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PROTACs Suppression of CDK4/6, Crucial Kinases for Cell Cycle Regulation in Cancer

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PROTACs based on two selective, FDA approved, CDK4/6 inhibitors were formed. One of them, based on palbociclib, potently initiates degradation of these CDK proteins, and suppresses phosphorylation of retinoblastoma protein (Rb) leading to cell cycle arrest. These PROTACs are active at nanomolar concentrations, and appear to be the first for CDK4/6.

Cyclin dependent kinases (CDKs) are critical for cell cycle regulation. Cell cycle arrest in cancer could suppress tumor growth and metastasis, so inhibition of the CDKs is an active research area.[1,2] The first inhibitors had little or no selectivity between the various members of the series, and they were relatively unsuccessful in pharmaceutical development. Emergence of the first *selective* CDK4/6 inhibitors, however, changed the research landscape completely.[3-5] In 2015, palbociclib (IBRANCE®) received *accelerated* approval from the US Federal Drug Administration for use in a combination therapy for estrogen receptor-positive, HER2-negative advanced breast cancer; such approval is only granted when substances show exceptional efficacy in treatment of diseases for which there is an urgent unmet need. A second selective CDK inhibitor, ribociclib (KISQALI®), was approved in 2017 for treatment of the same cancer sub-type. As of today, palbociclib and ribociclib feature in at least 20 ongoing clinical trials for treatment of cancers of breast metastases, non-small cell lung, prostate, pancreas and brain (glioblastoma), so there is justifiable optimism surrounding the likely long-term clinical impact of these drugs.

Both palbociclib and ribociclib selectively inhibit CDK 4 and 6 over others. CDK4/6 are important in the G1-S phase of cell cycling, wherein they trigger a sequence of events leading to phosphorylation of retinoblastioma (Rb) protein. Failure to phosphorylate Rb leads to arrested cell development in ways that have been proven to be valuable in chemotherapy.[6]

PROteolysis TArgeting Chimeras (PROTACs) are hybrids of two molecular fragments: one to bind a protein targeted for ubiquitination, and another to dock with an E3 ubiquitin ligase.[7-9] These chimeras can force the targeted protein and E3 ligase to become proximal, this can initiate ubiquitination then proteolysis of the target in the proteasome. A compelling feature of PROTACs is their potential to act *catalytically* in the cell, hence they can be even more effective than suicide inhibitors that permanently inactivate enzymes with 1:1 inhibitor:protein stoichiometries.[10]

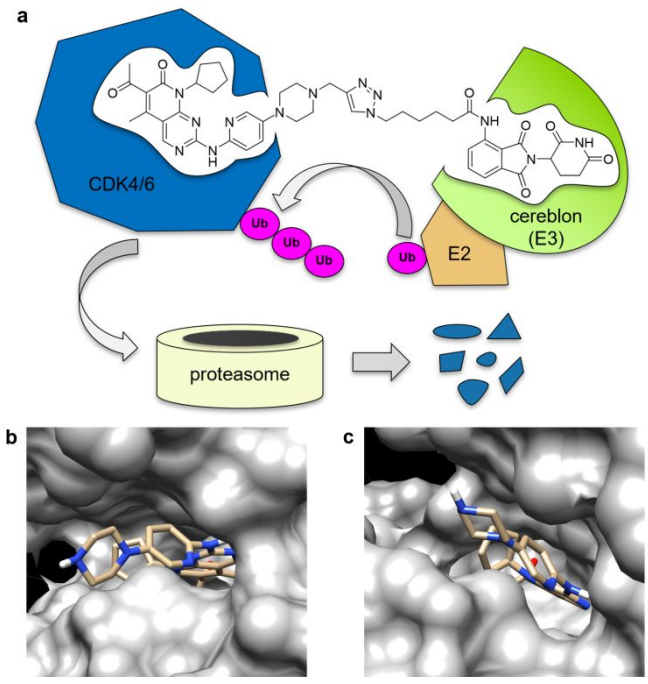

Fig. 1 (a) Central hypothesis of this paper is depletion of CDK4/6 via PROTACs. X-ray structures of CDK6 co-crystallized with palbociclib (b, PDB: 5L2I) and ribociclib (c, PDB: 5L2T).

