Published in final edited form as:

ACS Chem Biol. 2015 December 18; 10(12): 2680–2686. doi:10.1021/acscchembio.5b00368.

Chemoproteomics reveals novel protein and lipid kinase targets of clinical CDK4/6 inhibitors in lung cancer

Natalia J. Sumi^{1,2}, Brent M. Kuenzi^{1,2}, Claire E. Knezevic¹, Lily L. Remsing Rix¹, and Uwe Rix¹

¹Department of Drug Discovery, H. Lee Moffitt Cancer Center & Research Institute, Tampa, Florida 33612-9497, United States

²Cancer Biology Ph.D. Program, University of South Florida, Tampa, Florida 33620, United States

Abstract

Several selective CDK4/6 inhibitors are in clinical trials for non-small cell lung cancer (NSCLC). Palbociclib (PD0332991) is included in the phase II/III Lung-MAP trial for squamous cell lung carcinoma (LUSQ). We noted differential cellular activity between palbociclib and the structurally related ribociclib (LEE011) in LUSQ cells. Applying an unbiased mass spectrometry-based chemoproteomics approach in H157 cells and primary tumor samples, we here report distinct proteome-wide target profiles of these two drug candidates in LUSQ, which encompass novel protein and, for palbociclib only, lipid kinases. In addition to CDK4 and 6, we observed CDK9 as a potent target of both drugs. Palbociclib interacted with several kinases not targeted by ribociclib, such as casein kinase 2 and PIK3R4, which regulate autophagy. Furthermore, palbociclib engaged several lipid kinases, most notably PIK3CD and PIP4K2A/B/C. Accordingly, we observed modulation of autophagy and inhibition of AKT signaling by palbociclib, but not ribociclib.

The recent clinical success and subsequent FDA approval of the CDK4/6 inhibitor palbociclib (PD0332991) in breast cancer has revitalized the field of cell cycle inhibitors. (1-3) Several CDK4/6 inhibitors are in clinical trials for a range of malignancies. The most advanced CDK4/6 inhibitors, palbociclib, abemaciclib (LY2835219) and ribociclib (LEE011) are also in clinical trials for lung cancer. For instance, palbociclib is included in the phase II/III Lung-MAP umbrella trial in squamous cell lung cancer (LUSQ). (4) In contrast to lung adenocarcinoma (LUAD) where identification of activating *EGFR* mutations and *EML4-ALK* rearrangements has led to breakthrough therapies with tyrosine kinase inhibitors, no targeted therapies are yet approved for LUSQ. However, CDK4/6-Rb-E2F pathway alterations are commonly observed in LUSQ, (5) making targeting CDK4/6 an attractive therapeutic option in this subset.

Supporting Information

Additional data and methods. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Competing financial interests

The authors declare no competing financial interests.

Kinase inhibitors can have broad cellular target selectivity profiles,(6-9) which can impair drug safety, but can also enhance efficacy (“polypharmacology”).(10, 11) The *in vitro* kinase binding profile of abemaciclib against a near kinome-wide panel showed several additional targets.(12) Similarly comprehensive information on kinase selectivity for palbociclib and ribociclib is not available.(13, 14) In light of the potential clinical relevance of these drugs in LUSQ, we investigated the target spectrum of palbociclib and ribociclib to determine if additional targets contribute to or impinge on their anticancer mechanism of action in LUSQ cells. Using a chemical proteomics approach, we describe herein the global target profiles of palbociclib and ribociclib in the H157 LUSQ cell line and primary tumor samples from LUSQ patients.

