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Chemically induced degradation of CDK9 by a proteolysis targeting chimera (PROTAC)

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Abstract

Cyclin-dependent kinase 9 (CDK9), a member of the cyclin-dependent protein kinase (CDK) family, is involved in transcriptional elongation of several target genes. CDK9 is ubiquitously expressed and has been shown to contribute to a variety of malignancies such as pancreatic, prostate and breast cancers. Here we report the development of a heterobifunctional small molecule proteolysis targeting chimera (PROTAC) capable of cereblon (CRBN) mediated proteasomal degradation of CDK9. In HCT116 cells, it selectively degrades CDK9 while sparing other CDK family members. This is the first example of a PROTAC that selectively degrades CDK9.

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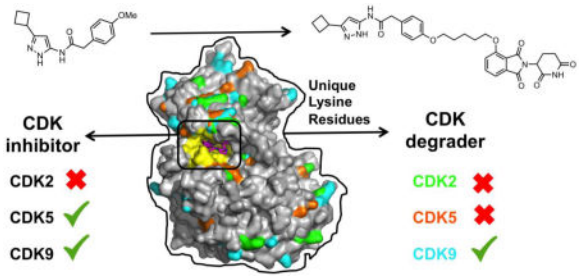

In this manuscript, for the first time, we report the synthesis of a PROTAC that selectively degrades cyclin dependent kinase 9 (CDK9).

The cyclin-dependent kinases (CDKs) are a family of more than 13 serine-threonine kinases. CDK1–4, CDK6 and CDK11 regulate cell cycle, while CDK7–9 are involved in transcription regulation.[1–4] Over the past two decades, a number of CDK inhibitors have entered clinical trials for the treatment of cancer.[5–11] Off-target dose limiting toxicities are a significant problem with the development of CDK inhibitors.[12–14] In 2015, FDA approved Palbociclib as the first selective CDK4/6 inhibitor for treatment of metastatic breast cancer.[15] As with other members of the family, CDK9 requires activation by the binding of a cyclin partner. It forms heterodimeric complexes with cyclin T1, T2a, T2b and K. CDK9 is ubiquitously expressed in all tissues and is highly expressed in terminally differentiated cells.[16] Preclinical studies indicate that selective CDK9 inhibitors may have therapeutic potential for the treatment of various cancers and other human diseases.[17, 18] CDK9 regulates the levels of among others the prosurvival protein Mcl-1. Inhibition of CDK9 leads to reduced phosphorylation of Ser2 on RPB1, which results in the reduction of Mcl-1 levels.[19–21]

Proteolysis targeting chimera (PROTAC) concept,[22] pioneered by the Crews laboratory has recently been used to target a BRD4.[23–28] This broad based interest is the direct result of the seminal work by the Bradner lab on the development of bromodomain and extra-terminal domain (BET) degrader.[23] PROTACs are heterofunctional bispecific molecules connected via a linker wherein one fragment interacts with the protein of interest and the other binds to a component of a ubiquitin ligase. The PROTAC forms a ternary complex by binding to both the target protein and either a component of E3 ubiquitin ligase or the E2 ligase itself. This results in the poly-ubiquitination of that target protein, which is then proteosomally degraded (Figure 1).[22–24, 26, 27, 29–37] FKBP12,[23] BET,[23, 26, 28] BCR-ABL,[34] and Sirt2[37] proteins have been successfully targeted for degradation using small molecule bifunctional degraders that bind cereblon/Cullin4A E3 ubiquitin ligase. Degradation of the target protein makes this a catalytic process. Furthermore, studies with BET degraders demonstrated potent inhibition of cancer cell growth and the induction of apoptosis when compared to the corresponding BET inhibitors.[24, 25, 27, 38]

Aminopyrazole analogs were synthesized and evaluated as CDK2 inhibitors.[39] A majority of these inhibitors are ATP competitive in nature due to a unique donor-acceptor-donor architecture of nitrogen atoms of the aminopyrazole core which mimics the ATP adenine core. Analyses of the X-ray crystal structure reveal that the three nitrogen atoms of the

aminopyrazole core interact with the hinge region residues of CDK. Substituents at the 3' position on the aminopyrazole core occupy the adjacent hydrophobic pocket. Substitutions on the exocyclic amine are solvent exposed (Figure 2).[39]

