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Selective CDK6 degradation mediated by cereblon, VHL, and novel IAP-recruiting PROTACs

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Abstract

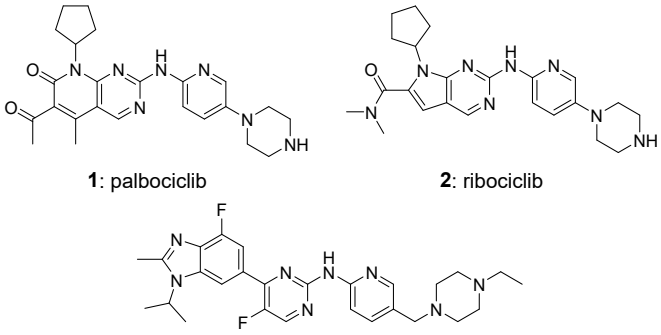
Inhibitors of CDK4 and CDK6 have emerged as important FDA-approved treatment options for breast cancer patients. The properties and pharmacology of CDK4/6 inhibitor medicines have been extensively profiled, and investigations into the degradation of these targets via a PROTAC strategy have also been reported. PROTACs are a novel class of small-molecules that offer the potential for differentiated pharmacology compared to traditional inhibitors by redirecting the cellular ubiquitin-proteasome system to degrade target proteins of interest. We report here the preparation of palbociclib-based PROTACs that incorporate binders for three different E3 ligases, including a novel IAP-binder, which effectively degrade CDK4 and CDK6 in cells. In addition, we show that the palbociclib-based PROTACs in this study that recruit different E3 ligases all exhibit preferential CDK6 vs. CDK4 degradation selectivity despite employing a selection of linkers between the target binder and the E3 ligase binder.

Keywords

CDK4; CDK6; PROTAC; palbociclib

Cyclin-dependent kinase (CDK) 4 and CDK6 are cell-cycle regulatory kinases that can become dysregulated in cancer.[1-3] Along with cyclin D, they play key roles in regulating the transition point between the G1- and S-phase during mitosis. In isolation, CDK4 and CDK6 lack intrinsic catalytic kinase activity, but their kinase activity becomes functional when these proteins bind to members of the cyclin D family. When activated, CDK4-cyclin D and CDK6-cyclin D complexes function by initiating the phosphorylation of retinoblastoma protein (Rb). Rb is a tumor suppressor protein that normally exerts a repressive effect on cell cycling until the cell is ready to enter mitosis.[4, 5] However, upon phosphorylation, Rb no longer suppresses cell cycle progression, and inappropriate Rb phosphorylation by CDK4 and CDK6 complexes can be an important oncogenic driver.[6] Inhibition of CDK4 and CDK6 has proven to be a clinically validated strategy for the treatment of metastatic and advanced ER+ HER- breast cancer, and palbociclib, ribociclib, and abemaciclib are FDA-approved CDK4/6 inhibitor medicines currently available to patients (**Figure 1**).

Figure 1: FDA-approved CDK4/6 inhibitors.



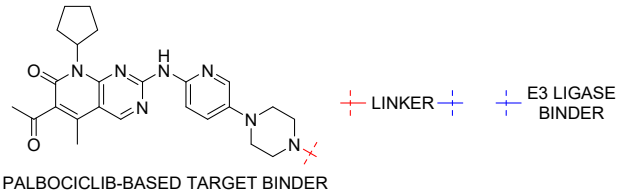
In addition to the described kinase-dependent functions of both CDK4 and CDK6, important kinase-independent biology associated with CDK6 has been reported, including correlation of CDK6 levels with tumor blood vessel density and with *Vegf-A* mRNA and protein levels in cancer cell lines.[7] Since inhibitors would not be expected to have any effect on these or other kinase-independent scaffolding functions of CDK6, we were interested in exploring the degradation of this protein through the use of a PROTAC (Proteolysis Targeting Chimera).[8]

PROTACs have emerged as an important new small-molecule modality to study the biology of medicinally important targets, and as potential medicines in their own right.[9-13] They function by redirecting the cellular ubiquitin-proteasome system (UPS) to tag specifically selected proteins for proteasomal degradation via E3 ligase complex-mediated ubiquitin transfer, thereby effecting a chemically-induced knock-down of the target protein. Cereblon-recruiting PROTACs based on the dual CDK4/6 inhibitor palbociclib have been reported,[14-17] and intriguingly were demonstrated to selectively degrade CDK6 over CDK4, despite palbociclib itself having approximately equal inhibitory potency against both CDK4- and CDK6-cyclin D complexes.[18]