Palbociclib and ribociclib are close structural analogues with equal potency for CDK4 inhibition (IC_{50} [palbociclib]: 9-11 nM; IC_{50} [ribociclib]: 10 nM).(1) Interestingly, we observed differential cellular potency in several LUSQ cell lines (**Fig. 1A**). This is likely not due to differences in cellular uptake, as both drugs effectively inhibited Rb phosphorylation at S780, a canonical substrate of CDK4, in H157 cells (**Fig. 1B**), consistent with observations in other cell lines.(13, 14) Abemaciclib, which is about 5-fold more potent for CDK4 than palbociclib and ribociclib,(1, 12) showed stronger effects on viability (**Fig. S1**). The difference in cellular potency between abemaciclib and ribociclib is consistent with their differential activity on CDK4; however, palbociclib is somewhat more active than ribociclib and this enhanced cellular activity may be due to additional palbociclib targets beyond CDK4/6. Although the attainable patient plasma levels of palbociclib of up to 433 nM(15) (not reported yet for ribociclib) suggest limited single agent activity and the need for drug combinations in LUSQ, accumulation in tumor tissue may be much higher as palbociclib concentrations in mouse tumors were as high as 52 μ M.(16) Thus, even moderate cellular activities may translate into clinical relevance.

To identify palbociclib and ribociclib targets in LUSQ cells, we applied a chemical proteomics strategy, which is a mass spectrometry (MS)-based drug affinity approach that allows for the proteome-wide identification of cellular drug targets.(17) To this end, we generated the coupleable drug analogues c-palbociclib (**3**) and c-ribociclib (**4**) for immobilization on affinity beads (**Fig. 1C**; **Fig. S2**). Based on available co-crystal data for palbociclib and CDK6 (pdb: 2EUF),(18) we hypothesized that modification of the piperazine moiety shared by both drugs would be compatible with target binding. Introduction of an aminopropyl linker would furthermore retain availability of the free electron pair of the amino group, which contributes to CDK6 binding.(18) Subsequent validation of these analogues by *in vitro* kinase assays for CDK4/cyclin D1 confirmed conservation of their CDK4 activity (**Fig. 1D**), which, based on our previous experience with kinase inhibitors, should translate well to other kinase targets.(19)

We next performed chemical proteomics experiments with **3** and **4** using H157 total cell lysates. Negative control experiments with CDK4/6 inhibitor beads incubated with CDK4/6 inhibitor-pretreated lysates and with ampicillin beads allowed for identification and prioritization of a large number of potential kinase targets (**Fig. 2A,B**). Confirming the suitability of our probe molecules, both drugs prominently enriched for the cognate targets CDK4 and CDK6. Strikingly, palbociclib was significantly less selective and interacted with

more than twice as many kinases as ribociclib (**Fig. S3A,B**). Several of these, such as CDK9 and FER, were identified with both drugs. There were only few kinases that were exclusive for ribociclib, namely cyclin G- associated kinase (GAK), TNK1 and aurora kinase A (AURKA). Although TNK1 was also weakly identified with palbociclib (**Table S1**), palbociclib pretreatment did not result in competition and TNK1 therefore did not pass our stringent selection criteria. AURKA was also prominently observed with ribociclib, but not palbociclib. However, AURKA was assigned a lower priority as pretreatment caused only partial competition, which can indicate a weaker drug-protein interaction and/or higher cellular expression levels. Conversely, a large number of kinases interacted specifically with the palbociclib matrix. The most prominent palbociclib- specific targets were casein kinase 2 α 1/2 (CSNK2 α 1/2, CK2 α 1/2), AMPK α 1, PIK3R4, JNK1, TBK1 and GSK3 β . Other kinases, such as JNK2, ERK2 and FAK, were also abundant in the drug affinity eluate, but only partially competed. Further target prioritization using the established normalized spectral abundance factor (NSAF) and significance analysis of interactome (SAINT) methods, highlighted GAK as a new ribociclib-specific and CSNK2 α 1, CSNK2 α 2 and PIK3R4 as the most prominent new palbociclib-specific targets (**Fig. 2C,D**). The specificities for these target candidates were confirmed by dose-dependent competition with soluble drug (**Fig. S3C**). Interestingly, we also observed several lipid kinases to exclusively interact with the palbociclib affinity matrix, namely PIK3C3, PIP4K2A/B/C and PIK3CD. These lipid kinases were all strongly competed yielding high SAINT scores. Immunoblot analysis of the palbociclib and ribociclib affinity eluates confirmed the MS data (**Fig. 2E**).