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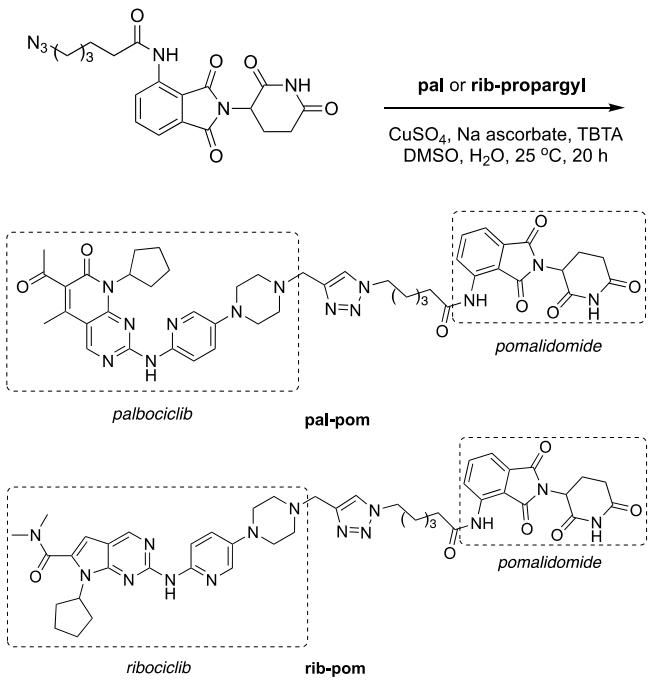
Approaches involving PROTACs are mechanistically distinct from others involving inhibition of kinases, because they instead involve its destruction. Recently, there have been reports of PROTACs applied to CDK9,[11] and some degraders of CDK8.[12] These reports prompted us to reveal our studies on PROTACs based on palbociclib and ribociclib (Fig. 1a). Fig. 1b and 1c show crystallographic data for palbociclib and ribociclib bound to CDK6. Both kinase inhibitors project a free piperazine-*N* into solvent, leading us to hypothesize that site could be conjugated to an E3 ligase ligand. Consequently, we set out to prepare conjugates of pomalidomide (**pom**, a E3 ligase ligand) to palbociclib and to ribociclib, and assay them for accelerated homeostasis of CDK4/6 and suppression of Rb-phosphorylation.

In actuality, the PROTACs for this study were prepared by cycloadding a known azide[13] derived from pomalidomide to *N*-propargyl derivatives of palbociclib or ribociclib as shown in Scheme 1.



Scheme 1 Syntheses of PROTACs for this study, **pal-pom** and **rib-pom**.

PROTACs of palbociclib and ribociclib were examined in MDA-MB-231, a triple negative (estrogen receptor, progesterone receptor and HER2 negative) breast cancer cell line. Fig. 2a and 2b show **pal-pom** dose-dependently depleted CDK4 and CDK6 when incubated with MDA-MB-231 cells for 18 h, but the extent of depletion was not linear for **rib-pom**. Also, both **pal-pom** and **rib-pom** degraded CDK4 more efficiently than CDK6, with a DC_{50} (the concentration for 50% protein degradation) of ~15 and ~100 nM to CDK4, respectively (Fig. 2c, 2d). Observant readers may notice in Fig. 2a and 2b that treatment of the cells with **pal** or **rib** consistently gives an increased level of CDK4/6. The reasons for this reproducible finding are unclear, but it seems the cells somehow compensate for the inhibition. PROTACs are sensitive to linker length; we investigated modification of **pal-pom** and **rib-pom** with a slightly shorter linker, but this had no significant effect (Fig. S1). Since **pal-pom** induced degradation was more efficient than **rib-pom**, the rest of this study focused on only the palbociclib derivative with the linker shown in Scheme 1.

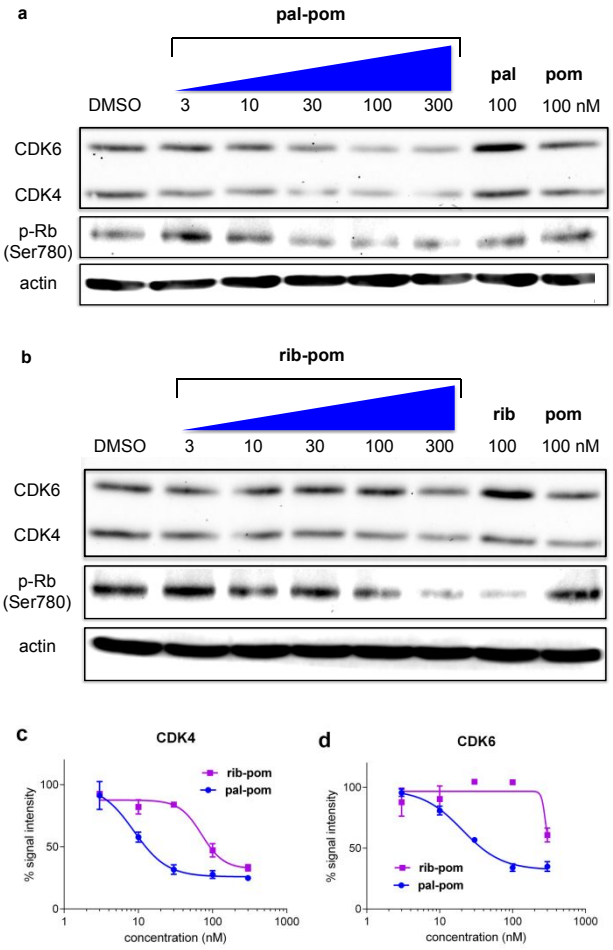


Fig. 2 (a, b) Degradation of CDK4/6 on MDA-MB-231 cell line by **pal-pom** and **rib-pom**, respectively. (c, d) Quantified data for both PROTACs (normalized to DMSO control set as 100% protein concentration).

Phosphorylation of retinoblastoma protein (p-Rb) is regulated by CDK4 and CDK6. Consequently, depletion of these cyclin dependent kinases should reduce the level of the p-Rb. Blotting experiments in Fig. 2a and 2b show that the PROTACs did indeed reduce the phosphorylation of Rb in a dose dependent way.

A time course study demonstrated that treatment of the same triple negative breast cell line with 100 nM **pal-pom** gave optimal CDK4 degradation after 4 h, whereas maximal decomposition of CDK6 took 6 h or more (Fig. 3). Similar experiments but with a higher PROTACs concentration (300 nM **pal-pom**) showed both CDK4 and CDK6 were degraded from the cell slightly faster (Fig. S2).

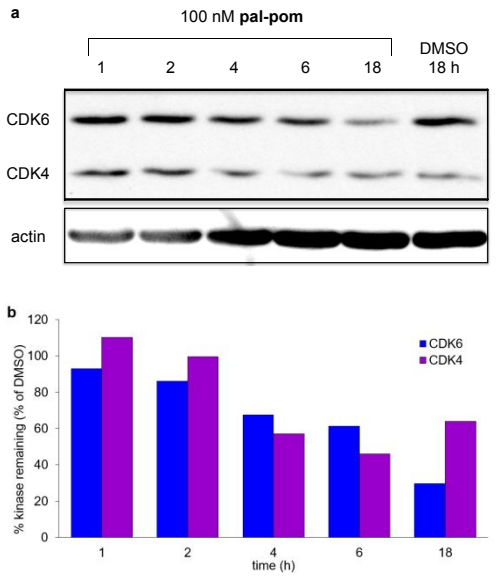


Fig. 3 (a) Kinetics for depletion of CDK4 and CDK6 in response to 100 nM **pal-pom**. (b) Quantified data for (a), normalized to DMSO control set as 100%.

Several "rescue" experiments were performed to confirm the mechanism of the observed CDK4/6 degradation by **pal-pom**, (Fig. 4a). The first of these used palbociclib (10 μM) or pomalidomide (10 μM) to compete with **pal-pom** for the binding to CDK4/6 or to cereblon, respectively. Under these conditions, the kinase and ligase active sites should be blocked by the excess monovalent ligands, rendering the PROTACs impotent. Again, we observed that pretreatment with **pal** increased the levels of CDK4/6 (see above). Neddylation inhibitor MLN4924 (1 μM) was added in another experiment because it inhibits NEDD8-activating enzyme (NAE) and therefore prevents activation of cullin-RING ligases, which are critical for proteasome-mediated protein degradation.[14] Incidentally, pretreatment with MLN4924 caused an increased level of CDK4, similar to that caused by pretreatment with **pal**. The final experiment featured 20 μM of the proteasome inhibitor MG-132.[15] In the event, most CDK4/6 remained after 18 h PROTACs treatment as compared to a sample that was pretreated with 0.1% DMSO. This outcome suggests mechanism of PROTACs **pal-pom** involves binding to CDK4/6 and cereblon, and proteasome-mediated degradation.

Fig. 4c shows data from "washout" experiments to see if CDK4/6 degradation is reversible wherein cells depleted in CDK4/6 by treatment with **pal-pom** were given 24 h in new media to facilitate efflux of the PROTACs. Concentrations of CDK4/6 were restored to at least their original levels.

Catalysis is the compelling reason to study PROTACs, but proving this occurs is difficult. One indicator is if the PROTACs DC_{50} in cell viability experiments is *lower* than the dissociation constant (K_d) of ligand that binds the parent protein. Consequently, K_d values were measured for **pal-pom** bound to CDKs 4 and 6 via KINOME*scan*™. KINOME*scan*™ is performed by combining DNA-tagged kinase, immobilized ligand, and test compounds; it is a competition binding assay featuring an immobilized, active-site directed ligand in which abilities of test samples to compete with immobilized ligand are measured via quantitative PCR of the DNA-tagged kinase (Table 1, Fig. S3). DC_{50} values for CDK4/6 suppression (deduced from Fig. 2) are indeed lower than the K_d values for kinase-bound **pal-pom** indicating the degradation is catalytic. Throughout, the K_d values for the inhibitor are less than those for the corresponding PROTACs, as anticipated because the PROTACs are significantly larger.

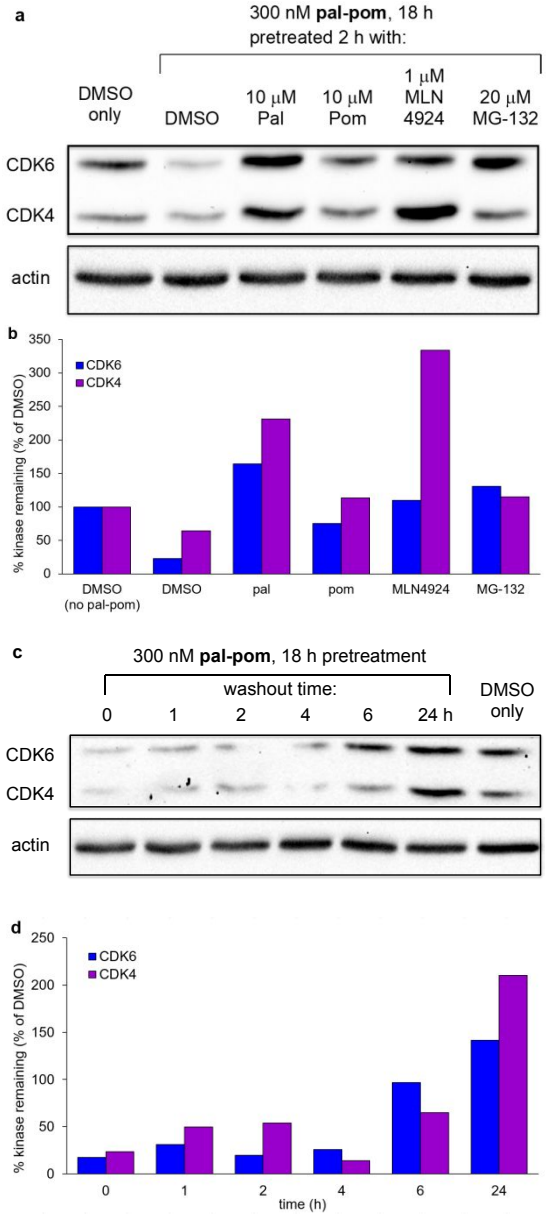


Fig. 4 (a) Pre-treatment with 10 μM palbociclib or pomalidomide, 1 μM neddylation inhibitor MLN4924, 20 μM proteasome inhibitor MG-132 insulated the cells from CDK4/6 degradation by the PROTACs. (b) Quantified data for (a), normalized to DMSO control set as 100%. (c) Washout of the PROTACs from the cells facilitates recovery of CDK4/6 levels after 24 h. (d) Quantified data for (c), normalized to DMSO control set as 100%.

Table 1. Comparison of kinase affinity (K_d) and DC_{50} for CDK4/6 depletion for the featured PROTACs.

kinase	pal K_d (nM)	rib K_d (nM)	pal-pom		rib-pom	
			K_d (nM)	DC_{50} (nM)	K_d (μM)	DC_{50} (nM)
CDK4	77±2	530±90	900±100	12.9±3.5	1.65±0.05	97±9.9
CDK6	9.7±0.4	255±5	235±15	34.1±7.3	6.1±0.1	~300[a]

[a] Estimated value based on quantification of Western blot in Fig. 2d.

Kinase inhibitors such as palbociclib and ribociclib are intended to induce cell senescence rather than cytotoxicity.[16] Cytotoxicities of **pal-pom** and **rib-pom** on MDA-MB-231 cells were determined to be modest (IC_{50} estimated to be in the 10 – 50 μM range, Fig. S4a), though definitive quantitation was limited by the solubilities of these compounds.

The work above features CDK4/6 PROTACs on a triple negative breast cancer cell line instead of the approved therapeutic end-point: estrogen receptor-positive, HER2-negative breast cancer. To further investigate the application of these PROTACs, similar experiments were performed on MCF-7 breast cancer cells, which *are* estrogen receptor-positive, HER2-negative; the PROTAC **pal-pom** worked, though, curiously, not as effectively as it did on MDA-MB-231 cells in protein degradation and cytotoxicity assay (Fig. S4b and S5a). However, it did inhibit the MCF-7 cell proliferation over extended periods (up to 6 days, Fig. S6). This observation motivated us to try a cell line for a completely different type of cancer: U87 cells corresponding to glioblastoma. In this case, both **pal-pom** and **rib-pom** at 20 - 200 nM gave significant depletion of CDK4 (Fig. S5b).

In summary, as far as we are aware, this is the first report of a PROTACs that acts on CDK4/6. This finding is more significant than the closest work in the area, *ie* PROTACs suppression of CDK8/9, because only CDK4/6 are validated targets for cancer, and the PROTACs here feature FDA approved kinase inhibitors. Moreover, the potency of lead compound, **pal-pom** (DC_{50} 20 – 50 nM) are conspicuously higher than for the compounds reported to affect CDK8 and 9 (we estimate DC_{50}s of 1 – 10 μM from the data reported, Table S1). For further calibration, Table S1 also summarizes DC_{50}s of PROTACs on other completely different kinases and related proteins,[11-13,17-19] and this supports our assertion that the compounds reported here are relatively potent. Finally, observation of PROTACs activity for **pal-pom** and **rib-pom** on U87 glioblastoma cells bodes well for application of the same compounds to other cancer types.

Conflicts of interest

There are no conflicts to declare.

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