Discovery of Potent and Selective Bivalent CDK2 Degraders that Demonstrate Activity in CCNE1^{amp} Driven Tumors

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Abstract

Introduction: The Cyclin-Dependent Kinases (CDKs) with their cyclin binding partners, are associated with cell cycle progression and transcriptional regulation. Targeting CDKs is a key oncology therapeutic strategy with CDK4/6 inhibitors demonstrating significant clinical benefit in HR$^+$/HER2$^-$ metastatic breast cancer, the most prevalent subtype. However, 30% of patients develop acquired resistance in the clinic. Cyclin E1 (CCNE1) amplification/overexpression and CDK2 activation is a major resistance mechanism in CDK4/6i breast cancer therapy. Additionally, CCNE1/CDK2 activation has also been associated with poor prognosis in ovarian and endometrial cancers. Currently, targeting CDK2 using small molecule inhibitor-based approaches have advanced into the clinic. Targeted protein degradation of CDK2 provides an alternative strategy that has the potential to eliminate the activity of the CCNE1/CDK2 complex, as well as CDK2 complexed with other cyclins, including cyclin A, and provide improved clinical benefit.

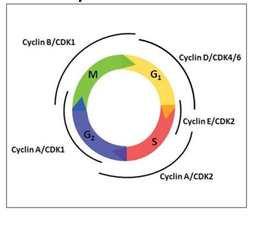
Results: Discovery efforts at Plexium have identified CDK2 bivalent degraders that consist of a CDK2-binding moiety, a linker and a high affinity cereblon-binding ligand. Cereblon binding potency was found to correlate with potent and deep degradation of CDK2. Degradation was blocked in the presence of a proteasome inhibitor as well as in a cereblon knock-out cell line, confirming that CDK2 degradation is mediated by the ubiquitin proteasome system and through engaging cereblon. Proteome-wide analysis demonstrated that CDK2 was selectively depleted without significantly modulating other CDKs or known cereblon neo-substrates. Degradation selectivity was achieved in the absence of CDK inhibition selectivity and was confirmed to be dependent on the formation of a CRBN-CDK2 ternary complex. Dose dependent CDK2 degradation resulted in dose dependent inhibition of Rb phosphorylation, cell cycle arrest, cell senescence and antiproliferative activity in CCNE1 amplified cancer cell lines. The *in vitro* data was used to evaluate the PK/PD drug exposure-response relationship *in vivo* and deep CDK2 degradation was demonstrated both *in vitro* and *in vivo.*

Conclusions: These data provide validation for CDK2 degradation as a therapeutic approach. Potent and selective CDK2 bivalent degraders were exploited as tools for studying the sensitivity of CCNE1 amplified tumor models to CDK2 degradation and dependence on E3 ligase. This proof-of concept study supports Plexium's current approach of discovering novel CDK2 molecular glue degraders for the treatment of CDK4/6 inhibitor-naïve and -resistant HR$^+$/HER2$^-$ breast cancer, and CCNE1 amplified ovarian and endometrial cancers.

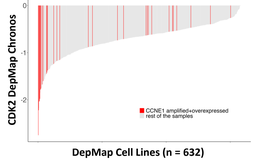
Introduction

- CDK2 associates with cyclin E (CCNE1) to regulate cell cycle progression. Aberrant Cyclin E levels activate CDK2 and correlate with poor survival (subset of Ovarian and Breast)
- Tumor cell lines with CCNE1 amplifications are sensitive to loss of CDK2
- CDK2 degrader has potential for superiority over inhibitors by (1) selectivity degrading CDK2 without modulating other CDKs and (2) disrupting CDK2 complexes
- Human cancers with cyclin E overexpression/amplification may be sensitive to loss of CDK2. Patients on CDK4/6 inhibitors for ER+HER- breast cancer develop resistance which can be driven by cyclin E overexpression

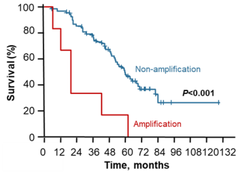
CDK2 Regulates G1/S Cell Cycle Transition



Tumor Cell Lines with CCNE1^{amp} Sensitive to CDK2



CCNE1^{amp} Correlates with Poor Survival (ovarian)