To determine, if the differential target profiles observed between palbociclib and ribociclib in the H157 cell line translated to primary tumor tissues, we next performed chemical proteomics with two separate tumor samples from LUSQ patients (T1 and T2) (**Tables S2,S3**). As the smaller samples available for T1 and T2 did not allow for ampicillin pulldowns, negative controls consisted only of drug competition with palbociclib or ribociclib. Subsequent MS analysis revealed substantial overlap with the target profiles obtained from H157 cells. Indeed, the majority of kinase targets were observed in all three samples (**Fig. 3A,B**). Some kinases, for instance PYK2, were only observed in tumor samples. We have recently made similar observations for other kinase inhibitors and shown that such targets likely originate from the tumor microenvironment.(20) As PYK2 is predominantly expressed in hematopoietic cells, this may also be the case here. Subsequent validation of the most prominent target candidates by *in vitro* kinase assays showed that CDK9 and TNK1 are indeed new and potent ribociclib targets. Similarly, CDK9, PIK3CD and CSNK2 α 2 are new palbociclib targets. Importantly, these targets display IC₅₀'s that suggest at least partial inhibition under physiological conditions. GAK, PIK3R4 and PIP4K2C were not tested due to lack of commercially available assays, but dose-dependent binding competition suggests potent interactions of ribociclib with GAK and palbociclib with PIK3R4 (**Fig. S3C**).

Some candidates were only weakly inhibited. For AURKA this was consistent with the lower priority assignment due to incomplete competition by ribociclib. Based on the MS results, CSNK2 α 1 and PIK3C3 (IC₅₀ = 14.8 μ M) were high confidence candidates with high NSAF values and SAINT scores. We therefore postulated these proteins to be specific, albeit

indirect palbociclib binders that were recovered through protein-protein interactions (PPIs) with another kinase. Querying known PPIs within the set of identified high confidence proteins using the ConsensusPathDB database we generated a hybrid drug-protein/protein-protein interaction network (**Fig. 4A**). This network corroborated our hypothesis as CSNK2 α 1 is known to form a functional protein kinase complex with CSNK2 α 2 and the regulatory subunit CSNK2B. Similarly, PIK3C3 (Vps34) is well characterized as an important component of protein complexes with PIK3R4 (Vps15).⁽²¹⁾ Given that PIK3R4, one of the most prominent palbociclib target candidates in H157, is a protein kinase that phosphorylates and activates PIK3C3, it is likely that PIK3C3 is indirectly engaged by palbociclib via PIK3R4.^(22, 23) Interestingly, two non-kinase proteins of this complex, namely beclin (BECN1) and UVRAG, which complement PIK3C3 complex II, were also found.

To evaluate the functional relevance of the new targets, we next used the bioinformatic platform DAVID to survey the KEGG database for pathways that were potentially inhibited (**Table S4**). As expected, one of the most significantly represented pathways was cell cycle signaling, which was engaged by both drugs (**Fig. 4A**). In addition, a number of palbociclib-specific targets mapped to the autophagy and phosphatidylinositol signaling pathways. Palbociclib, but not ribociclib, specifically interacted with several lipid kinases in the phosphatidylinositol signaling pathway. PIP4K2A/B/C catalyze the formation of phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P₂).⁽²⁴⁾ PIK3CD in turn generates phosphatidylinositol 3,4,5-trisphosphate (PIP₃) from PtdIns(4,5)P₂, a critical event in the activation of the AKT signaling cascade. Accordingly, we observed that palbociclib, but not ribociclib, dose-dependently inhibits phosphorylation of AKT S473 and its downstream target GSK3 β with moderate potency (**Fig. 4B**). Considering the relative insensitivity regarding viability of H157 and H520 cells to the potent PI3K inhibitors BKM120 and CAL-101 (**Fig. S4**), inhibition of PIK3CD and PIP4K2A/B/C likely does not contribute to palbociclib's overall anticancer effects in these cells. However, PIK3CD is a *bona fide* target in some hematopoietic malignancies, such as mantle cell lymphoma (MCL), where palbociclib has been found to overcome drug resistance and is entering a clinical trial in combination with ibrutinib.⁽²⁵⁾ As PIK3CD is a relatively weak palbociclib target, the relevance of this interaction in MCL depends on if sufficiently high drug concentrations accumulate in malignant or supporting microenvironment cells, which based on preclinical studies could be possible (see above).⁽¹⁶⁾