The kinase domain and specifically the ATP binding site of the CDKs are structurally similar, which makes it challenging to develop selective CDK inhibitors. On the other hand, the shape of the surface and the distribution of lysine residues on the surface among the CDKs are different (Figure S1). This provides a unique opportunity to develop a selective CDK degrader using the PROTAC strategy because an appropriately placed surface exposed lysine residue is required for ubiquitination and proteosomal degradation.[40] We hypothesized that a PROTAC utilizing a pan CDK inhibitor might lead to the development of a selective CDK degrader.

We designed a focused library of aminopyrazole analogs and screened for CDK activities to identify selective inhibitors (manuscript in preparation). Structure activity relationship (SAR) studies revealed that the para position on the phenyl ring that is surface exposed is amenable to substitution without loss of activity. To explore this position with a linker to conjugate thalidomide, we generated compounds **1** and **2** (Figure 3). HCT116 cells were treated with 10 μM of **1** and **2** for 6 hours and probed for CDK2, 5 and 9 levels and the phosphorylation state of their substrates RB, FAK and RPB1 respectively. We observed the inhibition of phosphorylation of FAK and RPB1 indicating selectivity for CDK5 and CDK9 over CDK2 (Figure S2). Based on this observation, we synthesized degrader **3** by conjugating **2** and thalidomide (**4)**, which was previously demonstrated as a bonafide ligand for cereblon/Cullin4A E3 ubiquitin ligase.[41]

The synthetic route to compound **1** and **2** are summarized in Scheme 1. Regioselective *t*-butoxycarbamate (Boc) protection of the ring nitrogen atom in the aminopyrazole **5** was accomplished following a reported procedure, to yield **6**.[39, 42] Coupling of **6** with commercially available 4-methoxyphenylacetic acid using propylphosphonic anhydride solution (T3P, 50% in DMF) and *N,N*-Diisopropylethylamine gave **7**. Removal of Boc protecting group under acidic condition resulted in **1**. Alkylation of hydroxyl group in **8** with 1,5-dibromopentane yielded **9**. Compound **10** was obtained by hydrolysis under basic conditions, which was then subjected to amide coupling with **6** using T3P to yield **11**. Removal of the Boc group under acid condition yielded **2**.

The PROTAC **3** was synthesized following the route described in Scheme 2. A Finkelstien reaction with compound **9** resulted in **12** and a base catalyzed hydrolysis yielded fragment **13**. Coupling of aminopyrazole **6** and **13** using T3P yielded **14**. Condensation of 3-hydroxyphthalic anhydride (**15**) with 3-aminopiperidine-2,6-dione hydrochloride afforded the intermediate **4**. Alkylation of the hydroxyl group on **4** with **14** an intermediate, which was subjected to Boc group deprotection under acidic condition to yield degrader **3**.

To determine if **3** is a CDK degrader we treated HCT116 cells with increasing concentrations of **3** for 6 hours and the lysates were subjected to western blot analyses with antibodies for a panel of six kinases (Figure 4A). Thalidomide analog (**4**) and the inhibitor (**2**) were included as controls in this assay. Remarkably, **3** degraded CDK9 in a dose

dependent manner. Inhibitor (**2)** and thalidomide analog (**4)** showed no effect on the levels of any of the kinases (Figure 4A). Interestingly, CDK2 and CDK5 levels and the levels of the other kinases IKKβ, Akt and FAK were unaltered suggesting selective CDK9 degradation. Given the high concentrations of **3** used in these studies, the lack of degradation of CDK2, CDK5, FAK, Akt and IKKβ could be attributed either the inability of form the ternary complex due to sterics or the absence of a proximal lysine residue that can be ubiquitinated.

