induce BRD4 degradation, confirming the necessity of the covalent warhead. Pretreatment with MLN-4924 or MG132 abolished BRD4 degradation, suggesting that ZZ5 promotes BRD4 degradation via the UPS (Figure 4C). Additionally, co-treatment with the parental BET inhibitor GSK778 rescued BRD4 degradation, indicating the requirement of initial binding to the first bromodomain of BRD4 (Figure 4C).

We used a DCAF knockout HiBiT-BRD4 degradation assay to investigate which specific DCAF E3 ubiquitin ligase was responsible for the degradation process. The screening results indicated that DCAF16 is the primary E3 ligase driving the degradation (Figure 4D). We further confirmed the degradation was DCAF16-dependent by performing Western blotting in DCAF16 knockout K-562 cells (Figure 4E). To identify the cysteine covalently labeled by ZZ5, we screened BRD4 degradation across various DCAF16 mutant cell lines. This led us to identify Cys58 as required for ZZ5-induced BRD4 degradation (Figure 4E). Furthermore, previous work demonstrated that Ala53, Cys177, and Cys179 are crucial for the E3 ligase function of DCAF16, which is consistent with our findings (Figure 4E).[27]

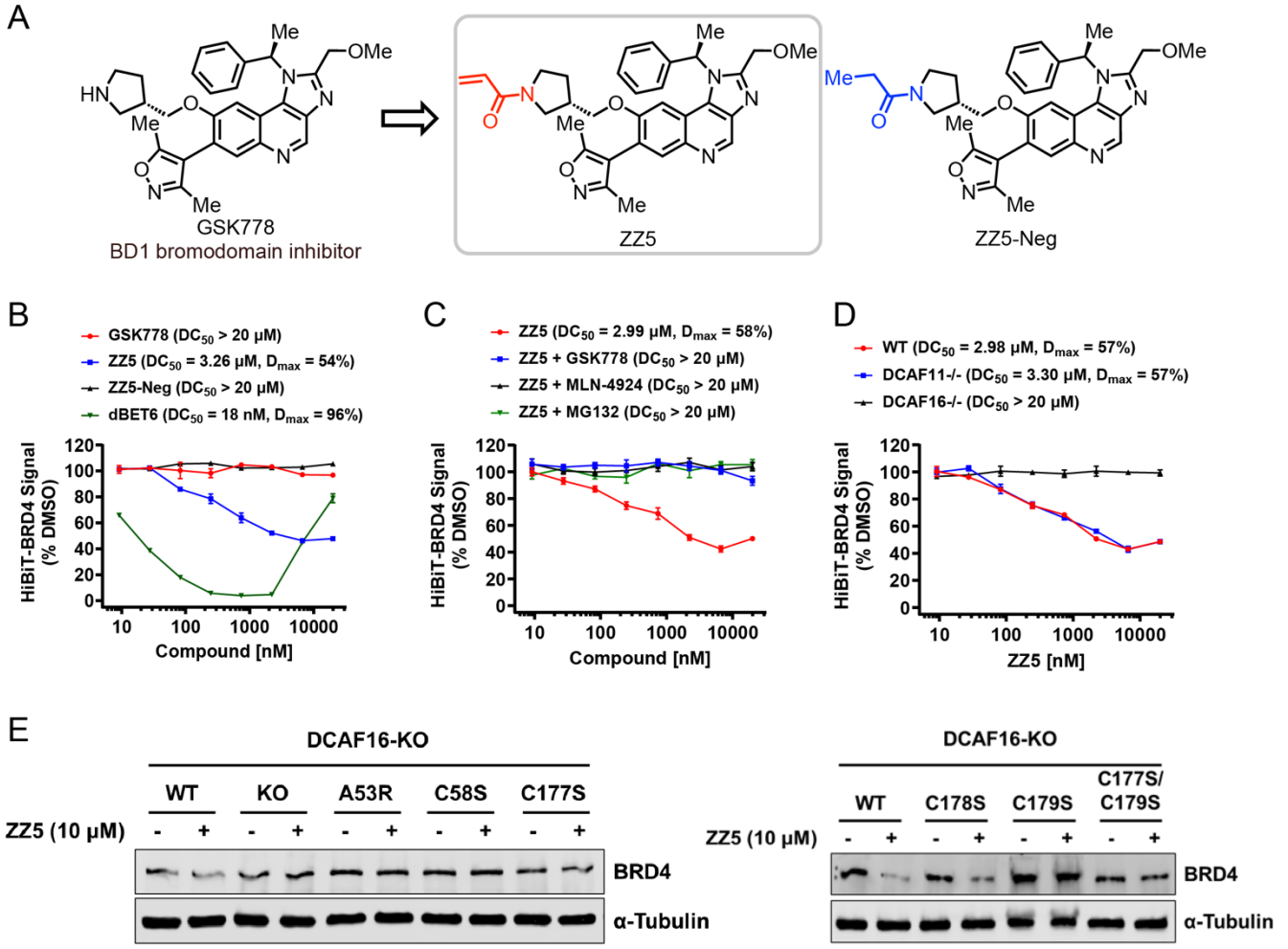


Figure 4. Chemocentric approach yields a molecular glue degrader for a pre-defined target BRD4. (A) Chemical structures of the parental BD1 bromodomain inhibitor (GSK778), its derivative (ZZ5) featuring a covalent warhead conferring degrader activity, and a negative control of ZZ5 (ZZ5-Neg) that negates activity. (B) HiBiT-BRD4 assay results for Jurkat cells treated with the indicated compounds for 6 h. (C) HiBiT-BRD4 assay results for Jurkat cells pre-treated with the indicated inhibitors for 1 h, followed by treatment with ZZ5 for 6 h. (D) HiBiT-BRD4 assay results for WT, DCAF11-KO, or DCAF16-KO Jurkat cells treated with ZZ5 for 6 h. (E) Western blots showing BRD4 degradation in DCAF16-WT, KO, and mutant K-562 cells treated with 10 μM ZZ5 for 6 h.

Designing molecular glue degraders has proven to be challenging and often relies on serendipity. Recent studies have shown that modifying surface-exposed moieties of known inhibitors can transform them into protein degraders by recruiting E3 ligase components.[25-27] Using this approach, we developed a potent CDK12/13 molecular glue degrader, ZZ3, from a CDK12/13 inhibitor by attaching hydrophobic gluing moieties to recruit DDB1. The resulting compounds exhibited comparable CDK12/13 degradation potencies a CRBN-based PROTAC derived from the same parental inhibitor, but with lower molecular weights. Through SAR study, we discovered of ZZ4, which maintains comparable potency. Following the same logic of inhibitors derivatization, we additionally demonstrated that employing a cysteine-reactive warhead as a gluing moiety can yield a BRD4 molecular glue degrader, ZZ5, by recruiting the DCAF16 E3 ligase.