AMPK, CK2 and the PIK3R4/PIK3C3/BECN1/UVRAG complex play important roles in autophagy.^(21, 26, 27) Indeed, palbociclib treatment increased lipidation of LC3 (LC3-II), a canonical readout of autophagy modulation, in H157 and H520 cells (**Fig. 4C**). In contrast to ribociclib, palbociclib also increased the number of autophagic vesicles (**Fig. 4D**). Given the overall similar behavior to the autophagy inhibitor hydroxychloroquine (HCQ) (**Fig. S5**), and that AMPK, CK2 and the PIK3C3 complex II *positively* regulate autophagy, palbociclib likely also inhibits autophagy. It is notable that PIK3R4 is amplified/mutated in about 9% of LUSQ (**Figure S6**),⁽⁵⁾ but not LUAD (**Figure S7**), and that PIK3R4 gene amplification statistically significantly co-occurs with amplification of GSK3 β (**Table S5**). Likewise, USP13, which deubiquitinates BECN1 and stabilizes PIK3C3 complexes,⁽²⁸⁾ is amplified

in LUSQ. This suggests that modulation of this pathway, which is affected specifically by palbociclib, may have functional consequences for a subset of LUSQ patients. Accordingly, inhibiting CDK4/6 in combination with compound C (AMPK inhibitor) or LY294002 (PIK3C3 inhibitor, used in lieu of available PIK3R4 inhibitors) causes a further reduction of H157 and H520 viability (**Fig. S8**). This suggests that inhibition of AMPK and PIK3R4 by palbociclib may augment its main CDK4/6- mediated anticancer activity in these cells. Notably, as these compounds affect also several other targets, they are not optimal, albeit the only suitable, probes for AMPK and PIK3C3. LY294002 broadly inhibits also PI3K and BET proteins.(29) However, comparison with bioequivalent doses of the pan-PI3K inhibitor BKM120 and the BET inhibitor I-BET151 (**Fig. S4**) for several PI3K and BET isoforms still support a contribution of PIK3C3 (and accordingly PIK3R4) towards LUSQ cell viability. Further studies are required to determine the exact effects of palbociclib on autophagy and the cellular consequences of this.

In summary, we here describe the proteome-wide target profiles of the two clinical CDK4/6 inhibitors palbociclib and ribociclib in LUSQ. We identified palbociclib, but not the more specific ribociclib, as a dual protein and lipid kinase inhibitor, which also modulates the PI3K/AKT and autophagy pathways in addition to canonical CDK4/6-Rb-E2F signaling. Considering the prevalence and relevance of alterations in these pathways in LUSQ and other cancers, palbociclib may in some cases have beneficial polypharmacology in addition to its primary targets CDK4/6.

Methods

Please see Supporting Methods.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This work was supported by the H. Lee Moffitt Cancer Center and Research Institute and the Moffitt Lung Cancer Center of Excellence. We furthermore wish to acknowledge the Moffitt Chemical Biology (Chemistry Unit) and Proteomics Core Facilities, which are supported by the National Cancer Institute (Award No. P30-CA076292) as a Cancer Center Support Grant. Proteomics is also supported by the US Army Medical Research and Material Command (Award No. W81XWH-08-2-0101) for a National Functional Genomics Center, the Moffitt Foundation, and the Bankhead-Coley Cancer Research program of the Florida Department of Health (09BE- 04).