RPB1 is a direct substrate of CDK9, therefore, we probed the membrane for the phosphorylation status of Ser2 on RPB1 using a phosphospecific antibody. Consistent with CDK9 inhibition and degradation, we observed inhibition of phosphorylation with **2** and **3** respectively (Figure 4B). Since, CDK9 activity regulates the levels of pro-survival protein Mcl-1,[43, 44] we investigated the effects of **2** and **3** on Mcl-1. As anticipated, we observed a dose-dependent decrease in Mcl-1 levels with **3** and reduction of Mcl-1 levels with **2** (Figure 4C). Quantification of the western blots showed that at 10 and 20 μM degrader **3** reduced the levels of CDK9 by ~56% and ~65%, respectively (Figure 4D). In order to determine if the degrader inhibits CDK5 we conducted an *in vitro* kinase assay and show that **3** indeed inhibits the kinase activity of CDK5 (Figure S3). It is important to note that although inhibitor **2** and degrader **3** have similar effects on the kinase activity of CDK9 (Figure 4B), the effect of the degrader **3** on the Mcl-1 levels is more pronounced than the inhibitor **2** (Figure 4C). This suggests the existence of a potential kinase-independent function of CDK9 associated with the regulation of Mcl-1.

In conclusion, we report the development of a PROTAC (**3**) for selective degradation of CDK9. **1** and **2** inhibits CDK5 and CDK9 whereas degrader **3** is selective for CDK9. This suggests that selective CDK degraders can be developed by exploiting differentially displayed surface lysine residues among the CDKs. Degrader **3** is a novel tool that can be used to dissect the role of CDK9 in various diseased states. CDK9 degradation by **3** resulted in reduced phosphorylation of Ser2 on RPB1, a CDK9 substrate and reduced the levels of Mcl-1 a prosurvival protein, regulated by CDK9 activity. Studies to investigate the anticancer effects through the induction of apoptosis by degrader **3** in combination with Bcl-xL inhibitors is currently underway and will be reported in due course.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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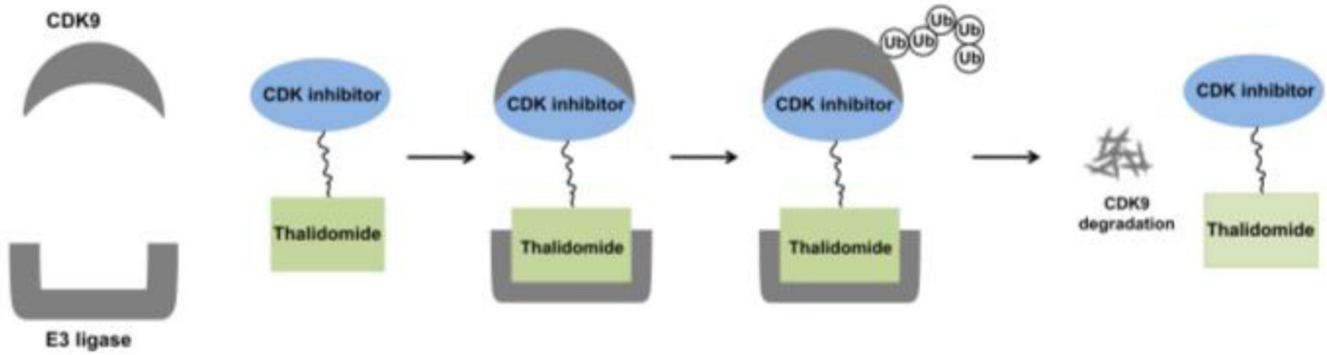


Figure 1.
PROTAC mechanism of action.