Previous studies have demonstrated that PROTAC composition plays a critical role in determining degradation selectivity in the case of non-specific target protein binders.[19, 20] Selectivity differences are presumably influenced by the stability and accessible conformations of the requisite ternary complex between the bound protein, the PROTAC molecule, and the recruited E3 ligase complex,[21-23] and differential ternary complex formation has been invoked to explain CDK6 vs. CDK4 degradation selectivity.[14] With selective CDK6 degradation having been previously reported for cereblon-recruiting palbociclib-based PROTACs, we sought to investigate what effect the choice of E3 ligase would have on the degradation of these targets by von Hippel-Lindau (VHL)- and Inhibitor of Apoptosis (IAP)-recruiting palbociclib-based PROTACs.

To probe the PROTAC-mediated CDK4 and CDK6 degradation effects of different E3 ligases, we prepared a series of compounds (**Figure 2**) incorporating various linkers and known binders to VHL (PROTACs **4-6**), and cereblon (PROTACs **10**,[15] **11**, and **12**[15]), as well as a novel IAP binder (PROTACs **7-9**). As has been previously described by others, we viewed the palbociclib piperazine moiety as a suitable linker design vector for PROTAC construction.

Figure 2: PROTACs prepared to test CDK4 and CDK6 degradation. Each individual PROTAC consists of the palbociclib-based target binder combined with a linker and an E3 ligase binder according to the connectivity indicated. PROTACs **4-6** contain the VHL binder shown, PROTACs **7-9** contain the novel IAP binder shown, and PROTACs **10-12** contain the cereblon binder shown.



PALBOCICLIB-BASED TARGET BINDER

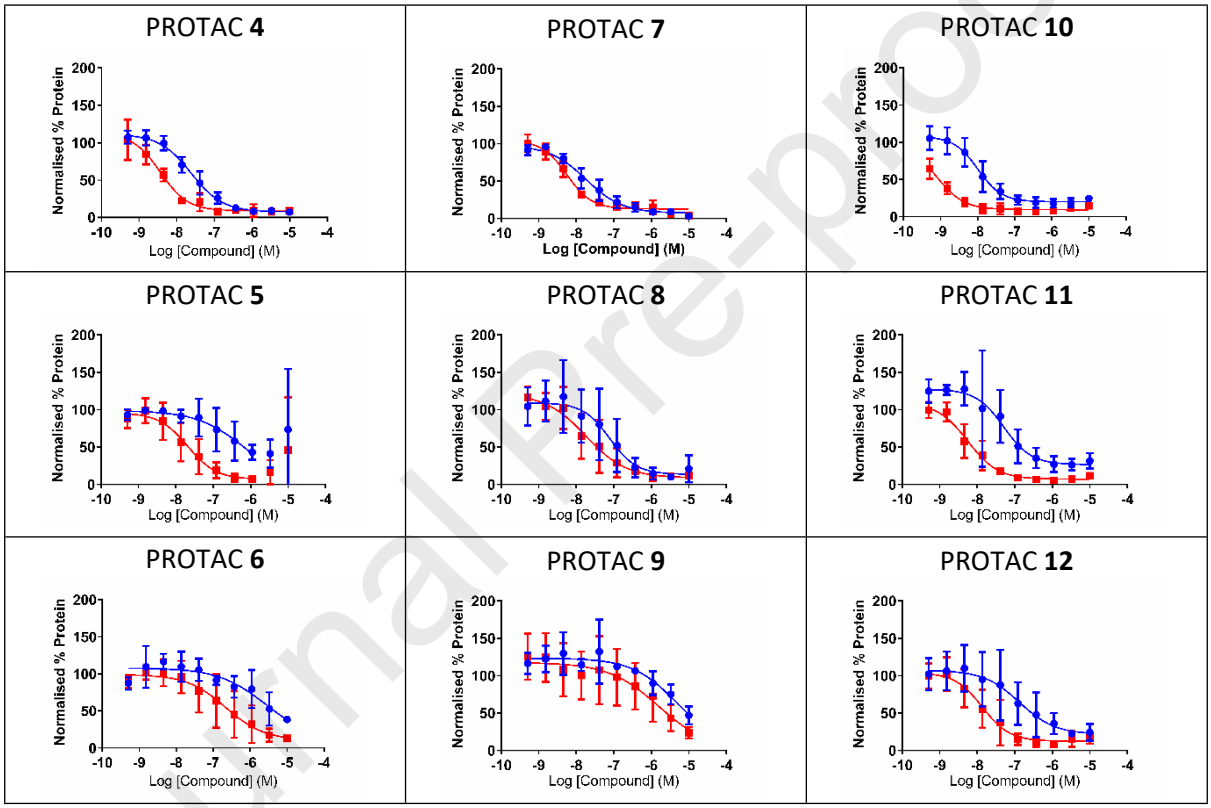
E3 LIGASE BINDER	**VHL** (PROTACs **4-6**)	**IAP** (PROTACs **7-9**)	**Cereblon** (PROTACs **10-12**)
LINKER	PROTAC **4**	PROTAC **7**	PROTAC **10**[15]
LINKER	PROTAC **5**	PROTAC **8**	PROTAC **11**
LINKER	PROTAC **6**	PROTAC **9**	PROTAC **12**[15]