References

1. Asghar U, Witkiewicz AK, Turner NC, Knudsen ES. The history and future of targeting cyclin-dependent kinases in cancer therapy. *Nat Rev Drug Discov.* 2015; 14:130–146. [PubMed: 25633797]
2. Dickson MA. Molecular pathways: CDK4 inhibitors for cancer therapy. *Clin Cancer Res.* 2014; 20:3379–3383. [PubMed: 24795392]
3. Finn RS, Crown JP, Lang I, Boer K, Bondarenko IM, Kulyk SO, Ettl J, Patel R, Pinter T, Schmidt M, Shparyk Y, Thummala AR, Voytko NL, Fowst C, Huang X, Kim ST, Randolph S, Slamon DJ. The cyclin-dependent kinase 4/6 inhibitor palbociclib in combination with letrozole versus letrozole alone as first-line treatment of oestrogen receptor-positive, HER2-negative, advanced breast cancer

- (PALOMA-1/TRIO-18): a randomised phase 2 study. *Lancet Oncol.* 2015; 16:25–35. [PubMed: 25524798]
4. Herbst RS, Gandara DR, Hirsch FR, Redman MW, LeBlanc M, Mack PC, Schwartz LH, Vokes E, Ramalingam SS, Bradley JD, Sparks D, Zhou Y, Miwa C, Miller VA, Yelensky R, Li Y, Allen JD, Sigal EV, Wholley D, Sigman CC, Blumenthal GM, Malik S, Kelloff GJ, Abrams JS, Blanke CD, Papadimitrakopoulou VA. Lung Master Protocol (Lung-MAP)-A Biomarker-Driven Protocol for Accelerating Development of Therapies for Squamous Cell Lung Cancer: SWOG S1400. *Clin Cancer Res.* 2015; 21:1514–1524. [PubMed: 25680375]
 5. Hammerman PS, Hayes DN, Wilkerson MD, Schultz N, Bose R, Chu A, Collisson EA, Cope L, Creighton CJ, Getz G, Herman JG, Johnson BE, Kucherlapati R, Ladanyi M, Maher CA, Robertson G, Sander C, Shen R, Sinha R, Sivachenko A, Thomas RK, Travis WD, Tsao MS, Weinstein JN, Wigle DA, Baylin SB, Govindan R, Meyerson M. Comprehensive genomic characterization of squamous cell lung cancers. *Nature.* 2012; 489:519–525. [PubMed: 22960745]
 6. Bantscheff M, Eberhard D, Abraham Y, Bastuck S, Boesche M, Hobson S, Mathieson T, Perrin J, Raida M, Rau C, Reader V, Sweetman G, Bauer A, Bouwmeester T, Hopf C, Kruse U, Neubauer G, Ramsden N, Rick J, Kuster B, Drewes G. Quantitative chemical proteomics reveals mechanisms of action of clinical ABL kinase inhibitors. *Nat Biotechnol.* 2007; 25:1035–1044. [PubMed: 17721511]
 7. Godl K, Wissing J, Kurtenbach A, Habenberger P, Blencke S, Gutbrod H, Salassidis K, Stein-Gerlach M, Missio A, Cotten M, Daub H. An efficient proteomics method to identify the cellular targets of protein kinase inhibitors. *Proc Natl Acad Sci U S A.* 2003; 100:15434–15439. [PubMed: 14668439]