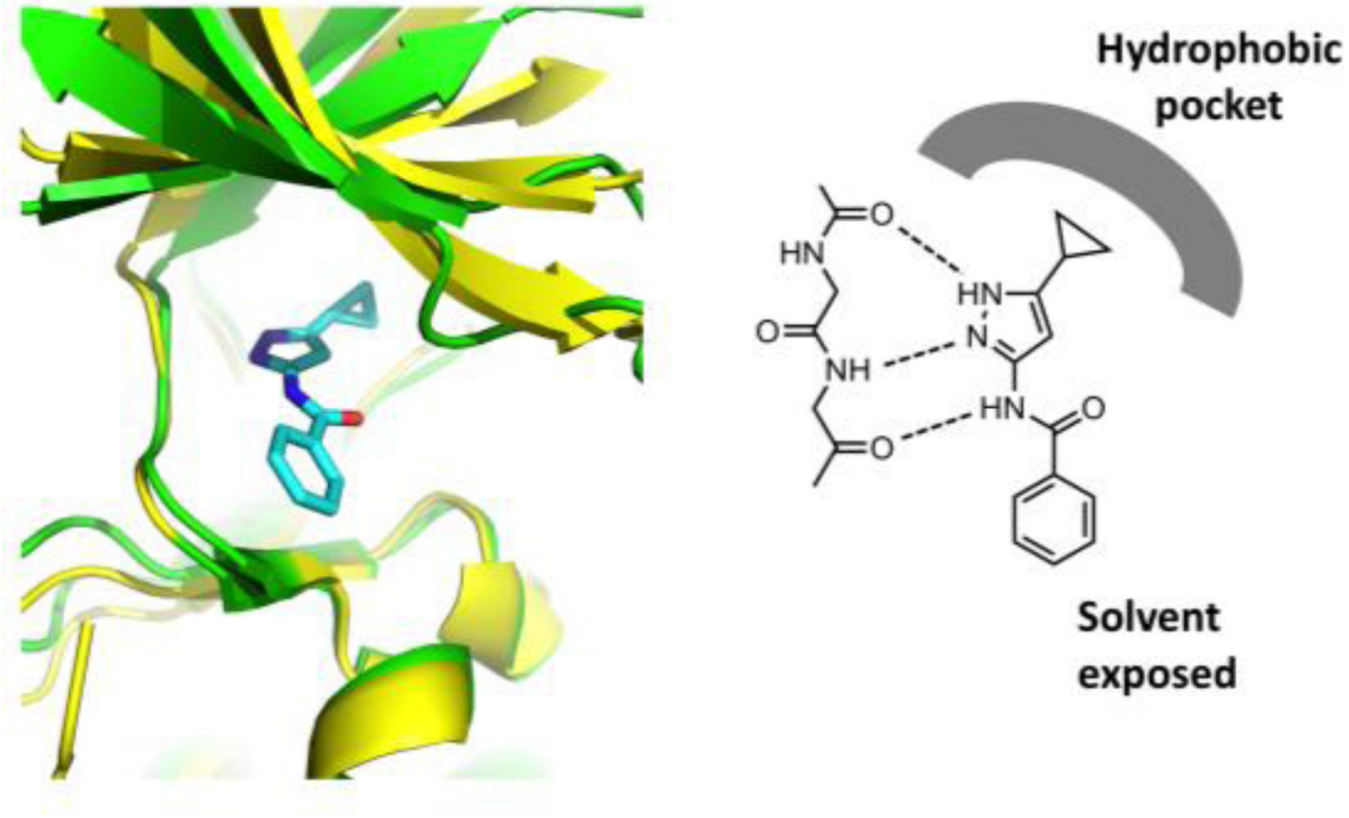


Figure 2.

Left: Overlay of CDK2 (PDB id 1VYZ, yellow) and CDK9 (PDB id 4BCG, green). Right: Hinge region residues of CDK2 interacting with the nitrogen atoms of aminopyrazole core. The potential hydrogen bonds between the CDK2 hinge region and the inhibitor are shown dashed lines.