Following preparation of PROTACs **4-12**, we evaluated the degradation of CDK4 and CDK6 in Jurkat cells after 24 h compound treatment in a dose-response manner using capillary electrophoresis combined with antibody labelling to measure protein levels (**Figure 3**). As expected, palbociclib **1** did not cause any significant degradation of CDK4 or CDK6 (**Figure SI-1**). However, we observed potent degradation of CDK4 for seven of the nine PROTACs studied, and of CDK6 for eight of the nine PROTACs studied, encompassing VHL, IAP, and cereblon E3 ligase binders. The exceptions to the potent target degradation were PROTACs **6** and **9**, which were found to weakly degrade CDK4 with pDC_{50} values between 5-6 (**Table 1**). Additionally, PROTAC **9** was also found to be a weak degrader of CDK6, with a pDC_{50} of 5.8. The relatively weak target degradation potency of PROTACs **6** and **9** may be related to the linker connecting the palbociclib-based target binder and the E3 ligase binders, since the analogous VHL-recruiting PROTACs **4** and **5**, as well as the analogous IAP-recruiting PROTACs **7** and **8** were each found to be potent degraders of both CDK4 and CDK6. With the exception of PROTACs **6** and **9**, there were no meaningful differences found between the ability of

the PROTACs incorporating VHL, IAP, or cereblon ligase binders to cause the degradation of CDK4 and CDK6 as measured by their respective pDC_{50} values.

The most potent CDK6 degrader that we observed was the previously reported short linker cereblon-recruiting PROTAC **10**,[15] with a CDK6 pDC_{50} of 9.1. This compound was also found to potently cause the degradation of CDK4, with a pDC_{50} of 8.0. It is noteworthy that this degradation selectivity preference for CDK6 over CDK4 was maintained for each compound studied ranging from a Δ pDC_{50} of 0.5 to 1.5 (pDC_{50} CDK6 – pDC_{50} CDK4), and that even the weak CDK4/6 degrader PROTAC **9** was found to selectively degrade CDK6 over CDK4 with a Δ pDC_{50} of 0.5 (**Table 1**).

Figure 3. Dose-response protein level curves for CDK4 (blue symbols) and CDK6 (red symbols) in Jurkat cells measured after 24 h treatment with PROTACs **4-12**. Individual data points are the average of three experiments. Error bars indicate standard deviation.