 8. Ong SE, Schenone M, Margolin AA, Li X, Do K, Doud MK, Mani DR, Kuai L, Wang X, Wood JL, Tolliday NJ, Koehler AN, Marcaurelle LA, Golub TR, Gould RJ, Schreiber SL, Carr SA. Identifying the proteins to which small-molecule probes and drugs bind in cells. *Proc Natl Acad Sci U S A.* 2009; 106:4617–4622. [PubMed: 19255428]
 9. Winter GE, Rix U, Carlson SM, Gleixner KV, Grebien F, Gridling M, Muller AC, Breitwieser FP, Bilban M, Colinge J, Valent P, Bennett KL, White FM, Superti-Furga G. Systems-pharmacology dissection of a drug synergy in imatinib-resistant CML. *Nat Chem Biol.* 2012; 8:905–912. [PubMed: 23023260]
 10. Li J, Rix U, Fang B, Bai Y, Edwards A, Colinge J, Bennett KL, Gao J, Song L, Eschrich S, Superti-Furga G, Koomen J, Haura EB. A chemical and phosphoproteomic characterization of dasatinib action in lung cancer. *Nat Chem Biol.* 2010; 6:291–299. [PubMed: 20190765]
 11. Remsing Rix LL, Kuenzi BM, Luo Y, Remily-Wood E, Kinose F, Wright G, Li J, Koomen JM, Haura EB, Lawrence HR, Rix U. GSK3 Alpha and Beta Are New Functionally Relevant Targets of Tivantinib in Lung Cancer Cells. *ACS chemical biology.* 2014; 9:353–358. [PubMed: 24215125]
 12. Gelbert LM, Cai S, Lin X, Sanchez-Martinez C, Del Prado M, Lallena MJ, Torres R, Ajamie RT, Wishart GN, Flack RS, Neubauer BL, Young J, Chan EM, Iversen P, Cronier D, Kreklau E, de Dios A. Preclinical characterization of the CDK4/6 inhibitor LY2835219: in-vivo cell cycle-dependent/independent anti-tumor activities alone/in combination with gemcitabine. *Investigational new drugs.* 2014; 32:825–837. [PubMed: 24919854]
 13. Fry DW, Harvey PJ, Keller PR, Elliott WL, Meade M, Trachet E, Albassam M, Zheng X, Leopold WR, Pryer NK, Toogood PL. Specific inhibition of cyclin-dependent kinase 4/6 by PD 0332991 and associated antitumor activity in human tumor xenografts. *Mol Cancer Ther.* 2004; 3:1427–1438. [PubMed: 15542782]
 14. Rader J, Russell MR, Hart LS, Nakazawa MS, Belcastro LT, Martinez D, Li Y, Carpenter EL, Attiyeh EF, Diskin SJ, Kim S, Parasuraman S, Caponigro G, Schnepf RW, Wood AC, Pawel B, Cole KA, Maris JM. Dual CDK4/CDK6 inhibition induces cell-cycle arrest and senescence in neuroblastoma. *Clin Cancer Res.* 2013; 19:6173–6182. [PubMed: 24045179]
 15. Schwartz GK, LoRusso PM, Dickson MA, Randolph SS, Shaik MN, Wilner KD, Courtney R, O'Dwyer PJ. Phase I study of PD 0332991, a cyclin-dependent kinase inhibitor, administered in 3-week cycles (Schedule 2/1). *Br J Cancer.* 2011; 104:1862–1868. [PubMed: 21610706]
 16. Nguyen L, Zhong WZ, Painter CL, Zhang C, Rahavendran SV, Shen Z. Quantitative analysis of PD 0332991 in xenograft mouse tumor tissue by a 96-well supported liquid extraction format and