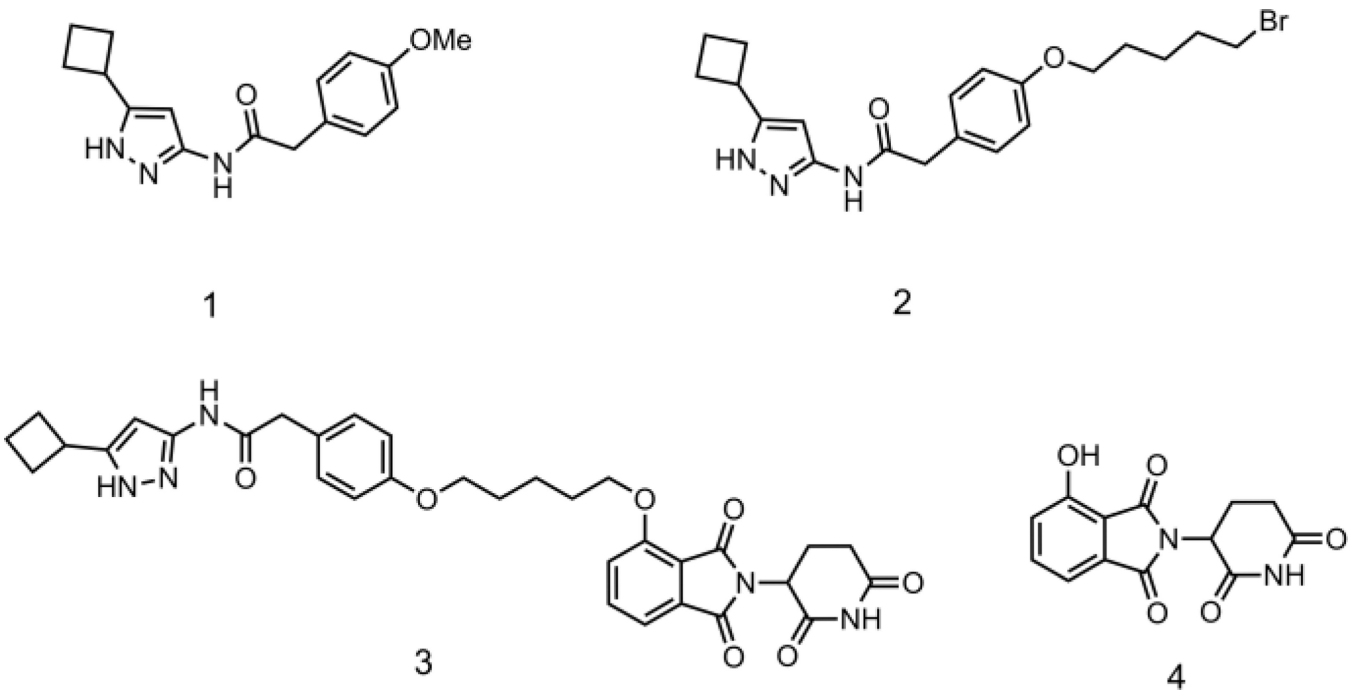


Figure 3.
Structures of aminopyrazole inhibitors, thalidomide and a PROTAC degrader.

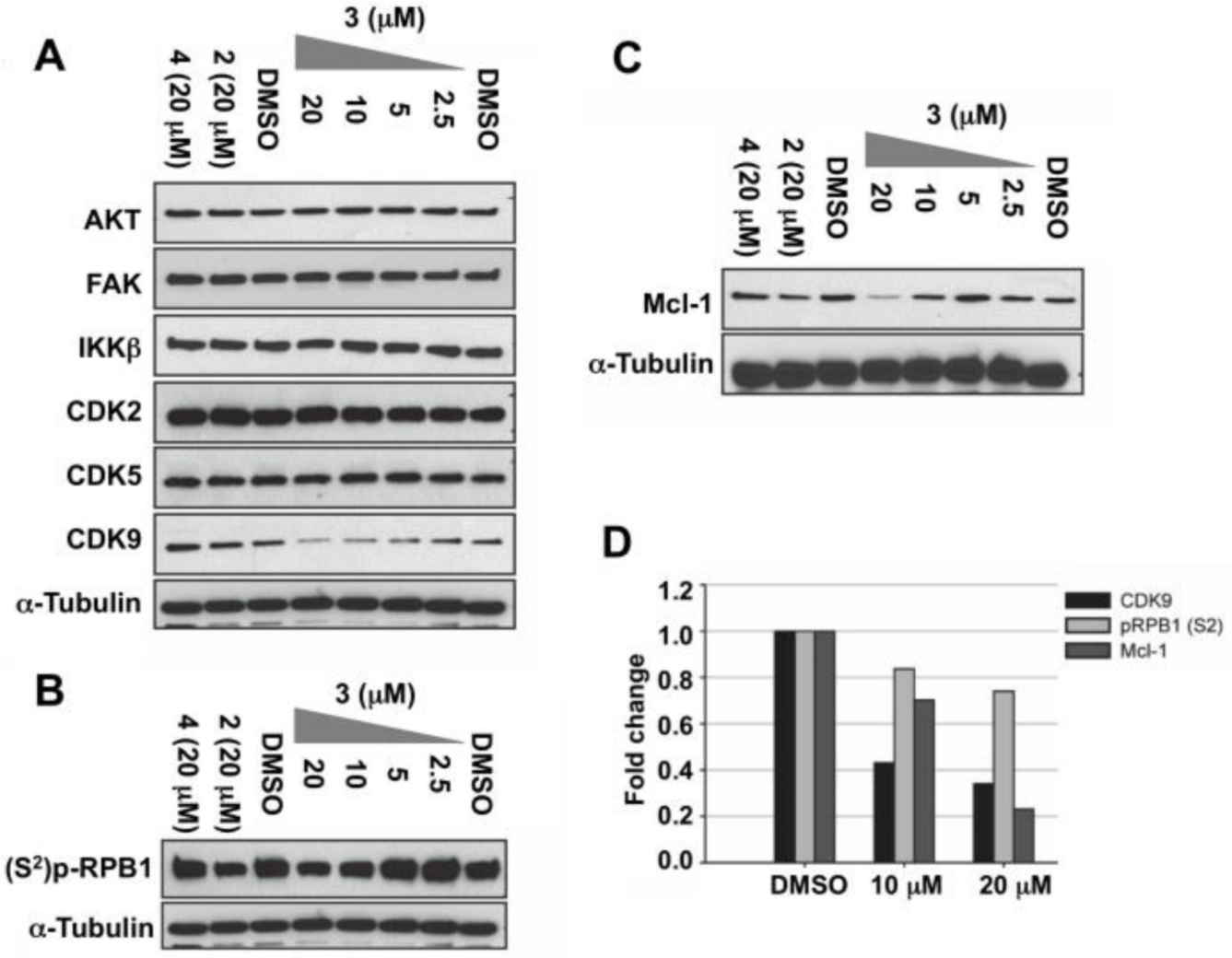
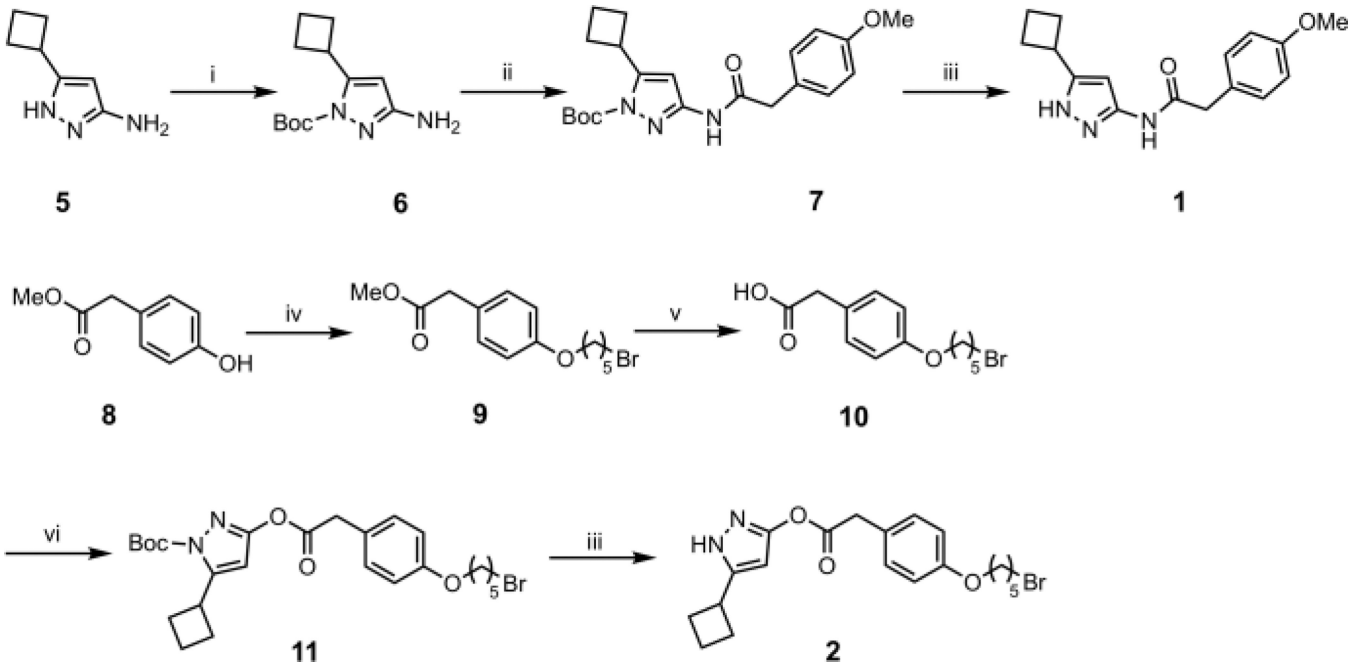


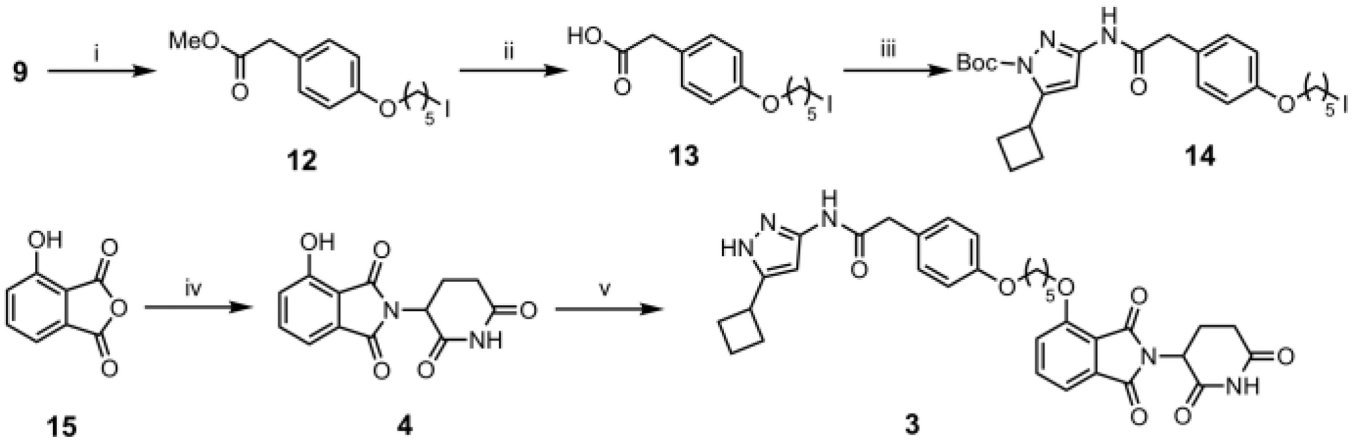
Figure 4.

Effect of the aminopyrazole inhibitor, thalidomide and the PROTAC on a panel of kinases. (A) Western blot analyses of a panel of kinases with lysates generated from HCT116 cells treated with **2, 3** and **4**. (B) The effect of **2, 3**, and **4** on phosphorylation status of RPB1. (C) The effect of **2, 3**, and **4** on Mcl-1 levels. (D) Quantification of CDK9, *p*-RPB1 and Mcl-1 levels at 10 and 20 μM.



Scheme 1.

(i). $(Boc)_2O$, KOH, DCM: water, 3h. (ii). 4-methoxyphenylacetic acid, 50% T3P in DMF, DIEA, 50 °C, 3h. (iii). TFA, DCM, 0 °C, 3h. (iv). 1,5-dibromopentane, K_2CO_3, acetone reflux, 72h. (v). LiOH, Ethanol: water, 16h. (vi). **6**, 50% T3P in DMF, DIEA, 50 °C, 3h.



Scheme 2.

(i). NaI, acetone, reflux, 16h. (ii). LiOH, Ethanol: water, 16h. (iii). **6**, 50% T3P in DMF, DIEA, 50 °C, 3h. (iv). 3-aminopiperidine-2,6-dione hydrochloride, KOAc, Acetic acid, reflux, 24h. (v). (a) $NaHCO_3$, DMF, 70 °C, 6h; (b). TFA, DCM, 0 °C, 3h.