The π-cation interaction between the hydrophobic gluing moiety of the CDK12/13 molecular glue and Arg928 of DDB1 is essential for forming the productive CDK12-DDB1 ternary complex. CDK12 has an innate, albeit low-affinity, protein-protein interaction with DDB1.[10, 18] Molecular glues leverage this interaction by first occupying the ATP pocket and subsequently promoting the π-cation interaction with DDB1 through a surface-exposed hydrophobic moiety. Similarly, the complementarity of the BRD4-DCAF16/11 protein-protein interface is a key factor in designing of DCAF-dependent BRD4 molecular glue degraders.

This work illustrates that minor modifications to parental inhibitors through the incorporation of gluing moieties can lead to protein degradation. Both hydrophobic and covalent chemical gluing moieties are capable of recruiting E3 ligase components, with the target-ligase pairing primed by pre-existing protein interface complementarity. Importantly, these prototype small molecules demonstrate drug-like properties with relatively low molecular weights, which is advantageous for drug development. We envision that this strategy may be applicable to the development of other first-in-class molecular glue degraders beyond CDK12/13 and BRD4.

ASSOCIATED CONTENT

Supporting Information

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

Full experimental details and characterization of new compounds (PDF)

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Notes

The authors declare the following competing financial interest(s): N.S.G. is a founder, science advisory board member (SAB) and equity holder in Syros, C4, Allorion, Lighthorse, Voronoi, Inception, Matchpoint, Shenandoah (board member), Larkspur (board member) and Soltego (board member). The Gray lab receives or has received research funding from Novartis, Takeda, Astellas, Taiho, Jansen, Kinogen, Arbella, Deerfield, Springworks, Interline and Sanofi. E.S.F. is a founder, scientific advisory board (SAB) member, and equity holder of Civetta Therapeutics, Proximity Therapeutics, and Neomorph, Inc. (also board of directors). He is an equity holder and SAB member for Avilar Therapeutics, Photys Therapeutics, and Ajax Therapeutics and an equity holder in Lighthorse Therapeutics. E.S.F. is a consultant to Novartis, EcoR1 capital, Odyssey and Deerfield. The Fischer lab receives or has received research funding from Deerfield, Novartis, Ajax, Interline, Bayer and Astellas. K.A.D. receives or has received consulting fees from Kronos Bio and Neomorph Inc. All other authors declare no competing interests.

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