- liquid chromatography/mass spectrometry. *Journal of pharmaceutical and biomedical analysis*. 2010; 53:228–234. [PubMed: 20236782]
17. Rix U, Superti-Furga G. Target profiling of small molecules by chemical proteomics. *Nat Chem Biol*. 2009; 5:616–624. [PubMed: 19690537]
18. Lu H, Schulze-Gahmen U. Toward understanding the structural basis of cyclin-dependent kinase 6 specific inhibition. *J Med Chem*. 2006; 49:3826–3831. [PubMed: 16789739]
19. Remsing Rix LL, Rix U, Colinge J, Hantschel O, Bennett KL, Stranzl T, Muller A, Baumgartner C, Valent P, Augustin M, Till JH, Superti-Furga G. Global target profile of the kinase inhibitor bosutinib in primary chronic myeloid leukemia cells. *Leukemia*. 2009; 23:477–485. [PubMed: 19039322]
20. Gridling M, Ficarro SB, Breitwieser FP, Song L, Parapatics K, Colinge J, Haura EB, Marto JA, Superti-Furga G, Bennett KL, Rix U. Identification of kinase inhibitor targets in the lung cancer microenvironment by chemical and phosphoproteomics. *Mol Cancer Ther*. 2014; 13:2751–2762. [PubMed: 25189542]
21. Levine B, Kroemer G. Autophagy in the pathogenesis of disease. *Cell*. 2008; 132:27–42. [PubMed: 18191218]
22. Stack JH, DeWald DB, Takegawa K, Emr SD. Vesicle-mediated protein transport: regulatory interactions between the Vps15 protein kinase and the Vps34 PtdIns 3-kinase essential for protein sorting to the vacuole in yeast. *The Journal of cell biology*. 1995; 129:321–334. [PubMed: 7721937]
23. Stack JH, Herman PK, Schu PV, Emr SD. A membrane-associated complex containing the Vps15 protein kinase and the Vps34 PI 3-kinase is essential for protein sorting to the yeast lysosome-like vacuole. *Embo J*. 1993; 12:2195–2204. [PubMed: 8387919]
24. Emerling BM, Hurov JB, Poulogiannis G, Tsukazawa KS, Choo-Wing R, Wulf GM, Bell EL, Shim HS, Lamia KA, Rameh LE, Bellinger G, Sasaki AT, Asara JM, Yuan X, Bullock A, Denicola GM, Song J, Brown V, Signoretti S, Cantley LC. Depletion of a putatively druggable class of phosphatidylinositol kinases inhibits growth of p53-null tumors. *Cell*. 2013; 155:844–857. [PubMed: 24209622]
25. Chiron D, Di Liberto M, Martin P, Huang X, Sharman J, Bleucia P, Mathew S, Vijay P, Eng K, Ali S, Johnson A, Chang B, Ely S, Elemento O, Mason CE, Leonard JP, Chen-Kiang S. Cell-cycle reprogramming for PI3K inhibition overrides a relapse-specific C481S BTK mutation revealed by longitudinal functional genomics in mantle cell lymphoma. *Cancer Discov*. 2014; 4:1022–1035. [PubMed: 25082755]
26. Liang J, Shao SH, Xu ZX, Hennessy B, Ding Z, Larrea M, Kondo S, Dumont DJ, Gutterman JU, Walker CL, Slingerland JM, Mills GB. The energy sensing LKB1-AMPK pathway regulates p27(kip1) phosphorylation mediating the decision to enter autophagy or apoptosis. *Nature cell biology*. 2007; 9:218–224.
27. Matsumoto G, Wada K, Okuno M, Kurosawa M, Nukina N. Serine 403 phosphorylation of p62/SQSTM1 regulates selective autophagic clearance of ubiquitinated proteins. *Mol Cell*. 2011; 44:279–289. [PubMed: 22017874]
28. Liu J, Xia H, Kim M, Xu L, Li Y, Zhang L, Cai Y, Norberg HV, Zhang T, Furuya T, Jin M, Zhu Z, Wang H, Yu J, Li Y, Hao Y, Choi A, Ke H, Ma D, Yuan J. Beclin1 controls the levels of p53 by regulating the deubiquitination activity of USP10 and USP13. *Cell*. 2011; 147:223–234. [PubMed: 21962518]
29. Dittmann A, Werner T, Chung CW, Savitski MM, Falth Savitski M, Grandi P, Hopf C, Lindon M, Neubauer G, Prinjha RK, Bantscheff M, Drewes G. The Commonly Used PI3-Kinase Probe LY294002 Is an Inhibitor of BET Bromodomains. *ACS chemical biology*. 2014; 9:495–502. [PubMed: 24533473]

Figure 1. Differential cellular activity of palbociclib and ribociclib and generation of drug analogues