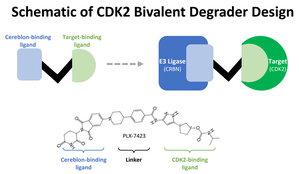


Tumor Type	CCNE1^{amp} Frequency*
Uterine	40%
Ovarian	19%
Gastric	11%
Esophageal	8%
Endometrial	8%
Sarcoma	8%
Bladder	6%
NSCLC	5%

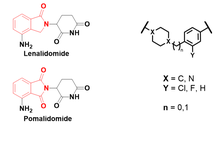
*cBioPortal frequency of CCNE1 amplification

Identification of Selective Cereblon-based Bivalent CDK2 Degraders

Schematic of CDK2 Bivalent Degrader Design



CRBN Binding Motifs

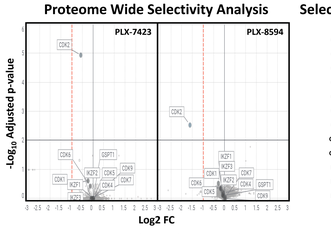


Linker

PLX#	CRBN Binding Moiety	Target Engagement IC_{50}, µM	HiBiT Degradation DC_{50}, µM (Dmax %)			Kinase Inhibition IC_{50}, µM		
		CRBN	CDK2	CDK2	CDK1	CDK9	CDK2-CCNE1	CDK1-CCNA2	CDK9-CCNT1
PLX-7423	Pom	0.116	0.006	0.156 (79)	> (44)	>10 (2)	0.007	0.414	0.14
PLX-8594	Pom	0.129	0.112	0.013 (87)	0.053 (61)	>10 (8)	0.003	0.138	0.002
PLX-8865	Pom	0.116	0.034	0.014 (85)	0.032 (55)	>10 (15)	0.006	0.055	0.036
PLX-8641 (Non-Deg)	Pom	>10	0.103	>10 (2)	>10 (20)	>10 (5)	0.005	0.092	0.021
PLX-5771 (Non-sel deg)	Pom	0.048	0.014	0.0016 (88)	0.005 (60)	0.0023 (96)	0.002	-	0.008
PLX-9365	Len	0.013	0.034	0.007 (84)	0.011 (53)	>10 (29)	0.005	0.124	0.006
PLX-9405	Len	0.013	0.045	0.007 (82)	0.005 (52)	>10 (30)	0.005	0.069	0.004

Table 1. Characterization of selective bivalent CDK2 degraders. Target engagement of cereblon (CRBN) and CDK2 in HEK293 cells was quantitated using NanoBRET™ Target Engagement assay platform (Promega). CDK2/1/9 degradation was measured using HiBiT technology in HEK293 cells (Promega) after 6h treatment with indicated compounds, max degradation (Dmax) is shown in parentheses. CDK2/1/9 inhibition was assayed using biochemical kinase activity assays (NanoSyn). 'Non-deg' is a non-degrader analog of PLX-8594 that is incapable of binding cereblon.

Proteome Wide Selectivity Analysis



Selectivity for CDKs and CRBN NeoSubstrates

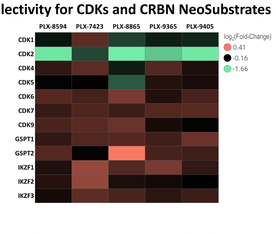


Figure 1. Selectivity profiling of CDK2 bivalent degraders. (*Left*) Quantitative proteomic profile of Jurkat cell line treated for 6h with 0.1 µM of select CDK2 bivalent degraders. Volcano plot represents the relationship between the log_2 fold-change and the $-log_{10}$(*P value)*. (*Right*) Heat map of Log2 Fold-Change in levels of selected proteins, based on proteomics analysis.

Mechanism of CDK2 Degradation

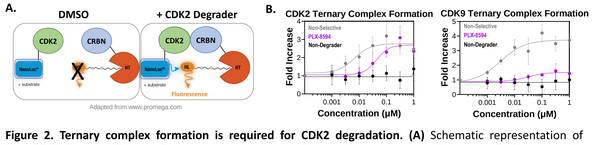


Figure 2. Ternary complex formation is required for CDK2 degradation. (A) Schematic representation of NanoBRET Cereblon Ternary Complex formation assay. Cartoon is adapted from Promega. **(B)** Non-selective degrader (PLX-5771) induces a ternary complex with CRBN and either CDK2/CCNE1 and CDK9/CCNT1, where as the CDK2 selective degrader, PLX-8594, only forms a ternary complex with CRBN and CDK2. The non-degrader (PLX-8641) does not induce ternary complex formation. Fold increase relative to DMSO.