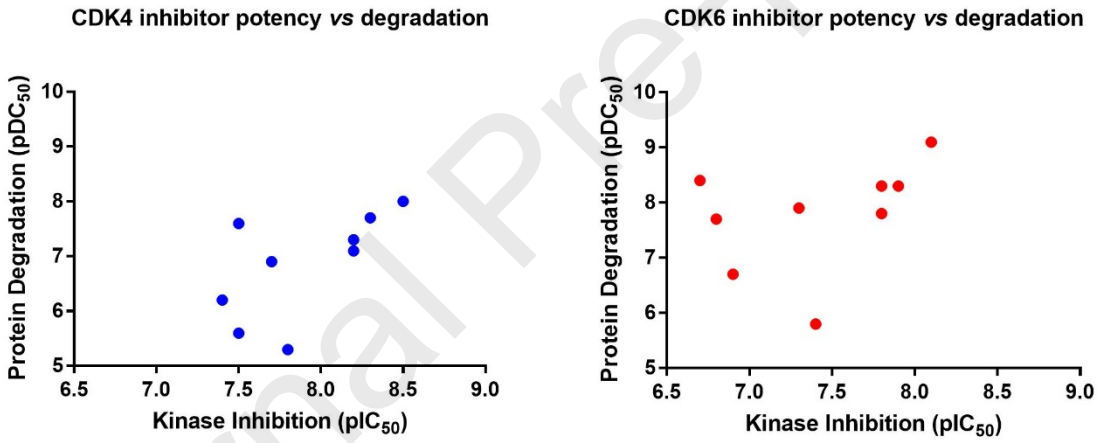


To explore whether the differences in the observed CDK4 and CDK6 degradation potency for these compounds might be related to differences in CDK4 and CDK6 inhibitory potencies, we determined the inhibitory potencies of PROTACs **4-12** as well as palbociclib **1** against CDK4-cyclin D1 and CDK6-cyclin D3 (**Table 1**). Although the measured inhibitory potency of palbociclib **1** against CDK4-cyclin D1 (pIC_{50} 7.5) and CDK6-cyclin D3 (pIC_{50} 7.0) in our assay was reduced as compared to the reported inhibitory potencies for this compound (CDK4-cyclin D1 pIC_{50} 8.0, CDK6-cyclin D2 pIC_{50} 7.8),[18] we observed sub-micromolar inhibition of CDK4-cyclin D1 and CDK6-cyclin D3 for all compounds tested. However, there was little correlation between CDK4/6-cyclin D inhibitory potency and CDK4/6 degradation potency (**Figure 4**), and it is notable that the weak CDK4/6 degrader PROTAC **9** was still a potent inhibitor of both CDK4-cyclin D1 and CDK6-cyclin D3, and that the 200 nM IC_{50} CDK6-cyclin D3 inhibitor PROTAC **4** was surprisingly found to be a 4 nM DC_{50} CDK6 degrader.

Table 1. CDK4/6 inhibition[24] and degradation potency for palbociclib **1** and PROTACs **4-12**. Experimentally determined data are reported as the average of three experiments.

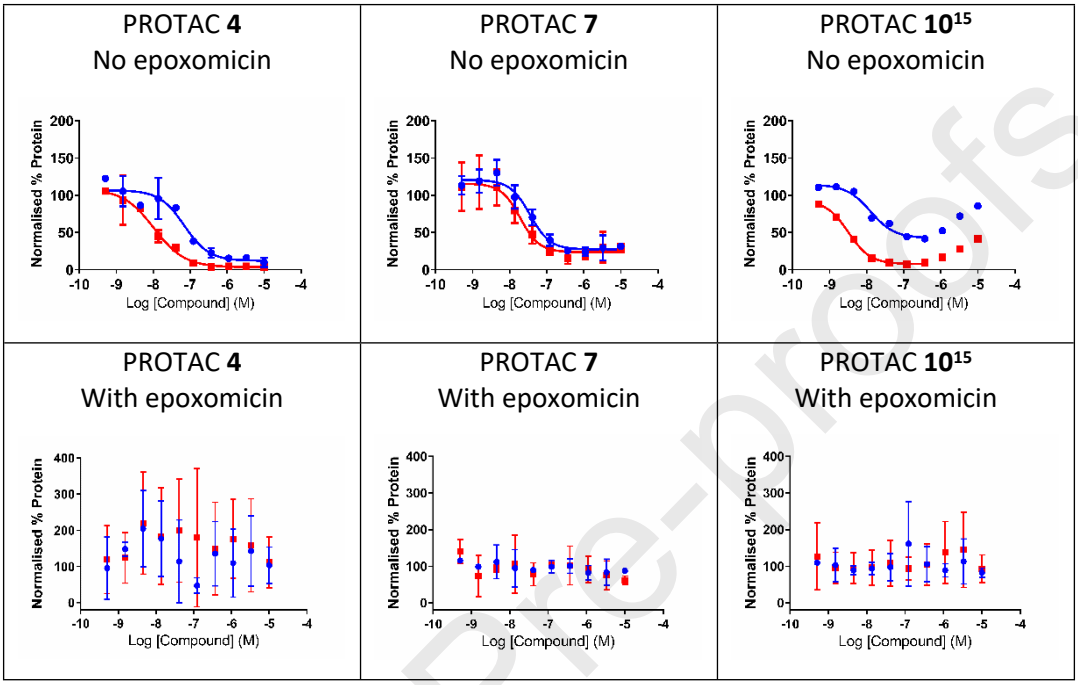
Compound:	1	4	5	6	7	8	9	10[15]	11	12[15]
CDK4-cyclin D1 pIC_{50}	7.5	7.5	7.4	7.5	8.3	8.2	7.8	8.5	8.2	7.7
CDK6-cyclin D3 pIC_{50}	7.0	6.7	6.8	6.9	7.9	7.8	7.4	8.1	7.8	7.3
CDK4 pDC_{50}	<4.0	7.6	6.2	5.6	7.7	7.1	5.3	8.0	7.3	6.9
CDK6 pDC_{50}	<4.0	8.4	7.7	6.7	8.3	7.8	5.8	9.1	8.3	7.9
CDK6-CDK4 Δ pDC_{50}	-	0.8	1.5	1.1	0.6	0.7	0.5	1.1	1.0	1.0