(A) Effects of palbociclib and ribociclib on viability of H157, H2170, H520 and H596 cells after 72 hours of drug treatment and IC₅₀ values for inhibition of cell viability. (B) Effects of 1 μM and 5 μM palbociclib and ribociclib on Rb Ser780 phosphorylation in H157 cells after 4 and 24 hours of drug treatment. Bottom panel: densitometry quantification of immunoblot signals for pSer780 Rb in relation to total Rb levels. (C) Chemical structures of palbociclib (1) and ribociclib (2) and their coupleable analogues. (D) *In vitro* kinase assays for CDK4/cyclin D1 activity.

Figure 2. Target profiles of palbociclib and ribociclib in H157 cells

Protein kinome map of palbociclib (A) and ribociclib (B) targets. Depicted are kinases that i) have an average exclusive unique spectral count of ≥ 2 , ii) show ≥ 2 -fold competition upon pretreatment with unmodified drug, iii) have an NSAF value higher than 0.001 and iv) are not detected in ampicillin controls. Kinases fulfilling all four criteria are depicted as large, those fulfilling three criteria as small nodes. Only kinases identified with at least 2 exclusive unique spectra per replicate are shown. Illustration reproduced courtesy of Cell Signaling Technology, Inc. (www.cellsignal.com). Prioritization of palbociclib (C) and ribociclib (D) protein and lipid kinase targets based on SAINT scores, determined via comparison with ampicillin and drug competition negative controls, and NSAF values. Targets with a SAINT score below 0.1 are not shown. Red (C) and blue (D) shaded areas denote high priority space. Node size is proportional to total unique spectral counts. (E) Western blot analysis of drug affinity eluates. TCL: total cell lysate; Amp: ampicillin beads; PT: pretreatment.

Figure 3. Comparison of palbociclib (A) and ribociclib (B) target profiles between H157 cells and primary tumor tissues from LUSQ patients

Left: overlapping and sample-specific targets. Box code adjacent to kinase name indicates, whether a kinase fulfilled all selection criteria (black), was observed, but did not fulfill all criteria (grey) or was not observed (white). Each box represents one sample; from left to right: H157, tumor tissue 1 (T1), tumor tissue 2 (T2). Only kinases that fulfilled all criteria in at least one sample are listed. Selection criteria are outlined in the legend for Fig. 2. In tumor tissues, criterion iv is omitted as ampicillin controls were not available. Kinases shown in red were selected for follow up. Right: *in vitro* kinase activity for selected kinases at two drug concentrations (in μM). Full dose-response curves and IC_{50} values were subsequently determined for potent targets. #average of IC_{50} (Reaction Biology) = 447 nM, IC_{50} (Eurofins) = 1848 nM.

Figure 4. Network and pathway analysis of proteome-wide drug interactomes in H157 cells
 (A) Direct and indirect interactions of palbociclib and ribociclib are represented by black and grey edges, respectively. Protein kinases are shown as red, lipid kinases as orange and other proteins as grey nodes. Known protein complexes are highlighted by red circles. Beige outlines denote proteins as part of a specific indicated pathway. (B) Representative pSer473 AKT and pGSK3 Western blots of H157 and H520 cells treated for 4 hours with indicated concentrations of palbociclib, ribociclib (in μM), BKM120 (2 μM) or CAL101 (5 μM). (C) Representative LC3 Western blots of H157 and H520 cells treated for 48 hours with indicated concentrations of palbociclib, ribociclib (in μM) or hydroxychloroquine (HCQ, 25 μM). Palbociclib and ribociclib concentrations in combination with hydroxychloroquine were 5 μM each. (D) Quantitative analysis of autophagic vesicles by Cyto-ID upon 3 hour treatment with the indicated concentrations (in μM) of palbociclib, ribociclib, HCQ (25 μM) and the mTOR inhibitor and autophagy inducer AZD8055 (4 μM). Values are normalized to DMSO and plotted as differences to DMSO.