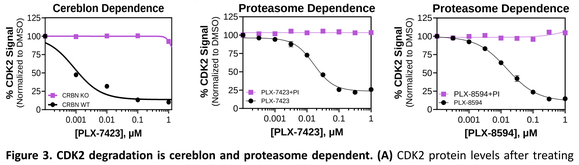
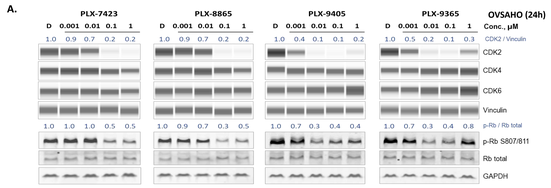
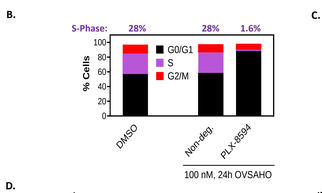
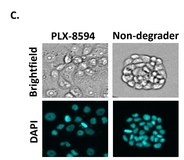


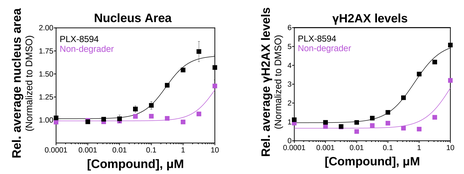
Figure 3. CDK2 degradation is cereblon and proteasome dependent. (A) CDK2 protein levels after treating with increasing concentrations of PLX-7423 for 24h in CRBN wt and KO Jurkat cells. CDK2 protein level was assayed by western blot. CDK2 HiBiT HEK293 cells were incubated +/- proteasome inhibitor (1 µM bortezomib) for 1h, followed by 6h treatment with varying concentrations of **(B)** PLX-7423 or **(C)** PLX-8594. CDK2 protein levels were detected by western blot analysis.

Selective CDK2 Degradation Leads to Cell Cycle Arrest and Cellular Senescence









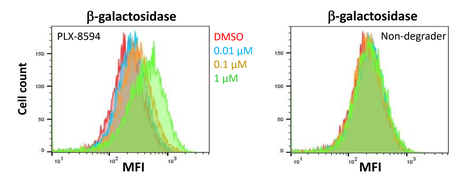


Figure 4. Selective CDK2 degradation correlates with down regulation of p-Rb, cell cycle arrest and senescence-related phenotypes. (A) CDK2 degradation correlates with down regulation of p-Rb. Western-blot analysis of CDK2/4/6 levels, total Rb and p-Rb in OVSAHO cells, after 24h treatment with compounds. **(B)** CDK2 degradation induces G1-arrest. Cell-cycle analysis by flow cytometry was assayed in OVSAHO cells, following 24h treatment with 0.1 µM PLX-8594 or the non-degrader analog. CDK2 degradation induced phenotypes associated with senescence. **(C)** Representative images of cell morphology (Brightfield) and DNA (DAPI) in OVSAHO cells treated for 6 days with 0.3 µM PLX-8594 or the non-degrader analog. **(D)** Nuclei average size, an indirect measure of cell size (left) and γH2AX levels, an early marker of senescence (right) were measured in OVSAHO cells. **(E)** Quantitative measure of senescence-associated β-galactosidase activity by flow cytometry using a cell permeable substrate which becomes fluorescent upon hydrolysis by β-galactosidase.

CDK2 Degradation Inhibits Proliferation in CCNE1 Amplified Cell Lines

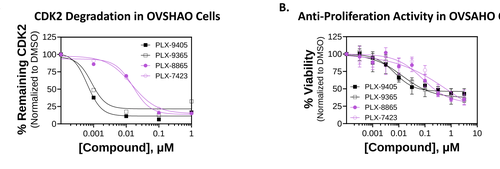


Figure 5. Increased CDK2 potency improves antiproliferation activity of degraders in cyclin E amplified cells. (A) OVSAHO cells treated with CDK2 bivalents degraders for 24h result in potent loss of CDK2 protein levels and **(B)** dose dependent anti-proliferative activity (6 days).

Anti-Proliferation Activity in CCNE1 Amplified Cells

Tumor model	PLX-8594 EC_{50}, µM	PLX-9365 EC_{50}, µM
OVSAHO	0.18	0.007
KLE	0.14	0.02
OVCAR-3	2.0	0.19

Anti-Proliferation Activity in CCNE1 Non-Amplified Cells

Tumor model	PLX-8594 EC_{50}, µM	PLX-9365 EC_{50}, µM
TOV-21G	2.2	1.0
COV644	4.5	4.3
SK-OV-3	13.0	4.3

Table 2. Increased sensitivity to CDK2 bivalent degraders is observed in cyclin E amplified tumor cells. Proliferation (EC_{50}) is measured in Cyclin E amplified (Left) and non-amplified (Right) tumor cells, treated for 6 days with either PLX-8594 or PLX-9365.

In Vivo Degradation of CDK2 by Bivalent Degraders

In vitro CDK2 Degradation Kinetics and Resynthesis Rate

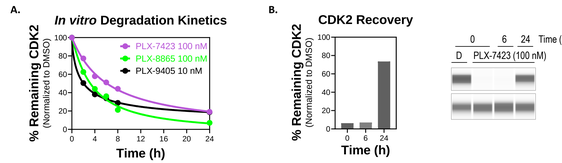
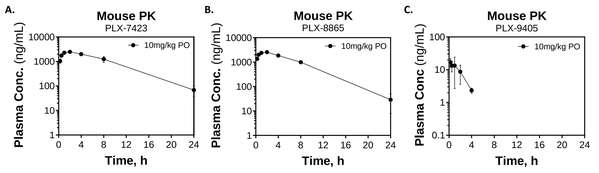


Figure 6. *In vitro* degradation and resynthesis kinetics of CDK2 protein levels. (A) Kinetics of CDK2 degradation with 3 different bivalent degraders (PLX-7423, PLX-8865, PLX-9405). MV-4-11 cells were treated with DC_{90} concentrations of compounds (depicted on graph). **(B)** After compound washout, ~70% of CDK2 was recovered within one doubling time of cells (~20 hours). MV-4-11 cells were treated for 24h with 100 nM (DC_{90}) PLX-7423. Compound was washed out and cells were supplemented with fresh media to allow recovery. CDK2 levels were assayed by western-blot immediately after washout (t0), then 6h and 24h later (*right*).

In vivo Profiling of CDK2 Bivalent Degraders



PK Parameter	PLX-7423	PLX-8865	PLX-9405
Cmax (ng/mL)	1523	1727	17.2
$T_{1/2}$ (h)	5.53	4.62	1.33
AUC_{last} (ng.h/mL)	12965	12517	32.9
%F	11.2	13.3	-

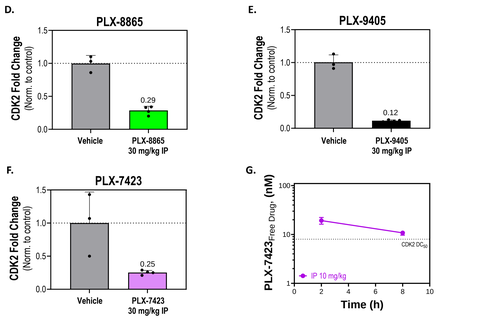


Figure 7. *In vivo* degradation of CDK2 in MV-4-11 CDX mouse model. (A-C) Plasma drug levels of select CDK2 bivalent degraders after administration of an oral (10 mg/kg) dose to C57BL/6J mice. PK parameters are summarized in the table. **(D-F)** CDK2 protein levels were significantly decreased in subcutaneous MV-4-11 tumors following treatment with bivalent degraders. Mice were dosed IP BID for 2 days with 30 mg/kg compound. CDK2 levels were measured by western blot 18h following the last dose. **(G)** Free drug concentrations following IP administration of PLX-7423 in female NOD SCID mice, relative to the DC_{50} value measured *in vitro* in MV-4-11 demonstrate sufficient exposure above the DC_{50} for ~8h..

Conclusions

- Potent and selective bivalent CDK2 degraders are identified and exploited at Plexium as tools for studying sensitivity of tumor models to CDK2 degradation, dependence on E3 ligase and differentiation of degradation from inhibition
- CDK2 bivalent degraders showed potent CDK2 degradation, inhibition of Rb phosphorylation and cell cycle progression independent of CDK2 inhibition
- CDK2 bivalent degraders showed anti-proliferative activity in CCNE1^{amp} cells *in vitro* and CDK2 degradation *in vivo*
- These data supported Plexium's current development of potent and selective CDK2 molecular glue degraders