Figure 4. Comparison of CDK4-cyclin D1 and CDK6-cyclin D3 inhibitor potency vs. CDK4 and CDK6 degradation potency.



To determine whether the observed CDK4 and CDK6 degradation was UPS-dependent, we pre-treated Jurkat cells with 1 μM of the known proteasome inhibitor epoxomicin, followed individually by PROTACs **4**, **7**, and **10**,[15] representing compounds that recruit VHL, IAP, and cereblon, respectively. Measuring CDK4 and CDK6 protein levels after 5 h treatment with each PROTAC following epoxomicin treatment clearly demonstrated an inhibition of protein loss (**Figure 5**), indicating that CDK4/6 protein level decreases caused by PROTAC treatment in the absence of epoxomicin was due to proteasomal degradation of these targets. Interestingly, we also observed a putative Hook effect after 5 h treatment with PROTAC **10**[15] in the absence of epoxomicin, as well as after 24 h treatment with PROTAC **5** (**Figure 3**). The appearance of a Hook effect is indicative of competing binary and ternary complex formation,[25] and provides additional evidence for a PROTAC-mediated ubiquitin transfer and degradation mechanism.

Figure 5. Dose-response protein level curves for CDK4 (blue symbols) and CDK6 (red symbols) in Jurkat cells measured after 5 h treatment without and with epoxomicin and with PROTACs **4**, **7**, and **10**.[15] No curves could be fitted to the data generated from treatment with epoxomicin and the PROTACs. Individual data points are the average of three experiments, except for the data points for PROTAC **10** with no epoxomicin treatment, which are the average of two experiments. Error bars indicate standard deviation, where present.



In contrast to a recent report indicating that neither CDK4 nor CDK6 was degraded with VHL or IAP PROTACs,[17] we observed potent degradation of both CDK4 and CDK6 with VHL- and with novel IAP-recruiting palbociclib-based PROTACs incorporating multiple linkers between the target binding and the E3 ligase binding moieties. Surprisingly, in addition to confirming the previously reported CDK6 vs. CDK4 degradation selectivity with cereblon-recruiting palbociclib-based PROTACs, we observed that the preference for CDK6 degradation over CDK4 degradation was maintained for VHL- and IAP-recruiting palbociclib-based PROTACs with all linkers studied. This preference for selective CDK6 vs. CDK4 degradation may be due to differential ternary complex effects related to the choice of palbociclib as the target binding moiety, as recent reports have shown that cereblon-recruiting ribociclib-based PROTACs induced preferential degradation of CDK4 over CDK6.[14, 17] Additional investigations studying differences in ternary complex formation and stability between palbociclib- and ribociclib-based PROTACs may be required to more fully understand the nature of degradation selectivity differences between CDK4 and CDK6 induced by these compounds.

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Supplementary Material

Full synthetic routes, experimental procedures, and **Figure SI-1** are available on-line in the Supplementary Information.

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Declaration of interests

☒ 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.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: