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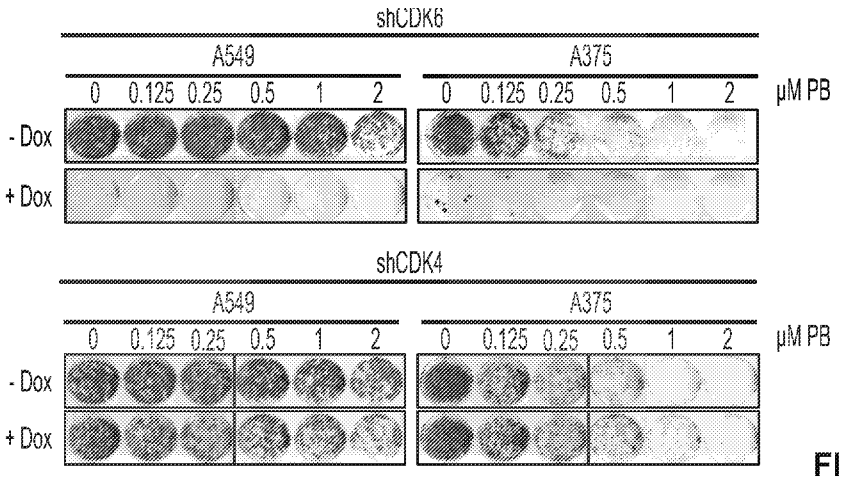


FIG. 2B

(57) Abstract: This disclosure features biomarkers, for, e.g., in diagnosis and treatment of cancer. Described herein are methods of treating cancer based on the levels of CDK6 and/or CDK4 in tumors. The disclosure also features methods for identifying compounds useful in the treatment of cancers, and kits for quantifying levels of CDK6 and/or CDK4.

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METHODS OF DIAGNOSING AND TREATING CANCER

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority and benefit from U.S. Provisional Patent Application 62/965,128, filed January 23, 2020; and U.S. Provisional Patent Application 63/094,014 filed October 20, 2020, the contents and disclosures of which are incorporated herein by reference in their entireties.

REFERENCE TO SEQUENCE LISTING

This instant application contains a Sequence Listing which has been submitted electronically in ASCII format and is hereby incorporated by reference in its entirety. Said ASCII copy, created on January 22, 2021, is named Sequence Listing.txt and is 12,288 bytes in size.

TECHNICAL FIELD

This disclosure relates generally to methods of diagnosing and treating cancer, based on CDK6 and CDK4 levels in tumors.

BACKGROUND

Cyclin-dependent kinase 4 (CDK4, also known as CMM3 and PSK-J3) (Matsushime et al, (1992) Cell Oct 16;71(2):323-34) and cyclin-dependent kinase 6 (CDK6, also known as MCPH12 and PLSTIRE) (Meyerson M. and Harlow E., (1994) Mol Cell Biol. Mar;14(3):2077-86) are related serine/threonine kinases (referred to together as "CDK4/6") that play a critical role in the cyclin D/CDK4/6/Rb/E2F signaling pathway ("CDK4/6/Rb signaling") (Sherr C.J. et al., (2016) Cancer Discov 6, 353-367). CDK4/6 regulate cell cycle progression by phosphorylating and inactivating the tumor suppressor Retinoblastoma protein (Rb) and thus have been targeted by small molecule inhibitors for cancer therapy (Sherr C.J. et al., (2016) Cancer Discov 6, 353-367; O'Leary B. Nature Reviews. Clinical Oncology (2016) 13, 417-430). CDK4/6 inhibitors ("CDK4/6i") are established therapeutics for metastatic breast cancer. CDK4/6 inhibitors (CDK4/6i) in combination with hormonal therapy showed significant clinical activity in

Rb-proficient metastatic ER positive breast cancers (B. O'Leary et al., (2016) Clinical Oncology 13, 417-430; Hortobagyi G. N. et al., (2016) N Engl J Med 375, 1738-1748), and three CDK4/6i, palbociclib (PB), abemaciclib (AB) and ribociclib, are FDA-approved for this indication (Finn R.S. et al., (2016) N Engl J Med 375, 1925-1936 (2016); Ingham, M. and Schwartz, G. K. (2017) J Clin Oncol, JCO2016690032; Goetz M. P. et al., (2017) J Clin Oncol 35, 3638-3646; Im S. A. et al., (2019) N Engl J Med 381, 307-316). Since the activity of CDK4/6 requires a functional RB protein, tumors that do not express functional Rb are resistant to these drugs (E. S. Knudsen, E.S. and Witkiewicz, A.K. (2017) Trends Cancer 3, 39-55). However, in many tumor types predominantly expressing wild-type RB1 (lung adenocarcinomas, melanomas, colon cancers etc) preclinical and clinical studies have shown only modest effectiveness of CDK4/6i (Hamilton, E. et al., (2016) Cancer Treat Rev 45, 129-138; Gong X. et al., (2017) Cancer Cell 32, 761-776; Kim S. et al., (2018) Oncotarget 9, 35226-35240), suggesting that other mechanisms limit their efficacy in these tumor types. Thus there is a need to determine mechanisms that limit the efficacy of the treatment of Rb-proficient tumors, and further determine the therapeutic options for such tumors.

SUMMARY

The present disclosure relates in general to the use of CDK6 and/or the CDK4:CDK6 ratio as a predictive biomarker for drug response of tumors to CDK4/6-directed therapies. Described herein are methods of treating subjects with particular CDK4/6-related therapies based on the subject's CDK6 level and/or CDK4 to CDK6 ratio. Also described herein are kits for measuring CDK6 and/or CDK4 levels.

In one aspect, the disclosure features a method for treating a cancer in a human subject, the method comprising: (a) obtaining a tumor sample from the subject; (b) determining a level of cyclin dependent kinase 6 (CDK6) in the tumor sample; and (c) comparing the level of CDK6 as determined in (b) with a reference level in a control; wherein when the level of CDK6 in the tumor is lower than the reference level of CDK6 in the control, the tumor is classified as a CDK4-dependent tumor and treated with a

therapeutically effective amount of one or more of a CDK4/6 inhibitor and a CDK4/6-directed PROTAC.

In a second aspect, the disclosure features for treating a cancer in a human subject, the method comprising: (a) obtaining a tumor sample from the subject; (b) determining levels of CDK6 and CDK4 in the tumor sample; and (c) determining the ratio of CDK4 to CDK6 levels; wherein (i) when the CDK6 level is below a threshold level and/or the CDK4 to CDK6 ratio is above a threshold ratio, the tumor is classified as a CDK4-dependent tumor and the subject is treated with a composition comprising a therapeutically effective amount of a CDK4/6-directed PROTAC; or (ii) when the CDK6 level is above a threshold level and/or the CDK4 to CDK6 ratio is below a threshold ratio, the tumor is classified as a CDK6-dependent tumor and the subject is treated with a composition comprising a therapeutically effective amount of a CDK4/6 inhibitor. In some embodiments, the CDK6 threshold level in the biological sample is below about 5.64 CPM, as determined by RNA sequencing.

In some embodiments of the above methods, the CDK4/6 inhibitor is one or more agents selected from a group consisting of abemaciclib (Verzenio), dinaciclib. palbociclib (Ibrance), ribociclib (Kisqali), trilaciclib (G1T28), G1T38, and the group of CDK4/6 inhibitor compounds referred to in International Patent Application Publications WO2016040858, WO2011130232, WO2011101409, WO2016025650, and WO2013006532 and the CDK4/6-directed PROTAC is one or more agents selected from MS140, the PROTACs referred to in **FIGs. 14-18**, and bivalent compounds referred to in International Patent Application Publication WO2018106870.

In some embodiments, the CDK4/6 inhibitor and/or CDK4/6-directed PROTAC is administered in combination with one or more additional therapeutic regimens selected from the group consisting of surgery, chemotherapy, radiation therapy, hormone therapy, targeted therapy, and immunotherapy. In some embodiments, the targeted therapy is with one or more agents selected from a group consisting of MEK inhibitors, ERK inhibitors, hormonal therapy, and RAS(G12C) inhibitors.

In some embodiments of the above aspects, the cancer is a solid tumor cancer. In some embodiments, the cancer is a cancer expressing functional retinoblastoma protein (Rb).

In a third aspect, the disclosure provides a method of treating a cancer in a human subject in need thereof, comprising administering to the human subject a therapeutically effective amount of a cancer therapy, wherein the human subject has been previously determined to have, in a biological sample obtained from the human subject, at least one of (a) a CDK6 level prior to initiation of the cancer therapy that is lower than a reference level in a control, and (b) a ratio of CDK4 to CDK6 levels prior to initiation of the cancer therapy that is higher than a reference level in a control.

In a fourth aspect, the disclosure provides a method for treating a human subject with cancer, comprising (a) measuring a level of CDK6 and optionally measuring a level of CDK4, and further, optionally determining a ratio of the CDK4 to CDK6 levels in a biological sample taken from the subject; and (b) treating the subject with a therapeutically effective amount of a cancer therapy if the measured levels of CDK6 and optionally the ratio of CDK4 to CDK6 indicate that the subject is a candidate for receiving the cancer therapy.

In some embodiments of the third and fourth aspects, the cancer therapy is one or more agents selected from a group consisting of abemaciclib (Verzenio), palbociclib (Ibrance), ribociclib (Kisqali), trilaciclib (G1T28), G1T38, and the group of CDK4/6 inhibitor compounds referred to in International Patent Application Publications WO2016040858, WO2011130232, WO2011101409, WO2016025650, and WO2013006532, MS140, the PROTACs referred to in **FIGs. 14-18**, and the group of bivalent compounds referred to in International Patent Application Publication WO2018106870.

In some embodiments of the third and fourth aspects, the cancer therapy is administered in combination with one or more additional therapeutic regimens selected from the group consisting of surgery, chemotherapy, radiation therapy, hormone therapy, targeted therapy, and immunotherapy. In some embodiments, the targeted therapy is with

one or more agents selected from a group consisting of MEK inhibitors, ERK inhibitors, hormonal therapy, and RAS(G12C) inhibitors.

In some embodiments of the third and fourth aspects, the cancer is a solid tumor cancer or a leukemia. In some embodiments, the cancer is a cancer expressing functional retinoblastoma protein (Rb).

In some embodiments of any of the aspects, the solid tumor cancer is selected from a group consisting of non-small-cell lung carcinoma (NSCLC), lung cancer, breast cancer, ewing sarcoma, central nervous system neoplasm, skin cancer, head and neck cancer, ovarian cancer, colon cancer, anal cancer, stomach cancer, gastrointestinal cancer, fallopian tube cancer, endometrial cancer, cervical cancer, vaginal cancer, vulvar cancer, esophageal cancer, endocrine cancer, thyroid cancer, parathyroid cancer, adrenal cancer, soft tissue sarcoma, urethral cancer, penile cancer, testicular cancer, brain stem glioma, pituitary cancer, adrenocortical cancer, gallbladder cancer, multiple myeloma, cholangiocarcinoma, fibrosarcoma, lymphoma, liver cancer, kidney cancer, bone cancer, bladder cancer, colorectal cancer, endometrial cancer, renal cell cancer, pancreatic cancer, prostate cancer, thyroid cancer, mesothelioma, neuroblastoma, retinoblastoma, and melanoma.

In some embodiments of the third and fourth aspects, the leukemia is acute myeloid leukemia (AML) and T-cell acute lymphocytic leukemia (T-ALL), Mantle Cell Lymphoma (MCL), and Multiple Myeloma (MM).

In a fifth aspect, the disclosure provides a method for predicting prognosis of a human subject with cancer, comprising (a) obtaining a biological sample of the cancer from the subject; (b) determining a level of CDK6 in the biological sample; and (c) comparing the level of CDK6 as determined in (b) with a reference level of CDK6 in a control; wherein a lower level of CDK6 in the biological sample compared to the CDK6 reference level in the control is an indication that the cancer in the subject will be successfully treated with a CDK4/6 inhibitor.

In a sixth aspect, the disclosure provides a method of predicting response to a cancer therapy or predicting disease progression in a human subject with cancer comprising: (a) obtaining a biological sample from the subject; (b) determining levels of

CDK4 and CDK6 in the sample and obtaining the ratio of CDK4/CDK6; (c) based on the determinations of step (b), determining a probability of response to the cancer therapy or a future risk of cancer progression in the subject.

In some embodiments of any of the above aspects, the levels of CDK6 and/or CDK4 are measured by determining one or more of the mRNA levels, cDNA levels and protein levels of CDK6 and/or CDK4.

In a seventh aspect, the disclosure provides a method of identifying a compound capable of treating cancer, or identifying a compound capable of reducing the risk of developing cancer, or identifying a compound capable of reducing the risk of cancer recurrence or development of metastatic cancer, comprising: (a) providing a cell expressing CDK6; (b) contacting the cell with a candidate compound; and (c) determining whether the candidate compound reduces the expression or activity of CDK6; wherein the reduction observed in the presence of the compound indicates that the compound is capable of treating cancer, or reducing the risk of developing cancer, or reducing the risk of cancer recurrence or development of metastatic cancer.

In another aspect, the disclosure provides a kit comprising means for quantifying the levels of CDK6 and/or CDK4. The kit comprises reagents for specifically measuring the levels of CDK6 and/or CDK4 in a biological sample. The reagents are nucleic acid molecules or antibodies.

The details of one or more embodiments of this disclosure are set forth in the accompanying drawings and the description below. Other features, objects, and advantages of the disclosure will be apparent from the description and drawings, and from the claims.

DESCRIPTION OF DRAWINGS

FIG. 1A shows a bar graph of substantial growth inhibition (GI_{90}) values based on cell growth crystal violet assays for the indicated cell lines treated with increasing concentrations of Palbociclib (PB) for 10-15 days. The CDK4/6i-sensitive or resistant cell lines are indicated.

FIG. 1B shows immunoblots of the indicated antibodies in lysates of the indicated cell lines treated with 1 μM PB for 24, 48 and 72 hr.

FIG. 1C shows immunoblots of the indicated antibodies in lysates of the indicated cell lines treated with 3 μM Ribociclib (RB) for 24, 48 and 72 hr.

FIG. 1D shows immunoblots of the indicated antibodies in lysates of the indicated cell lines treated with 0.3 μM Abemaciclib (AB) for 24, 48 and 72 hr.

FIG. 1E shows immunoblots of the indicated antibodies for cell cycle regulators (pRb, CDK6, CDK4, pT172-CDK4, CRBN, CDK2, cyclins D1-D3, cyclin E2, p21, p27, p16) and the control (actin) in lysates of the indicated cell lines.

FIG. 2A shows A549 and A375 cell lines expressing Doxycycline (Dox)-inducible short hairpin CDK4 (shCDK4) or shCDK6 treated with 0.1 μg/ml doxycycline for 36 hr, followed by the indicated concentrations of PB for 24 hr. Cell lysates were immunoblotted with the indicated antibodies for cell cycle regulators and the control (actin).

FIG. 2B shows cell growth crystal violet assay for A549 and A375 cell lines expressing Dox-inducible shCDK4 or shCDK6 in the presence or absence of 0.1 μg/ml doxycycline and increasing concentrations of PB for 10 days.

FIG. 2C top panel shows cell growth crystal violet assay for the indicated cell lines (A549, SK-MEL-2, Calu-6, and HCT116) expressing Dox-inducible shCDK4 or shCDK6 in the presence or absence of 0.1 μg/ml doxycycline for 10 days followed by crystal violet staining. The bottom panel shows immunoblots of the indicated antibodies for cell cycle regulators and control (actin) in lysates of the indicated cell lines treated with or without 0.1 μg/ml doxycycline for 72 hr.

FIG. 2D top panel shows cell growth crystal violet assay for the indicated cell lines (A375, A549, Calu-6, and HCT116) expressing Dox-inducible shCDK6 or shRNA-resistant form of CDK6 (shr-CDK6) in the presence or absence of 0.05 μg/ml doxycycline for 10 days followed by crystal violet staining. The bottom panel shows immunoblots of the indicated antibodies for cell cycle regulators and control (actin) in lysates of the indicated cell lines treated with or without 0.05 μg/ml doxycycline for 72 hr.

FIG. 2E top panel shows cell scatterplots of DEMETER score (DepMap RNAi; DEMETER2 Data v5) and expression for CDK4 and CDK6. The bottom panel shows scatterplots of CERES (DepMap CRISPR;Public 20Q1) score and the mass spectrometry-based proteomics levels of CDK4 and CDK6 (Nusinow DP et al, 2020). All expression values are in log2(TPM +1). Proteomic levels are shown as normalized log2-transformed ratios to the bridge sample in each Tandem Mass Tags (TMT) 10-plex as previously described (Nusinow DP and Gygi SP, 2020; BIORXIV: doi:10.1101/2020.02.03.932384). Cell lines harboring COSMIC hotspot mutations to RB1 are annotated in light dots. P-values were calculated based on linear regression analysis.

FIG. 3A shows immunoblots of the indicated antibodies for cell cycle regulators and control (actin) in lysates of the indicated NSCLC cell lines transfected with control short interfering RNA (siRNA), siCDK4 and siCDK6 for 72 hr. H1792, H2087, H2291, HCC827, and H1915, are CDK4/6i-sensitive cell lines. Calu6, and HC1666 are CDK4/6i-resistant cell lines.

FIG. 3B shows immunoblots of the indicated antibodies for cell cycle regulators and control (actin) in lysates of the indicated NSCLC cell lines. The ratio of CDK4:CDK6 in the various cell lines is also indicated. MCF7, H1792, H358, H2087, H2291, and HCC827 are CDK4/6i-sensitive cell lines. Calu6, A549, PC9 and HC1666 are CDK4/6i-resistant cell lines.

FIG. 3C shows immunoblots of the indicated antibodies for cell cycle regulators in lysates of the indicated NSCLC cell lines treated with increasing concentrations of PB for 24 hr. H358, H2291, H2087, H1792, and HCC827 are CDK4/6i-sensitive cell lines. PC9 is a CDK4/6i-resistant cell line.

FIG. 3D shows cell growth crystal violet assays for the indicated NSCLC cell lines treated with increasing concentrations of PB for 10-15 days and stained with crystal violet. H358, H2291, H2087, H1792, and HCC827 are CDK4/6i-sensitive cell lines. PC9 is a CDK4/6i-resistant cell line.

FIG. 3E shows progression-free survival analysis of NSCLC/RAS-mutant patients that received abemaciclib in the JUNIPER trial based on CDK6-low versus

CDK6-medium/high tumors. ITT: Translational Research population; Abema: Abemaciclib

FIG. 3F shows overall survival analysis of NSCLC/RAS-mutant patients that received abemaciclib in the JUNIPER trial based on CDK6-low versus CDK6-medium/high tumors. HR: Hazard Ratio; ITT: Translational Research population; Abema: Abemaciclib

FIG. 4A shows the chemical structure of the bifunctional CDK4/6 inhibitor-degrader MS140.

FIG. 4B shows the IC_{50} of *in vitro* kinase activity assays for PB and MS140 against CDK4/cyclin D1 and CDK6/cyclin D1.

FIG. 4C shows immunoblots of the indicated antibodies for CDK4, CDK6 and control (actin) in lysates of Colo205 cells pretreated with either the proteasome inhibitor 100 nM bortezomib (BOR), 10 μM PB, 10 μM pomalidomide (POM), the Nedd8-activating enzyme inhibitor 1 μM MLN4924 (MLN), or control (DMSO) for 4 hr, followed by treatment with MS140 (100 nM/3 hr).

FIG. 4D shows immunoblots of the indicated antibodies for CDK4, CRBN and control (actin) in lysates of ZR-75-1 wild-type and CRBN-deficient cells (ZR-75-1 sgCRBN) treated with the indicated concentrations of MS140 for 5 hr.

FIG. 4E shows immunoblots of the indicated antibodies and control (actin) in lysates of Colo205 cells treated with DMSO (control), MS140-ve and MS140 for 5 hr.

FIG. 4F shows a volcano plot of the protein log2 ratios which represents the quantitative dynamics of 4,822 proteins in MS140 and MS140 negative control (140-ve) treated Colo205 samples (0.3 μM, 5 hr). The experiment was conducted in duplicate.

FIG. 4G shows a cell growth crystal violet assay in the presence of varying concentrations of PB or MS140 for 10-15 days.

FIG. 4H shows immunoblots of the indicated antibodies and control (actin) in lysates of the indicated cell lines treated with increasing concentrations of PB or MS140 for 24 hr.

FIG. 4I shows immunoblots of the indicated antibodies and control (actin) in lysates of KMS-12-PE and Pfeiffer cells treated with increasing concentrations of PB or MS140 for 24 hr. Lysates were immunoblotted with the indicated antibodies.

FIG. 4J shows cell viability of KMS-12-PE and Pfeiffer cells treated with PB or MS140 for 72-96 hr. Cell viability was assayed using 0.1 mg/ml resazurin solution. IC_{50} values were determined by nonlinear regression curve fit in Graphpad Prism in six replicates.

FIG. 4K shows immunoblots of the indicated antibodies and control (actin) in lysates of tumor samples from mice carrying JeKo-1 xenografts. The mice were treated with vehicle or MS140 (25 mg/kg, b.i.d) or PB (50 mg/kg, q.d.) for 3 days.

FIG. 4L shows scatter plot of fold change in tumor growth in JeKo-1 tumor xenografts in mice treated with vehicle, MS140 (25 mg/kg, b.i.d) or PB (50 mg/kg, q.d.) for 21 days. Each treatment contained 8 animals (n=8). Data represent mean ± S.D., paired two-tailed t-test.

FIG. 5A shows cell growth crystal violet assays for the indicated cell lines treated with increasing concentrations of PB or MS140 for 10-12 days.

FIG. 5B shows immunoblots of the indicated antibodies in lysates of A375, SKMEL2 and Calu6 cells were treated with increasing concentrations of PB or MS140 for 24 hr.

FIG. 5C shows immunoblots of the indicated antibodies in lysates of A549 or A375 expressing Dox-inducible shCDK6 were treated with either the indicated concentrations of doxycycline (A375: 0.1 μg/ml) or MS140 for 72 hr.

FIG. 5D shows cell growth crystal violet assays for the indicated cell lines treated with either doxycycline or MS140 for 10 days. Colonies were stained with crystal violet.

FIG. 5E shows immunoblots of the indicated antibodies in lysates of Rb-proficient cell lines treated with MS140 (3 nM) for the indicated time points.

FIG. 5F shows graphs of relative band intensities (%) derived by immunoblot analysis of CDK6 and actin expression using Image J. The indicated cell lines were treated with PB (1 μM/2 hr) followed by a Cellular Thermal Shift Assay (CETSA).

FIG. 5G shows graphs of relative band intensities (%) derived by immunoblot analysis of CDK6 and actin expression using Image J. The indicated cell lines were treated with MS140-ve (15 μM/2 hr) followed by CETSA assay.

FIG. 5H shows immunoblots of the indicated antibodies in lysates of MV4-11 and SKMEL2 cells pretreated with increasing concentrations of PB for 2 hr and the lysates subjected to a desthiobiotin-ADP enrichment assay for CDK6.

FIG. 6A shows immunoblots of the indicated antibodies for cell cycle regulators in lysates from CDK6-dependent cell lines.

FIG. 6B top panel shows a volcano plot of the CDK6-interacting proteins in KMS-12-PE and Calu6. Proteins in large circles were annotated as HSP90/CDC37-related. The bottom panel shows a comparison of total peptide-spectrum match (PSM) for the HSP90 protein family and CDC37 in KMS-12-PE and Calu6.

FIG. 6C shows immunoblots from the lysates of indicated cell lines which were either subjected to co-immunoprecipitation with a CDK6 antibody followed by immunoblotting with HSP90, CDC37 and CDK6, or immunoblotted with the indicated antibodies.

FIG. 6D shows immunoblots of the indicated antibodies in lysates of cell lines treated with the HSP90 inhibitor Ganetespib (GAN, 30 nM) at the indicated time points.

FIG. 6E shows immunoblots representing CDK6 thermal stability assay (CETSA) in lysates of CDK4/6i-sensitive (KMS-12-PE, MV4-11, Pfeiffer, and Colo205) and CDK4/6i-resistant (Calu6, A375, A549, SKMEL2 and the Rb-null BT549) cell lines heat-treated at increasing temperature end points.

FIG. 6F shows immunoblots of the indicated antibodies in lysates of indicated cell lines treated with 100 μg/ml CHX at the indicated time points.

FIG. 6G shows immunoblots of the indicated antibodies in lysates of A375 cells transfected with either WT or CDK6 (S178P) followed by treatment with increasing concentrations of MS140 for 24 hr.

FIG. 6H shows relative mRNA expression by qPCR analysis of the indicated Rb/E2F target genes in tumors, kidney and liver from mice bearing JeKo-1 tumors treated

with vehicle or MS140 (25 mg/kg, b.i.d) or PB (50 mg/kg, q.d.) for 3 days. Data represents mean ± S.D. of triplicates.

FIG. 6I shows neutrophil counts in C57BL/6 mice before treatment and post treatment with PB (50 mg/kg, q.d., n=8) or MS140 (25 mg/kg, b.i.d., n=7) for 21 days. Data represent mean ± S.D., paired two-tailed t-test.

FIG. 7 shows a model of CDK6 association with the HSP90 complex affecting tumor cell sensitivity to CDK4/6 inhibitors and degraders. **FIG. 7A** depicts that in CDK4/6 inhibitor and degrader-sensitive cells, CDK6 is associated with the HSP90 complex. CDK4/6 inhibitors or CDK6 degraders bind strongly to CDK6, and promote CDK6 inhibition or both CDK6 inhibition and degradation respectively. **FIG. 7B** depicts that in CDK4/6 inhibitor and degrader-resistant cells, CDK6 is weakly associated with the HSP90 complex. In these cells, CDK4/6 inhibitors and degraders bind CDK6 weakly, and thus fail to promote CDK6 inhibition or both inhibition and degradation, respectively.

FIG. 8A shows cell growth crystal violet assays for the indicated cell lines treated with increasing concentrations of PB for 10-16 days and stained with crystal violet. CDK4/6i-sensitive cell lines are in the left panel, CDK4/6i-resistant cell lines are in the right panel.

FIG. 8B shows immunoblots of the indicated antibodies in lysates from MCF7 and HCT116 cell lines treated with 1 μM PB for 24, 48 and 72 hr.

FIG. 8C shows immunoblots of the indicated antibodies in lysates from Colo205 cells were treated with 1 μM PB at the indicated time points.

FIG. 9A shows immunoblots of the indicated antibodies in lysates from A673 and TC-71 cells transfected with non-targeting control or siCDK4 or siCDK6 for 72 hr.

FIG. 9B shows the relationship between CDK4 and CDK6 expression (CCLE RNA-seq) and DepMap CRISPR–Cas9 single-gene knockout scores (CERES; 20Q1 public dataset). All expression values are in log2(TPM +1). Cell lines harboring COSMIC hotspot mutations to RB1 are annotated in light dots. P-values were calculated based on linear regression analysis.

FIG. 10A shows immunoblots of the indicated antibodies in lysates from cell lines treated with increasing concentrations of PB for 24 hr.

FIG. 10B shows GI_{90} values of PB and CDK4/6 dependency in NSCLC cell lines. Cell lines NCIH358, NCIH2087, NCIH1792, NCIH2291, AND HCC827 are CDK4/6-sensitive cell lines; Calu6, A549, H1666, and PC9 are CDK4/6-resistant cell lines.

FIG. 11A shows immunoblots of the indicated antibodies in lysates from Colo205 cells were treated with increasing concentrations of MS140 for 5 hr.

FIG. 11B shows immunoblots of the indicated antibodies in lysates from Colo205 treated with MS140 (0.5 μM) for the indicated time points.

FIG. 11C shows immunoblots of the indicated antibodies in lysates from T47D cells pretreated with either the proteasome inhibitor 100 nM bortezomib (BOR),10 μM PB, 10 μM pomalidomide (POM) or 1 μM MLN4924 (MLN) for 4 hr, followed by treatment with MS140 (100 nM/3 hr).

FIG. 11D shows the chemical structure of the MS140 negative control (MS140-ve) that does not bind CRBN.

FIG. 11E shows immunoblots of the indicated antibodies in lysates from U87MG and MCF7 treated with increasing concentrations of PB or MS140 for 24 hr.

FIG. 11F shows immunoblots of the indicated antibodies in lysates from H358 cells treated with increasing concentrations of MS140 for 24 hr.

FIG. 11G shows cell viability (%) of Mantle Cell Lymphoma (MCL) cells treated with PB or MS140 for 72-96 hr. The cell viability was assayed using 0.1 mg/ml resazurin solution. IC_{50} values were determined by nonlinear regression curve fit in Graphpad Prism in six replicates.

FIG. 11H shows immunoblots of the indicated antibodies in lysates from MCL cell lines treated with increasing concentrations of PB or MS140 for 24 hr.

FIG. 11I shows immunoblots of the indicated antibodies in lysates from MCL cell lines treated with 0.1 μM PB or MS140 at different time points.

FIG. 11J shows GI_{50} values of PB and MS140 in hematologic cancer cell lines.

FIG. 11K shows immunoblots of the indicated antibodies in lysates from tumor samples in mice carrying Colo205 xenografts. The mice were treated with vehicle or MS140 (30 mg/kg, b.i.d) for 3 days.

FIG. 11L shows scatter plot of fold change for an efficacy assay in Colo205 tumor xenografts in nude mice treated with vehicle or MS140 (30 mg/kg, b.i.d) for 21 days. Vehicle treatment contained 5 animals (n=5). MS140 treatment contained 8 animals (n=8). Data represent mean ± S.D., unpaired two-tailed t-test.

FIG. 11M shows body weight in mice bearing JeKo-1 tumors treated with vehicle (n=8) or PB (50 mg/kg, q.d., n=8) or MS140 (25 mg/kg, b.i.d., n=8) over the course of the experiment (21 days).

FIG. 12A shows immunoblots of the indicated antibodies in lysates from MV4-11 and A375 treated with MS140 (3 nM) or YKL-06-102 (3 nM) or BSJ-02-162 (3nM) at different time points.

FIG. 12B shows immunoblots of the indicated antibodies in lysates from KMS-12-PE and Calu6 treated MS140 (3 nM) or YKL-06-102 (3 nM) or BSJ-02-162 (3 nM) at different time points. Lysates were subjected to immunoblotting with the indicated antibodies.

FIG. 13A shows immunoblots of the indicated antibodies in lysates from the indicated cell lines were either subjected to co-immunoprecipitation with a CDK6 antibody followed by immunoblotting with or immunoblotted with the indicated antibodies.

FIG. 13B shows immunoblots of the indicated antibodies in lysates from the indicated cell lines treated with increasing concentrations of Ganetespib (GAN) for 24 hr.

FIG. 13C shows immunoblots of the indicated antibodies in lysates from the indicated cell lines treated with 40 nM Luminespib (LUM) at the indicated time points.

FIG. 13D shows immunoblots of the indicated antibodies in lysates from CDK4-dependent cell lines treated with 30 nM GAN for the indicated time points.

FIG. 13E shows immunoblots of the indicated antibodies in lysates from KMS-12-PE and Calu6 treated with increasing concentrations of GAN for 24 hr.

FIG. 13F shows immunoblots of the indicated antibodies in lysates from A375 cells ectopically expressing V5-CDK6 or V5-CDK6 S178p immunoprecipitated with a V5 antibody. The immunoprecipitates were subjected to kinase assay with recombinant Rb protein as substrate.

FIG. 13G shows immunoblots of the indicated antibodies in lysates from KMS-12-PE and BT549 cells treated with increasing concentrations of MS140 for 24 hr. Lysates were subjected to immunoblotting with the indicated antibodies.

FIG. 13H shows white blood cell, red blood cell and lymphocytes counts in C57BL/6 mice before treatment and post treatment with PB (50 mg/kg, q.d., n=8) or MS140 (25 mg/kg, b.i.d., n=7) for 21 days. Data represent mean ± S.D., paired two-tailed t-test.

FIG. 14A shows the chemical structures of various palbociclib-based PROTACs. The linkers are depicted for each named structure.

FIG. 14B shows the chemical structures of various ribociclib-based PROTACs. The linkers are depicted for each named structure.

FIG. 14C shows the chemical structures of abemaciclib-based PROTACs. The alternative linkers are depicted for the named PROTAC.

FIG. 15 shows the chemical structures of various palbociclib-based PROTACs. The linkers are depicted for each named structure.

FIG. 16 shows the chemical structures of various palbociclib-based PROTACs. The linkers are depicted for each named structure.

FIG. 17 shows the chemical structures of various palbociclib-based PROTACs. The linkers are depicted for each named structure.

FIG. 18 shows the chemical structures of three different PROTACs.

Like reference symbols in the various drawings indicate like elements.

DETAILED DESCRIPTION

This disclosure is based on the surprising findings that tumor response to CDK4/6i is critically determined by the expression of CDK6. Tumors expressing low levels of CDK6 are driven by CDK4, and are sensitive to CDK4/6i. When both CDK4 and CDK6 are expressed, tumors are selectively dependent on CDK6, which is expressed as either sensitive or resistant to CDK4/6i.

Unless otherwise defined herein, scientific and technical terms used in this application shall have the meanings that are commonly understood by those of ordinary skill in the art. Generally, nomenclature used in connection with, and techniques of, pharmacology, cell and tissue culture, molecular biology, cell and cancer biology, neurobiology, neurochemistry, virology, immunology, microbiology, genetics and protein and nucleic acid chemistry, described herein, are those well-known and commonly used in the art. In case of conflict, the present specification, including definitions, will control.

The practice of the present application will employ, unless otherwise indicated, conventional techniques of molecular biology (including recombinant techniques), microbiology, cell biology, biochemistry and immunology, which are within the skill of the art. Such techniques are explained fully in the literature, such as, Molecular Cloning: A Laboratory Manual, second edition (Sambrook et al., 1989) Cold Spring Harbor Press; Oligonucleotide Synthesis (M.J. Gait, ed., 1984); Methods in Molecular Biology, Humana Press; Cell Biology: A Laboratory Notebook (J.E. Cellis, ed., 1998) Academic Press; Animal Cell Culture (R.I. Freshney, ed., 1987); Introduction to Cell and Tissue Culture (J.P. Mather and P.E. Roberts, 1998) Plenum Press; Cell and Tissue Culture: Laboratory Procedures (A. Doyle, J.B. Griffiths, and D.G. Newell, eds., 1993-1998) J. Wiley and Sons; Methods in Enzymology (Academic Press, Inc.); Gene Transfer Vectors for Mammalian Cells (J.M. Miller and M.P. Calos, eds., 1987); Current Protocols in Molecular Biology (F.M. Ausubel et al., eds., 1987); PCR: The Polymerase Chain Reaction, (Mullis et al., eds., 1994); Sambrook and Russell, Molecular Cloning: A Laboratory Manual, 3rd. ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (2001); Ausubel et al., Current Protocols in Molecular Biology, John Wiley & Sons, NY (2002); Harlow and Lane Using Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1998); Coligan et al., Short Protocols in Protein Science, John Wiley & Sons, NY (2003); Short Protocols in Molecular Biology (Wiley and Sons, 1999).

The nomenclatures used in connection with, and the laboratory procedures and techniques of biochemistry, immunology, microbiology, molecular biology, and virology described herein are those well-known and commonly used in the art.

Throughout this specification and embodiments, the word comprise, or variations such as comprises or comprising, will be understood to imply the inclusion of a stated integer or group of integers but not the exclusion of any other integer or group of integers.

It is understood that wherever embodiments are described herein with the language "comprising," otherwise analogous embodiments described in terms of "consisting of" and/or "consisting essentially of" are also provided.

The term "including" is used to mean "including but not limited to." "Including" and "including but not limited to" are used interchangeably.

Any example(s) following the term "e.g." or "for example" is not meant to be exhaustive or limiting.

Unless otherwise required by context, singular terms shall include pluralities and plural terms shall include the singular.

The articles a, an, and "the" are used herein to refer to one or to more than one (i.e., to at least one) of the grammatical object of the article. By way of example, an element means one element or more than one element. Reference to "about" a value or parameter herein includes (and describes) embodiments that are directed to that value or parameter per se. For example, description referring to "about X" includes description of "X." Numeric ranges are inclusive of the numbers defining the range. As used herein, the term "about" permits a variation of ±10% within the range of the significant digit.

Notwithstanding that the disclosed numerical ranges and parameters are approximations, the numerical values set forth in the specific examples are reported as precisely as possible. Any numerical value, however, inherently contains certain errors necessarily resulting from the standard deviation found in their respective testing measurements. Moreover, all ranges disclosed herein are to be understood to encompass any and all subranges subsumed therein. For example, a stated range of "1 to 10" should be considered to include any and all subranges between (and inclusive of) the minimum value of 1 and the maximum value of 10; that is, all subranges beginning with a minimum value of 1 or more, e.g., 1 to 6.1, and ending with a maximum value of 10 or less, e.g., 5.5 to 10.

Where aspects or embodiments are described in terms of a Markush group or other grouping of alternatives, the present application encompasses not only the entire group listed as a whole, but each member of the group individually and all possible subgroups of the main group, and also the main group absent one or more of the group members. The present application also envisages the explicit exclusion of one or more of any of the group members in the Markush group or other grouping of alternatives.

Exemplary methods and materials are described herein, although methods and materials similar or equivalent to those described herein can also be used in the practice or testing of the various aspects and embodiments. The materials, methods, and examples are illustrative only and not intended to be limiting.

Definitions

In order that the disclosure may be more readily understood, certain terms are first defined. These definitions should be read in light of the remainder of the disclosure and as understood by a person of ordinary skill in the art. Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by a person of ordinary skill in the art. Additional definitions are set forth throughout the detailed description.

As used herein, and unless otherwise specified, the terms “treat”, “treating” and “treatment” and variations thereof refer to partially or completely alleviating, inhibiting, ameliorating, or relieving the disease or condition from which the subject is suffering. This means any manner in which one or more of the symptoms of a disease or disorder (e.g., cancer) are ameliorated or otherwise beneficially altered.

As used herein, amelioration of the symptoms of a particular disorder (e.g., cancer) refers to any lessening, whether permanent or temporary, lasting or transient that can be attributed to or associated with treatment by the compositions and methods of the present disclosure. In some embodiments, the treatment can promote or result in, for example, a decrease in the number of tumor cells (e.g., in a subject) relative to the number of tumor cells prior to treatment; a decrease in the viability (e.g., the average/mean viability) of tumor cells (e.g., in a subject) relative to the viability of tumor

cells prior to treatment; a decrease in the rate of growth of tumor cells; a decrease in the rate of local or distant tumor metastasis; or reductions in one or more symptoms associated with one or more tumors in a subject relative to the subject's symptoms prior to treatment.

As used herein, the term "treating cancer" or "treating a tumor" means causing a partial or complete decrease in the rate of growth of a tumor, and/or in the size of the tumor and/or in the rate of local or distant tumor metastasis, and/or the overall tumor burden in a subject, and/or any decrease in tumor survival, in the presence of a compound (e.g., an CDK4/6 inhibitor/degrader) described herein.

In certain embodiments, "treat" and its variations refers to slowing the progression or reversing the progression of cancer relative to an untreated control. Exemplary CDK4/6-mediated cancers that can be treated with the methods of this disclosure, include but are not limited to, solid tumors (e.g., breast cancer (e.g., ER+ breast cancer) and prostate cancer), leukemia (e.g., acute lymphoblastic leukemia, acute myeloid leukemia, chronic lymphocytic leukemia, chronic myelogenous leukemia, hairy cell leukemia, and myeloid leukemia), lymphoma (e.g., Burkitt's lymphoma, cutaneous T-cell lymphoma, diffuse large B-cell lymphoma (DLBCL), follicular lymphoma (FL), Hodgkin's lymphoma, mantel cell lymphoma, and non-Hodgkin's lymphoma (NHL)), adrenocortical cancer, AIDS- related cancer, anal cancer, astrocytoma, basal cell carcinoma, skin cancer (non-melanoma), bile duct cancer, bladder cancer, bone cancer (e.g., fibrosarcoma/osteosarcoma/malignant fibrous histiocytoma), brain tumor (e.g., cerebral astrocytoma, ependymoma, glioma, medulloblastoma, and supratentorial primitive neuroectodermal tumors (PNETs)), brainstem glioma, bronchial adenomas/carcinoids, carcinoid tumors, central nervous system neoplasms, cervical cancer, cholangiocarcinoma, chronic myeloproliferative disorder, colon cancer, endometrial cancer, esophageal cancer, melanoma (e.g., cutaneous or intraocular), gallbladder cancer, gastrointestinal cancer (e.g., colorectal, duodenal, and gastric (stomach) cancer), germ cell tumors, head and neck cancer, hepatocellular (liver) cancer, hypopharyngeal cancer, islet cell carcinoma, Kaposi's sarcoma, kidney (renal cell) cancer, laryngeal cancer, lip and oral cavity cancer, lung cancer (small cell and non-small cell),

Merkel cell carcinoma, mesothelioma, endocrine cancer (e.g., multiple endocrine neoplasia syndrome), multiple myeloma/plasma cell neoplasm mycosis fungoides, myelodysplastic syndrome, myeloproliferative disorders, nasal cavity and paranasal sinus cancer, nasopharyngeal cancer, neuroblastoma, oropharyngeal cancer, ovarian cancer, pancreatic cancer, parathyroid cancer, penile cancer, pituitary cancer, pleuropulmonary blastoma, rectal cancer, retinoblastoma, rhabdomyosarcoma, salivary gland cancer, Ewing sarcoma, soft tissue sarcoma, Sezary syndrome, squamous cell carcinoma, squamous neck cancer, synovial sarcoma, testicular cancer, thymoma, thymic carcinoma, thyroid cancer, transitional cell cancer, trophoblastic tumors, urethral cancer, uterine cancer, fallopian tube cancer, vaginal cancer, visual pathway and hypothalamic glioma, vulvar cancer, Waldenstrom's macroglobulinemia, and Wilms' tumor.

As used herein, the term "biological sample" refers to a sample obtained from a subject. As used herein, biological samples include any clinical samples useful in the detection of cancer in subjects, including, but not limited to, cells, tissues, and bodily fluids, such as: blood; derivatives and fractions of blood, such as serum; biopsied or surgically removed tissue, including tissues that are, for example, unfixed, frozen, fixed in formalin and/or embedded in paraffin; swabs; skin scrapes; urine; sputum; cerebrospinal fluid; prostate fluid; pus; or bone marrow aspirates. For example, a sample includes a solid tumor biopsy obtained from a human subject. In another instance, a sample includes cells, for example a group of cells collected as part of a tissue section.

As used herein, the term "expressed" or "expression" refers to the transcription from a gene to a ribonucleic acid (RNA) molecule at least complementary in part to a region of one of the two nucleic acid strands of the gene. Alternatively, the term "expressed" or "expression" may refer to the translation from the RNA molecule to give a protein, a polypeptide or a portion thereof.

1. Determining Levels of CDK6 and/or CDK4

CDK6 and/or CDK4 gene expression can be detected as, e.g., protein or RNA expression of a target gene. That is, the presence or expression level (amount) of a gene can be determined by detecting and/or measuring the level of mRNA or protein

expression of the gene. In some embodiments, gene expression can be detected as the activity of a protein encoded by the CDK6 gene. In some embodiments, gene expression can be detected as the activity of a protein encoded by the CDK4 gene.

In one embodiment, the expression of a gene (e.g., CDK6 and/or CDK4 gene) can be determined by detecting and/or measuring expression or concentration of a protein encoded by the gene. Methods of determining protein expression/concentration are well known in the art. A generally used method involves the use of antibodies specific for the target protein of interest. For example, methods of determining protein expression include, but are not limited to, western blot or dot blot analysis, immunohistochemistry (e.g., quantitative immunohistochemistry), immunocytochemistry, enzyme-linked immunosorbent assay (ELISA), enzyme-linked immunosorbent spot (ELISPOT; Coligan, J. E., et al., eds. (1995) Current Protocols in Immunology. Wiley, New York), radioimmunoassay, chemiluminescent immunoassay, electrochemiluminescence immunoassay, latex turbidimetric immunoassay, latex photometric immunoassay, immuno-chromatographic assay, and antibody array analysis (see, e.g., U.S. Publication Nos. 2003/0013208 and 2004/171068, the disclosures of each of which are incorporated herein by reference in their entirety). Further description of many of the methods above and additional methods for detecting protein expression can be found in, e.g., Sambrook et al. (supra).

In one example, the presence or amount of CDK6 and/or CDK4 protein expression of the CDK6 and/or CDK4 gene can be determined using a western blotting technique. For example, a lysate can be prepared from a surface skin sample, or the surface skin sample itself, can be contacted with Laemmli buffer and subjected to sodium-dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE-resolved proteins, separated by size, can then be transferred to a filter membrane (e.g., nitrocellulose) and subjected to immunoblotting techniques using a detectably-labeled antibody specific to the protein of interest. The presence or amount of bound detectably-labeled antibody indicates the presence or amount of protein in the surface skin sample.

In one embodiment, the SimplePlex platform is used to measure the levels of CDK6 and/or CDK4. SimplePlex is commercially available from Protein Simple (San Jose, CA, USA) (See Dysinger M, et al. J. Immunol. Methods. 451:1-10, 2017).

In another example, an immunoassay can be used for detecting and/or measuring the protein expression of a gene (e.g., CDK6 and/or CDK4 genes). As above, for the purposes of detection, an immunoassay can be performed with an antibody that bears a detection moiety (e.g., a fluorescent agent or enzyme). Proteins from a surface skin sample can be conjugated directly to a solid-phase matrix (e.g., a multi-well assay plate, nitrocellulose, agarose, sepharose, encoded particles, or magnetic beads) or it can be conjugated to a first member of a specific binding pair (e.g., biotin or streptavidin) that attaches to a solid-phase matrix upon binding to a second member of the specific binding pair (e.g., streptavidin or biotin). Such attachment to a solid-phase matrix allows the proteins to be purified away from other interfering or irrelevant components of the surface skin sample prior to contact with the detection antibody and also allows for subsequent washing of unbound antibody. Here, as above, the presence or amount of bound detectably-labeled antibody indicates the presence or amount of protein in the surface skin sample.

There is no particular restriction as to the form of the antibody and the present disclosure includes polyclonal antibodies, as well as monoclonal antibodies. The antiserum obtained by immunizing animals, such as rabbits with a protein or fragment thereof of this disclosure (i.e., a protein or an immunological fragment thereof of CDK6 and/or CDK4 protein), as well polyclonal and monoclonal antibodies of all classes, human antibodies, and humanized antibodies produced by genetic recombination, are also included.

An intact protein or its partial peptide may be used as the antigen for immunization. As partial peptides of the proteins, for example, the amino (N)-terminal fragment of the protein and the carboxy (C)-terminal fragment can be given.

A gene encoding a protein of interest (e.g., CDK6 and/or CDK4) or a fragment thereof (e.g., an immunological fragment) is inserted into a known expression vector, and, by transforming the host cells with the vector described herein, the desired protein or

a fragment thereof is recovered from outside or inside the host cells using standard methods. This protein can be used as the sensitizing antigen. Also, cells expressing the protein, cell lysates, or a chemically synthesized protein of the disclosures may be also used as a sensitizing antigen.

The mammal that is immunized by the sensitizing antigen is not restricted; however, it is preferable to select animals by considering the compatibility with the parent cells used in cell fusion. Generally, animals belonging to the orders rodentia, lagomorpha, or primates are used. Examples of animals belonging to the order of rodentia that may be used include, for example, mice, rats, and hamsters. Examples of animals belonging to the order of lagomorpha that may be used include, for example, rabbits. Examples of animals belonging to the order of primates that may be used include, for example, monkeys. Examples of monkeys to be used include the infraorder catarrhini (old world monkeys), for example, Macaca fascicularis, rhesus monkeys, sacred baboons, and chimpanzees.

Well-known methods may be used to immunize animals with the sensitizing antigen. For example, the sensitizing antigen is injected intraperitoneally or subcutaneously into mammals. Specifically, the sensitizing antigen is suitably diluted and suspended in physiological saline, phosphate-buffered saline (PBS), and so on, and mixed with a suitable amount of general adjuvant if desired, for example, with Freund's complete adjuvant. Then, the solution is emulsified and injected into the mammal. Thereafter, the sensitizing antigen suitably mixed with Freund's incomplete adjuvant is preferably given several times every 4 to 21 days. A suitable carrier can also be used when immunizing and animal with the sensitizing antigen. After the immunization, the elevation in the level of serum antibody is detected by usual methods.

Polyclonal antibodies against the proteins of the present disclosure can be prepared as follows. After verifying that the desired serum antibody level has been reached, blood is withdrawn from the mammal sensitized with antigen. Serum is isolated from this blood using conventional methods. The serum containing the polyclonal antibody may be used as the polyclonal antibody, or according to needs, the polyclonal antibody-containing fraction may be further isolated from the serum. For example, a

fraction of antibodies that specifically recognize the protein of the invention may be prepared by using an affinity column to which the protein is coupled. Then, the fraction may be further purified by using a Protein A or Protein G column in order to prepare immunoglobulin G or M.

To obtain monoclonal antibodies, after verifying that the desired serum antibody level has been reached in the mammal sensitized with the above-described antigen, immunocytes are taken from the mammal and used for cell fusion. For this purpose, splenocytes can be mentioned as preferable immunocytes. As parent cells fused with the above immunocytes, mammalian myeloma cells are preferably used. More preferably, myeloma cells that have acquired the feature, which can be used to distinguish fusion cells by agents, are used as the parent cell.

The cell fusion between the above immunocytes and myeloma cells can be conducted according to known methods, for example, the method by Milstein et al. (Galfre et al., Methods Enzymol. 73:3-46, 1981).

The hybridoma obtained from cell fusion is selected by culturing the cells in a standard selection medium, for example, HAT culture medium (medium containing hypoxanthine, aminopterin, and thymidine). The culture in this HAT medium is continued for a period sufficient enough for cells (non-fusion cells) other than the objective hybridoma to perish, usually from a few days to a few weeks. Then, the usual limiting dilution method is carried out, and the hybridoma producing the objective antibody is screened and cloned.

Other than the above method for obtaining hybridomas, by immunizing an animal other than humans with the antigen, a hybridoma producing the objective human antibodies having the activity to bind to proteins can be obtained by the method of sensitizing human lymphocytes, for example, human lymphocytes infected with the EB virus, with proteins, protein-expressing cells, or lysates thereof in vitro and fusing the sensitized lymphocytes with myeloma cells derived from human, for example, U266, having a permanent cell division ability.

The monoclonal antibodies obtained by transplanting the obtained hybridomas into the abdominal cavity of a mouse and extracting ascites can be purified by, for

example, ammonium sulfate precipitation, protein A or protein G column, DEAE ion exchange chromatography, an affinity column to which the protein of the present disclosure is coupled, and so on.

Monoclonal antibodies can be also obtained as recombinant antibodies produced by using the genetic engineering technique (see, for example, Borrebaeck C.A.K. and Larrick, J.W., THERAPEUTIC MONOCLONAL ANTIBODIES, Published in the United Kingdom by MACMILLAN PUBLISHERS LTD (1990)). Recombinant antibodies are produced by cloning the encoding DNA from immunocytes, such as hybridoma or antibody-producing sensitized lymphocytes, incorporating into a suitable vector, and introducing this vector into a host to produce the antibody. The present disclosure encompasses such recombinant antibodies as well.

Antibodies or antibody fragments specific for a protein encoded by one or more biomarkers (e.g., CDK6 and/or CDK4) can also be generated by in vitro methods such as phage display. Moreover, the antibody of the present disclosure may be an antibody fragment or modified-antibody, so long as it binds to a protein encoded by a biomarker of the disclosure (CDK6 and/or CDK4). For instance, Fab, F (ab') 2, Fv, or single chain Fv (scFv) in which the H chain Fv and the L chain Fv are suitably linked by a linker (Huston et al., (1998) Proc. Natl. Acad. Sci. USA, 85:5879-5883) can be given as antibody fragments. Specifically, antibody fragments are generated by treating antibodies with enzymes, for example, papain or pepsin. Alternatively, they may be generated by constructing a gene encoding an antibody fragment, introducing this into an expression vector, and expressing this vector in suitable host cells (see, for example, Co et al., J. Immunol., 152:2968-2976, 1994; Better et al., Methods Enzymol., 178:476-496, 1989; Pluckthun et al., Methods Enzymol., 178:497-515, 1989; Lamoyi, Methods Enzymol., 121:652-663, 1986; Rousseaux et al., Methods Enzymol., 121:663-669, 1986; Bird et al., Trends Biotechnol., 9:132-137, 1991).

The antibodies may be conjugated to various molecules, such as fluorescent substances, radioactive substances, and luminescent substances. Methods to attach such moieties to an antibody are already established and conventional in the field (see, e.g., US 5,057,313 and 5,156,840).

Examples of methods that assay the antigen-binding activity of the antibodies include, for example, measurement of absorbance, enzyme-linked immunosorbent assay (ELISA), enzyme immunoassay (EIA), radioimmunoassay (RIA), and/or immunofluorescence. For example, when using ELISA, a protein encoded by a biomarker of the invention is added to a plate coated with the antibodies of the present disclosure, and then, the antibody sample, for example, culture supernatants of antibody-producing cells, or purified antibodies are added. Then, secondary antibody recognizing the primary antibody, which is labeled by alkaline phosphatase and such enzymes, is added, the plate is incubated and washed, and the absorbance is measured to evaluate the antigen-binding activity after adding an enzyme substrate such as p-nitrophenyl phosphate. As the protein, a protein fragment, for example, a fragment comprising a C-terminus, or a fragment comprising an N-terminus may be used. To evaluate the activity of the antibody of the invention, BIAcore (Pharmacia) may be used.

By using these methods, the antibody and a sample presumed to contain a protein of the disclosure are contacted, and the protein encoded by a biomarker of the disclosure is detected or assayed by detecting or assaying the immune complex formed between the above-mentioned antibody and the protein.

Mass spectrometry based quantitation assay methods, for example, but not limited to, multiple reaction monitoring (MRM)-based approaches in combination with stable-isotope labeled internal standards, are an alternative to immunoassays for quantitative measurement of proteins. These approaches do not require the use of antibodies and so the analysis can be performed in a cost- and time- efficient manner (see, for example, Addona et al., Nat. Biotechnol., 27:633–641, 2009; Kuzyk et al., Mol. Cell Proteomics, 8:1860–1877, 2009; Paulovich et al., Proteomics Clin. Appl., 2:1386-1402, 2008). In addition, MRM offers superior multiplexing capabilities, allowing for the simultaneous quantification of numerous proteins in parallel. The basic theory of these methods has been well-established and widely utilized for drug metabolism and pharmacokinetics analysis of small molecules.

In another embodiment, the expression level of CDK6 and/or CDK4 is determined by measuring RNA levels. A variety of suitable methods can be employed to

detect and/or measure the level of mRNA expression of a gene. For example, mRNA expression can be determined using Northern blot or dot blot analysis, reverse transcriptase-PCR (RT-PCR; e.g., quantitative RT-PCR), in situ hybridization (e.g., quantitative in situ hybridization) or nucleic acid array (e.g., oligonucleotide arrays or gene chips) analysis. Details of such methods are described below and in, e.g., Sambrook et al., Molecular Cloning: A Laboratory Manual Second Edition vol. 1, 2 and 3. Cold Spring Harbor Laboratory Press: Cold Spring Harbor, New York, USA, Nov. 1989; Gibson et al. (1999) Genome Res., 6(10):995-1001; and Zhang et al. (2005) Environ. Sci. Technol., 39(8):2777-2785; U.S. Publication No. 2004086915; European Patent No. 0543942; and U.S. Patent No. 7,101,663; the disclosures of each of which are incorporated herein by reference in their entirety.

In one example, the presence or amount of one or more discrete mRNA populations in a biological sample (e.g., a tumor sample) can be determined by isolating total mRNA from the biological sample (see, e.g., Sambrook et al. (supra) and U.S. Patent No. 6,812,341) and subjecting the isolated mRNA to agarose gel electrophoresis to separate the mRNA by size. The size-separated mRNAs are then transferred (e.g., by diffusion) to a solid support such as a nitrocellulose membrane. The presence or amount of one or more mRNA populations in the surface skin sample can then be determined using one or more detectably-labeled-polynucleotide probes, complementary to the mRNA sequence of interest, which bind to and thus render detectable their corresponding mRNA populations. Detectable-labels include, e.g., fluorescent (e.g., umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride, allophycocyanin (APC), or phycoerythrin), luminescent (e.g., europium, terbium, Qdot™ nanoparticles supplied by the Quantum Dot Corporation, Palo Alto, CA), radiological (e.g., 125I, 131I, 35S, 32P, 33P, or 3H), and enzymatic (horseradish peroxidase, alkaline phosphatase, beta-galactosidase, or acetylcholinesterase) labels.

In another example, the presence or amount of discrete populations of mRNA (e.g., mRNA encoded by the CDK6 and/or CDK4 genes) in a biological sample can be determined using nucleic acid (or oligonucleotide) arrays. For example, isolated mRNA from a biological sample (e.g., a tumor sample) can be amplified using RT-PCR with,

e.g., random hexamer or oligo(dT)-primer mediated first strand synthesis. The amplicons can be fragmented into shorter segments. The RT-PCR step can be used to detectably-label the amplicons, or, optionally, the amplicons can be detectably-labeled subsequent to the RT-PCR step. For example, the detectable-label can be enzymatically (e.g., by nick-translation or kinase such as T4 polynucleotide kinase) or chemically conjugated to the amplicons using any of a variety of suitable techniques (see, e.g., Sambrook et al., supra). The detectably-labeled-amplicons are then contacted with a plurality of polynucleotide probe sets, each set containing one or more of a polynucleotide (e.g., an oligonucleotide) probe specific for (and capable of binding to) a corresponding amplicon, and where the plurality contains many probe sets each corresponding to a different amplicon. Generally, the probe sets are bound to a solid support and the position of each probe set is predetermined on the solid support. The binding of a detectably-labeled amplicon to a corresponding probe of a probe set indicates the presence or amount of a target mRNA in the surface skin sample. Additional methods for detecting mRNA expression using nucleic acid arrays are described in, e.g., U.S. Patent Nos. 5,445,934; 6,027,880; 6,057,100; 6,156,501; 6,261,776; and 6,576,424; the disclosures of each of which are incorporated herein by reference in their entirety.

Methods of detecting and/or for quantifying a detectable label depend on the nature of the label. The products of reactions catalyzed by appropriate enzymes (where the detectable label is an enzyme; see above) can be, without limitation, fluorescent, luminescent, or radioactive or they may absorb visible or ultraviolet light. Examples of detectors suitable for detecting such detectable labels include, without limitation, x-ray film, radioactivity counters, scintillation counters, spectrophotometers, colorimeters, fluorometers, luminometers, and densitometers.

Methods for detecting or measuring gene expression (e.g., protein or mRNA expression) can optionally be performed in formats that allow for rapid preparation, processing, and analysis of multiple samples. This can be, for example, in multi-welled assay plates (e.g., 96 wells or 386 wells) or arrays (e.g., nucleic acid chips or protein chips). Stock solutions for various reagents can be provided manually or robotically, and subsequent sample preparation (e.g., RT-PCR, labeling, or cell fixation), pipetting,

diluting, mixing, distribution, washing, incubating (e.g., hybridization), sample readout, data collection (optical data) and/or analysis (computer aided image analysis) can be done robotically using commercially available analysis software, robotics, and detection instrumentation capable of detecting the signal generated from the assay. Examples of such detectors include, but are not limited to, spectrophotometers, luminometers, fluorimeters, and devices that measure radioisotope decay. Exemplary high-throughput cell-based assays (e.g., detecting the presence or level of a target protein in a cell) can utilize ArrayScan® VTI HCS Reader or KineticScan® HCS Reader technology (Cellomics Inc., Pittsburgh, PA).

In some embodiments, the expression level of the CDK6 and/or CDK4 gene biomarkers of this disclosure can be assessed and/or measured. To aid in detecting the presence or level of expression of the biomarker genes of interest, any part of the nucleic acid sequence of the genes can be used, e.g., as hybridization polynucleotide probes or primers (e.g., for amplification or reverse transcription). The probes and primers can be oligonucleotides of sufficient length to provide specific hybridization to an RNA, DNA, cDNA, or fragments thereof isolated from a surface skin sample. Depending on the specific application, varying hybridization conditions can be employed to achieve varying degrees of selectivity of a probe or primer towards target sequence. The primers and probes can be detectably-labeled with reagents that facilitate detection (e.g., fluorescent labels, chemical labels (see, e.g., U.S. Patent Nos. 4,582,789 and 4,563,417), or modified bases).

Standard stringency conditions are described by Sambrook, et al. (supra) and Haymes, et al. Nucleic Acid Hybridization, A Practical Approach, IRL Press, Washington, D.C. (1985). In order for a nucleic acid molecule to serve as a primer or probe it need only be sufficiently complementary in sequence to be able to form a stable double-stranded structure under the particular hybridization conditions (e.g., solvent and salt concentrations) employed.

Hybridization can be used to assess homology between two nucleic acid sequences. A nucleic acid sequence described herein, or a fragment thereof, can be used as a hybridization probe according to standard hybridization techniques. The

hybridization of a probe of interest (e.g., a probe containing a portion of a nucleotide sequence described herein or its complement) to DNA, RNA, cDNA, or fragments thereof from a test source is an indication of the presence of DNA or RNA corresponding to the probe in the test source. Hybridization conditions are known to those skilled in the art and can be found in Current Protocols in Molecular Biology, John Wiley & Sons, N.Y., 6.3.1-6.3.6, 1991. Moderate hybridization conditions are defined as hybridization in 2X sodium chloride/sodium citrate (SSC) at 30°C, followed by a wash in 1 X SSC, 0.1% SDS at 50°C. Highly stringent conditions are defined as hybridization in 6X SSC at 45°C, followed by a wash in 0.2 X SSC, 0.1% SDS at 65°C.

Primers can be used in in a variety of PCR-type methods. For example, polymerase chain reaction (PCR) techniques can be used to amplify specific sequences from DNA as well as RNA, including sequences from total genomic DNA or total cellular RNA. The PCR primers are designed to flank the region that one is interested in amplifying. Primers can be located near the 5' end, the 3' end or anywhere within the nucleotide sequence that is to be amplified. The amplicon length is dictated by the experimental goals. For qPCR, the target length is closer to 100 base pairs and for standard PCR, it is near 500 base pairs. Generally, sequence information from the ends of the region of interest or beyond is employed to design oligonucleotide primers that are identical or similar in sequence to opposite strands of the template to be amplified. PCR primers can be chemically synthesized, either as a single nucleic acid molecule (e.g., using automated DNA synthesis in the 3' to 5' direction using phosphoramidite technology) or as a series of oligonucleotides. For example, one or more pairs of long oligonucleotides (e.g., >100 nucleotides) can be synthesized that contain the desired sequence, with each pair containing a short segment of complementarity (e.g., about 15 nucleotides) such that a duplex is formed when the oligonucleotide pair is annealed. DNA polymerase is used to extend the oligonucleotides, resulting in a single, double-stranded nucleic acid molecule per oligonucleotide pair.

In addition, the nucleic acid sequences or fragments thereof (e.g., oligonucleotide probes) can be used in nucleic acid arrays for detection and/or quantitation of gene expression.

The nucleic acid and amino acid sequences of human CDK6 and CDK4 biomarkers of this disclosure that can be measured using methods of this disclosure include the following sequences and variants thereof:

SEQ ID NO: 1: human CDK6 nucleic acid sequence (NM_001259.8)

GTTCGTTGCAACAAATTGATGAGCAATGCTTTTTTATAATGCCAACTTTGTACA
AAAAAGTTGGCATGGAGAAGGACGGCCTGTGCCGCGCTGACCAGCAGTACGA
ATGCGTGGCGGAGATCGGGGAGGGCGCCTATGGGAAGGTGTTCAAGGCCCGC
GACTTGAAGAACGGAGGCCGTTTCGTGGCGTTGAAGCGCGTGCGGGTGCAGA
CCGGCGAGGAGGGCATGCCGCTCTCCACCATCCGCGAGGTGGCGGTGCTGAG
GCACCTGGAGACCTTCGAGCACCCCAACGTGGTCAGGTTGTTTGATGTGTGCA
CAGTGTCACGAACAGACAGAGAAACCAAACTAACTTTAGTGTTTGAACATGT
CGATCAAGACTTGACCACTTACTTGGATAAAGTTCCAGAGCCTGGAGTGCCCA
CTGAAACCATAAAGGATATGATGTTTCAGCTTCTCCGAGGTCTGGACTTTCTTC
ATTCACACCGAGTAGTGCATCGCGATCTAAAACCACAGAACATTCTGGTGACC
AGCAGCGGACAAATAAAACTCGCTGACTTCGGCCTTGCCCGCATCTATAGTTT
CCAGATGGCTCTAACCTCAGTGGTCGTCACGCTGTGGTACAGAGCACCCGAA
GTCTTGCTCCAGTCCAGCTACGCCACCCCCGTGGATCTCTGGAGTGTTGGCTG
CATATTTGCAGAAATGTTTCGTAGAAAGCCTCTTTTTCGTGGAAGTTCAGATGT
TGATCAACTAGGAAAAATCTTGGACGTGATTGGACTCCCAGGAGAAGAAGAC
TGGCCTAGAGATGTTGCCCTTCCCAGGCAGGCTTTTCATTCAAAATCTGCCCA
ACCAATTGAGAAGTTTGTAACAGATATCGATGAACTAGGCAAAGACCTACTTC
TGAAGTGTTTGACATTTAACCCAGCCAAAAGAATATCTGCCTACAGTGCCCTG
TCTCACCCATACTTCCAGGACCTGGAAAGGTGCAAAGAAAACCTGGATTCCCA
CCTGCCGCCCAGCCAGAACACCTCGGAGCTGAATACAGCCTGCCCAACTTTCT
TGTACAAAGTTGGCATTATAAGAAAGCATTGCTTATCAATTTGTTGCAACGAAC

SEQ ID NO: 2: human CDK6 amino acid sequence (NP_001138778.1)

MEKDGLCRADQQYECVAEIGEGAYGKVFKARDLKNGGRFVALKRVRVQTGEEG
MPLSTIREVAVLRHLETFEHPNVVRLFDVCTVSRTDRETKLTLVFEHVDQDLTTYL

DKVPEPGVPTETIKDMMFQLLRGLDFLHSHRVVHRDLKPQNILVTSSGQIKLADF
GLARIYSFQMALTSVVVTLWYRAPEVLLQSSYATPVDLWSVGCIFAEMFRRKPLF
RGSSDVDQLGKILDVIGLPGEEDWPRDVALPRQAFHSKSAQPIEKFVTDIDELGK
DLLLKCLTFNPAKRISAYSALSHPYFQDLERCKENLDSHLPPSQNTSELNTA

SEQ ID NO: 3: human CDK4 nucleic acid sequence (NM_000075.4)
ATGGCTACCTCTCGATATGAGCCAGTGGCTGAAATTGGTGTCGGTGCCTATGGG
ACAGTGTACAAGGCCCGTGATCCCCACAGTGGCCACTTTGTGGCCCTCAAGA
GTGTGAGAGTCCCCAATGGAGGAGGAGGTGGAGGAGGCCTTCCCATCAGCAC
AGTTCGTGAGGTGGCTTTACTGAGGCGACTGGAGGCTTTTGAGCATCCCAATG
TTGTCCGGCTGATGGACGTCTGTGCCACATCCCGAACTGACCGGGAGATCAAG
GTAACCCTGGTGTTTGAGCATGTAGACCAGGACCTAAGGACATATCTGGACAA
GGCACCCCCACCAGGCTTGCCAGCCGAAACGATCAAGGATCTGATGCGCCAG
TTTCTAAGAGGCCTAGATTTCCTTCATGCCAATTGCATCGTTCACCGAGATCTG
AAGCCAGAGAACATTCTGGTGACAAGTGGTGGAACAGTCAAGCTGGCTGACT
TTGGCCTGGCCAGAATCTACAGCTACCAGATGGCACTTACACCCGTGGTTGTT
ACACTCTGGTACCGAGCTCCCGAAGTTCTTCTGCAGTCCACATATGCAACACC
TGTGGACATGTGGAGTGTTGGCTGTATCTTTGCAGAGATGTTTCGTCGAAAGC
CTCTCTTCTGTGGAAACTCTGAAGCCGACCAGTTGGGCAAAATCTTTGACCTG
ATTGGGCTGCCTCCAGAGGATGACTGGCCTCGAGATGTATCCCTGCCCCGTGG
AGCCTTTCCCCCCAGAGGGCCCCGCCCAGTGCAGTCGGTGGTACCTGAGATG
GAGGAGTCGGGAGCACAGCTGCTGCTGGAAATGCTGACTTTTAACCCACACA
AGCGAATCTCTGCCTTTCGAGCTCTGCAGCACTCTTATCTACATAAGGATGAAG
GTAATCCGGAGTGA

SEQ ID NO: 4: human CDK4 amino acid sequence (NP_000066.1)
MATSRYEPVAEIGVGAYGTVYKARDPHSGHFVALKSVRVPNGGGGGGGLPISTV
REVALLRRLEAFEHPNVVRLMDVCATSRTDREIKVTLVFEHVDQDLRTYLDKAPP
PGLPAETIKDLMRQFLRGLDFLHANCIVHRDLKPENILVTSGGTVKLADFGLARIY
SYQMALTPVVVTLWYRAPEVLLQSTYATPVDMWSVGCIFAEMFRRKPLFCGNSE

ADQLGKIFDLIGLPPEDDWPRDVSLPRGAFPPRGPRPVQSVVPEMEESGAQLLLE
MLTFNPHKRISAFRALQHSYLHKDEGNPE

In some instances, following the determination of CDK6 and/or CDK4 levels in a biological sample in a subject, the ratio (quotient) of CDK4 to CDK6 is determined by conventional means known in the art in which the level of one protein or RNA amount (CDK4) is divided by the level of the other protein or RNA amount (CDK6) to obtain the mathematical ratio of CDK4 to CDK6.

2. Controls and Threshold Level

As described above, the methods of the present disclosure can involve, measuring the expression level (e.g., mRNA, cDNA, or protein concentration) of CDK6 and/or CDK4 in a biological sample from a subject (e.g., a human subject with a tumor), wherein the expression level of CDK6 and/or CDK4 gene or protein, and/or the CDK4:CDK6 ratio compared to a control, predicts whether a CDK4/6-based therapy (e.g., a CDK4/6 inhibitor and/or a CDK4/6-directed PROTAC) will be successful in treating a subject with that tumor. The level of CDK6 and/or CDK4:CDK6 ratio in a tumor compared to a control or threshold level can predict the sensitivity of that tumor to a CDK4/6 inhibitor; the subject response to CDK4/6 inhibitors; and whether or not a subject will be a responder to treatment comprising a CDK4/6-based therapy.

As used herein, the term "control" refers to a single biological sample or multiple biological samples from which an average amount or level of biomarker (e.g., CDK6 and/or CDK4) can be determined. A "reference level in a control" refers to the average amount or level of that biomarker in single sample or a set of biological samples. The amount of the biomarker that is measured in the sample may be relative or absolute. In some embodiments the relative expression of mRNA or cDNA is measured in the test sample versus the control sample. In other embodiments, the absolute amount of protein biomarker is measured in the test sample versus the control sample.

In certain embodiments, when the level/concentration of CDK6 in a tumor sample from a subject is lower than that in a control, the subject is identified as likely to respond to CDK4/6 inhibitor based therapy and/or CDK4/6-directed PROTAC. In this context, the

term "control" refers to a threshold level of CDK6 mRNA as determined by quantitative PCR or RNA-seq, or CDK6 protein levels, as determined by Immunohistochemistry or mass spectrometry analysis.

In certain embodiments, the level/concentration of CDK6 in a tumor sample from a subject is measured by any RNA sequencing technology known in the art (see e.g., Kukurba KR and Montgomery SB, (2015) Cold Spring Harb Protoc. Nov; (11): 951–969; and Hrdlickova R et al., (2017) Wiley Interdiscip Rev RNA. Jan; 8(1): 10.1002/wrna.1364) or by quantitative PCR. In some embodiments, when the level/concentration of CDK6 in a tumor sample as measured by RNA sequencing is lower than 5.37 counts per million reads (CPM), the tumor is classified as "CDK6-low". Conversely, when the level/concentration of CDK6 in a tumor sample as measured by RNA sequencing is higher than 5.37 CPM, the tumor is classified as "CDK6-hgh".

The "threshold" expression level/concentration for a CDK4/6 mRNA, cDNA or protein and/or the threshold ratio of CDK4 to CDK6 may also be pre-established by an analysis of mRNA, cDNA, or protein expression in biological samples (e.g., tumor samples) from one or more (e.g., two, three, four, five, six, seven, eight, nine, 10, 15, 20, 25, 30, 35, or 40 or more) human subjects who have the same tumors. This pre-established reference value (which may be an average or median expression level/concentration taken from multiple subjects that have tumors) may then be used for the "control" concentration/expression level of the protein or nucleic acid in the comparison with the test sample. In such a comparison, the subject is predicted to be responsive to CDK4/6 inhibitor therapy and/or CDK4/6-directed PROTAC therapy if the expression level of the CDK6 being analyzed is lower than the pre-established reference and/or the ratio of CDK4 to CDK6 is higher than the pre-established reference.

In certain embodiments, the "control" is a pre-determined cut-off value. In some embodiments, the methods described herein include determining if the concentration of CDK6 and/or CDK4 to CDK6 ratio falls above or below a predetermined cut-off value (the threshold level). A cut-off value is typically a concentration of a protein above or below which is considered predictive of something - e.g., likely to be responsive to a therapy of interest. Thus, in accordance with the methods described herein, a reference

concentration of CDK6 mRNA, cDNA or protein is identified as a cut-off value, and/or CDK4 to CDK6 ratio above or below of which is predictive of a subject who shows responsiveness to a cancer therapy. Some cut-off values are not absolute in that clinical correlations can still remain significant over a range of values on either side of the cutoff; however, it is possible to select an optimal cut-off value (e.g. varying H-scores) of concentration of CDK6 and/or CDK4 mRNA, cDNA or protein for a particular sample type. Cut-off values determined for use in the methods described herein can be compared with, e.g., published ranges of CDK6 and/or CDK4 concentrations, but can be individualized to the methodology used and patient population. It is understood that improvements in optimal cut-off values could be determined depending on the sophistication of statistical methods used and on the number and source of samples used to determine reference level values for the different proteins, genes, and sample types. Therefore, established cut-off values can be adjusted up or down, on the basis of periodic reevaluations or changes in methodology or population distribution.

In certain embodiments, the control or threshold levels of CDK6 and/or CDK4 mRNA and/or protein levels are established using cell lines or xenografts. In some embodiments, the control or threshold levels of CDK6 and/or CDK4 protein levels are established using immunohistochemistry in preclinical samples.

The reference concentration of biomarkers of the present disclosure (i.e., CDK6 and CDK4) can be determined by a variety of methods. For instance, the reference level can be determined by comparison of the concentration of protein of interest in, e.g., populations of subjects (e.g., patients) that are responsive to a CDK4/6-directed therapy or not responsive to a CDK4/6-directed therapy. This can be accomplished, for example, by histogram analysis, in which an entire cohort of patients is graphically presented, wherein a first axis represents the concentration of a protein of interest and a second axis represents the number of subjects in the cohort whose sample contain one or more concentrations. Determination of the reference concentration of a protein can then be made based on an amount or concentration which best distinguishes these separate groups. The reference level can be a single number, equally applicable to every subject, or the reference level can vary, according to specific subpopulations of subjects. For

example, older subjects can have a different reference level than younger subjects. In addition, a subject with more severe disease can have a different reference value than one with a milder form of the disease (e.g., early stage vs late stage cancer).

The pre-established cut-off value can be a CDK6 and/or CDK4 protein concentration that is determined based on receiver operating characteristic (ROC) analysis. ROC curves are used to determine a cut-off value for a clinical test. Consider the situation where there are two groups of patients and by using an established standard technique one group is known to be responsive to a CDK4/6-directed therapy, and the other is known to not respond to the CDK4/6-directed therapy. A measurement using a biological sample (e.g., a tumor sample) from all members of the two groups is used to test for responsiveness to a CDK4/6-directed therapy. The test will find some, but not all, responders to respond to a CDK4/6-directed therapy. The ratio of the responders found by the test to the total number of responders (known by the established standard technique) is the true positive rate (also known as sensitivity). The test will find some, but not all, non-responders to not respond to a CDK4/6-directed therapy. The ratio of the non-responders found by the test to the total number of non-responders (known by the established standard technique) is the true negative rate (also known as specificity). The ROC curve analysis of the CDK4/6-directed therapy responsiveness test will find a cut-off value that will minimize the number of false positives and false negatives. A ROC is a graphical plot which illustrates the performance of a binary class stratifier system as its discrimination threshold is varied. It is created by plotting the fraction of true positives out of the positives versus the fraction of false positives out of the negatives, at various threshold settings.

In one embodiment, the CDK6 and/or CDK4 protein concentration is determined based on ROC analysis predicting response to a CDK4/6-driven therapy with a positive predictive value, wherein a concentration of a protein of interest (e.g., CDK6 and/or CDK4) equal to or below the pre-established cut off value is a low concentration of the protein of interest and a value higher than the pre-established cut-off value is a high concentration of the protein of interest.

In another embodiment, the CDK4 to CDK6 protein ratio is determined based on ROC analysis predicting response to a CDK4/6-driven therapy with a positive predictive value, wherein a CDK4 to CDK6 protein ratio equal to or below the pre-established cut off value is a low CDK4 to CDK6 ratio and a value higher than the pre-established cut-off value is a high CDK4 to CDK6 ratio.

The positive predictive value is the proportion of positive test results that are true positives; it reflects the probability that a positive test reflects the underlying condition being tested for. Methods of constructing ROC curves and determining positive predictive values are well known in the art.

In another embodiment, the pre-established cut-off value can be a CDK6 and/or CDK4 protein concentration that is determined based on simulation models predicting responsiveness to CDK4/6-driven therapy, and wherein a concentration of CDK6 and/or CDK4 equal to or below the pre-established cut-off value is a low concentration of the CDK6 and/or CDK4 and a value higher than the pre-established cut-off value is a high concentration of CDK6 and/or CDK4.

3. Rb-proficient cancers

The methods described herein can be used to treat proliferative disorders such as cancer, and in particular, a cancer expressing functional retinoblastoma protein (Rb)-proficient cancer. In some embodiments, the methods described herein can be used to treat a subject suffering from an Rb-positive cancer or other Rb-positive abnormal cellular proliferative disorder. In some embodiments, the cancer or cellular proliferation disorder is a CDK4/6-dependent cancer that requires the activity of CDK4/6 for replication or proliferation, or which may be treated by a CDK4/6 inhibitor and/or a CDK4/6-directed PROTAC. Cancers and disorders of such type can be characterized by the presence of a functional Retinoblastoma protein. Such cancers and disorders are classified as being Rb-positive. Rb-positive abnormal cellular proliferation disorders, and variations of this term as used herein, refer to disorders or diseases caused by uncontrolled or abnormal cellular division which are characterized by the presence of a functional Retinoblastoma protein, which can include cancers. In one aspect of the

disclosure, the methods described herein can be used to treat a non-cancerous Rb-positive abnormal cellular proliferation disorder. Examples of such disorders may include non-malignant lymphoproliferation, non-malignant breast neoplasms, psoriasis, arthritis, dermatitis, pre-cancerous colon lesions or pulps, angiogenesis disorders, immune mediated and non-immune mediated inflammatory diseases, arthritis, age-related macular degeneration, diabetes, and other non-cancerous or benign cellular proliferation disorders.

Targeted cancers suitable for treatment with the methods described herein include but are not limited to Rb-positive: estrogen-receptor positive cancer, HER2-negative advanced breast cancer, late-line metastatic breast cancer, liposarcoma, non-small cell lung cancer, liver cancer, ovarian cancer, glioblastoma, refractory solid tumors, retinoblastoma positive breast cancer as well as retinoblastoma positive endometrial, vaginal and ovarian cancers and lung and bronchial cancers, adenocarcinoma of the colon, adenocarcinoma of the rectum, central nervous system germ cell tumors, teratomas, estrogen receptor-negative breast cancer, estrogen receptor-positive breast cancer, familial testicular germ cell tumors, HER2-negative breast cancer, HER2-positive breast cancer, male breast cancer, ovarian immature teratomas, ovarian mature teratoma, ovarian monodermal and highly specialized teratomas, progesterone receptor-negative breast cancer, progesterone receptor-positive breast cancer, recurrent breast cancer, recurrent colon cancer, recurrent extragonadal germ cell tumors, recurrent extragonadal non-seminomatous germ cell tumor, recurrent extragonadal seminomas, recurrent malignant testicular germ cell tumors, recurrent melanomas, recurrent ovarian germ cell tumors, recurrent rectal cancer, stage III extragonadal non-seminomatous germ cell tumors, stage III extragonadal seminomas, stage III malignant testicular germ cell tumors, stage III ovarian germ cell tumors, stage IV breast cancers, stage IV colon cancers, stage IV extragonadal non-seminomatous germ cell tumors, stage IV extragonadal seminoma, stage IV melanomas, stage IV ovarian germ cell tumors, stage IV rectal cancers, testicular immature teratomas, testicular mature teratomas. In particular embodiments, the targeted cancers included estrogen-receptor positive, HER2-negative advanced breast cancer, late-line metastatic breast cancer, liposarcoma, non-small cell lung cancer, liver cancer, ovarian cancer, glioblastoma, refractory solid tumors,

retinoblastoma positive breast cancer as well as retinoblastoma positive endometrial, vaginal and ovarian cancers and lung and bronchial cancers, metastatic colorectal cancer, metastatic melanoma with CDK4 mutation or amplification, or cisplatin-refractory, and unresectable germ cell tumors.

In preferred embodiments, the Rb-positive cancer suitable to treat with the methods described herein include but are not limited to non-small-cell lung carcinoma (NSCLC), colorectal carcinoma (CRC), melanoma, Acute lymphoblastic leukemia (ALL), T-cell acute lymphocytic leukemia (T-ALL), Mantle Cell Lymphoma (MCL), and Multiple Myeloma (MM).

In some embodiments, the Rb-positive cancer is selected from an Rb-positive carcinoma, sarcoma, including, but not limited to, lung cancer, bone cancer, pancreatic cancer, skin cancer, cancer of the head or neck, cutaneous or intraocular melanoma, uterine cancer, ovarian cancer, rectal cancer, cancer of the anal region, stomach cancer, colon cancer, breast cancer, uterine cancer, carcinoma of the fallopian tubes, carcinoma of the endometrium, carcinoma of the cervix, carcinoma of the vagina, carcinoma of the vulva, cancer of the esophagus, cancer of the small intestine, cancer of the endocrine system, cancer of the thyroid gland, cancer of the parathyroid gland, cancer of the adrenal gland, sarcoma of soft tissue, cancer of the urethra, cancer of the penis, prostate cancer, cancer of the bladder, cancer of the kidney or ureter, renal cell carcinoma, carcinoma of the renal pelvis, neoplasms of the central nervous system (CNS), primary CNS lymphoma, spinal axis tumors, brain stem glioma, pituitary adenoma, or a combination of one or more of the foregoing cancers.

In some embodiments, the Rb-positive cancer is selected from the group consisting of Rb-positive: fibrosarcoma, myxosarcoma, chondrosarcoma, osteosarcoma, chordoma, malignant fibrous histiocytoma, hemangiosarcoma, angiosarcoma, lymphangiosarcoma. Mesothelioma, leiomyosarcoma, rhabdomyosarcoma, squamous cell carcinoma; epidermoid carcinoma, malignant skin adnexal tumors, adenocarcinoma, hepatoma, hepatocellular carcinoma, renal cell carcinoma, hypernephroma, cholangiocarcinoma, transitional cell carcinoma, choriocarcinoma, seminoma, embryonal cell carcinoma, glioma anaplastic; glioblastoma multiforme, neuroblastoma,

medulloblastoma, malignant meningioma, malignant schwannoma, neurofibrosarcoma, parathyroid carcinoma, medullary carcinoma of thyroid, bronchial carcinoid, pheochromocytoma, Islet cell carcinoma, malignant carcinoid, malignant paraganglioma, melanoma, Merkel cell neoplasm, cystosarcoma phylloide, salivary cancers, thymic carcinomas, bladder cancer, and Wilms tumor.

In more particular embodiments, the Rb-positive cancer or disorder includes a blood disorder or a hematologic malignancy, including, but not limited to, myeloid disorder, lymphoid disorder, leukemia, lymphoma, myelodysplastic syndrome (MDS), myeloproliferative disease (MPD), mast cell disorder, and myeloma (e.g., multiple myeloma), among others. Abnormal proliferation of T-cells, B-cells, and/or NK-cells can result in a wide range of diseases such as cancer, proliferative disorders and inflammatory/immune diseases. A host, for example a human, afflicted with any of these disorders can be treated with the methods described herein to achieve a decrease in symptoms or a decrease in the underlying disease.

Examples include T-cell or NK-cell lymphoma, for example, but not limited to: peripheral T-cell lymphoma; anaplastic large cell lymphoma, for example anaplastic lymphoma kinase (ALK) positive, ALK negative anaplastic large cell lymphoma, or primary cutaneous anaplastic large cell lymphoma; angioimmunoblastic lymphoma; cutaneous T-cell lymphoma, for example mycosis fungoides, Sézary syndrome, primary cutaneous anaplastic large cell lymphoma, primary cutaneous CD30+ T-cell lymphoproliferative disorder; primary cutaneous aggressive epidermotropic CD8+ cytotoxic T-cell lymphoma; primary cutaneous gamma-delta T-cell lymphoma; primary cutaneous small/medium CD4+ T-cell lymphoma, and lymphomatoid papulosis; Adult T-cell Leukemia/Lymphoma (ATLL); Blastic NK-cell Lymphoma; Enteropathy-type T-cell lymphoma; Hematosplenic gamma-delta T-cell Lymphoma; Lymphoblastic Lymphoma; Nasal NK/T-cell Lymphomas; Treatment-related T-cell lymphomas; for example lymphomas that appear after solid organ or bone marrow transplantation; T-cell prolymphocytic leukemia; T-cell large granular lymphocytic leukemia; Chronic lymphoproliferative disorder of NK-cells; Aggressive NK cell leukemia; Systemic EBV+ T-cell lymphoproliferative disease of childhood (associated with chronic active EBV

infection); Hydroa vacciniforme-like lymphoma; Adult T-cell leukemia/lymphoma; Enteropathy-associated T-cell lymphoma; Hepatosplenic T-cell lymphoma; or Subcutaneous panniculitis-like T-cell lymphoma.

In some embodiments, the methods disclosed herein can be used to treat a subject with a lymphoma or lymphocytic or myelocytic proliferation disorder or abnormality. For example, a Hodgkin Lymphoma (including, not limited to: Nodular Sclerosis Classical Hodgkin's Lymphoma (CHL); Mixed Cellularity CHL; Lymphocyte-depletion CHL; Lymphocyte-rich CHL; Lymphocyte Predominant Hodgkin Lymphoma; or Nodular Lymphocyte Predominant HL) or a Non-Hodgkin Lymphoma (including, but not limited to: an AIDS-Related Lymphoma; Anaplastic Large-Cell Lymphoma; Angioimmunoblastic Lymphoma; Blastic NK-Cell Lymphoma; Burkitt's Lymphoma; Burkitt-like Lymphoma (Small Non-Cleaved Cell Lymphoma); Chronic Lymphocytic Leukemia/Small Lymphocytic Lymphoma; Cutaneous T-Cell Lymphoma; Diffuse Large B-Cell Lymphoma; Enteropathy-Type T-Cell Lymphoma; Follicular Lymphoma; Hepatosplenic Gamma-Delta T-Cell Lymphoma; Lymphoblastic Lymphoma; Mantle Cell Lymphoma; Marginal Zone Lymphoma; Nasal T-Cell Lymphoma; Pediatric Lymphoma; Peripheral T-Cell Lymphomas; Primary Central Nervous System Lymphoma; T-Cell Leukemias; Transformed Lymphomas; Treatment-Related T-Cell Lymphomas; or Waldenstrom's Macroglobulinemia.

In some embodiments, the methods disclosed herein can be used to treat a subject with a specific B-cell lymphoma or proliferative disorder including, but not limited to: multiple myeloma; Diffuse large B cell lymphoma; Follicular lymphoma; Mucosa-Associated Lymphatic Tissue lymphoma (MALT); Small cell lymphocytic lymphoma; Mediastinal large B cell lymphoma; Nodal marginal zone B cell lymphoma (NMZL); Splenic marginal zone lymphoma (SMZL); Intravascular large B-cell lymphoma; Primary effusion lymphoma; or Lymphomatoid granulomatosis;; B-cell prolymphocytic leukemia; Hairy cell leukemia; Splenic lymphoma/leukemia, unclassifiable; Splenic diffuse red pulp small B-cell lymphoma; Hairy cell leukemia-variant; Lymphoplasmacytic lymphoma; Heavy chain diseases, for example, Alpha heavy chain disease, Gamma heavy chain disease, Mu heavy chain disease; Plasma cell myeloma; Solitary plasmacytoma of bone;

Extraosseous plasmacytoma; Primary cutaneous follicle center lymphoma; T cell/histiocyte rich large B-cell lymphoma; DLBCL associated with chronic inflammation; Epstein-Barr virus (EBV)+ DLBCL of the elderly; Primary mediastinal (thymic) large B-cell lymphoma; Primary cutaneous DLBCL, leg type; ALK+ large B-cell lymphoma; Plasmablastic lymphoma; Large B-cell lymphoma arising in HHV8-associated multicentric; Castleman disease; B-cell lymphoma, unclassifiable, with features intermediate between diffuse large B-cell lymphoma; or B-cell lymphoma, unclassifiable, with features intermediate between diffuse large B-cell lymphoma and classical Hodgkin lymphoma.

In some embodiments, the methods disclosed herein can be used to treat a subject with an acute or chronic leukemia of a lymphocytic or myelogenous origin, including, but not limited to: Acute lymphoblastic leukemia (ALL); Acute myelogenous leukemia (AML); Chronic lymphocytic leukemia (CLL); Chronic myelogenous leukemia (CIVIL); juvenile myelomonocytic leukemia (JMML); hairy cell leukemia (HCL); acute promyelocytic leukemia (a subtype of AML); large granular lymphocytic leukemia; or Adult T-cell chronic leukemia. In one embodiment, the patient suffers from an acute myelogenous leukemia, for example an undifferentiated AML (M0); myeloblastic leukemia (M1; with/without minimal cell maturation); myeloblastic leukemia (M2; with cell maturation); promyelocytic leukemia (M3 or M3 variant [M3V]); myelomonocytic leukemia (M4 or M4 variant with eosinophilia [M4E]); monocytic leukemia (M5); erythroleukemia (M6); or megakaryoblastic leukemia (M7).

The presence or normal functioning of the retinoblastoma (Rb) tumor suppressor protein (Rb-positive) can be determined through any of the standard assays known to one of ordinary skill in the art, including but not limited to Western Blot, ELISA (enzyme linked immunoadsorbent assay), IHC (immunohistochemistry), and FACS (fluorescent activated cell sorting). The selection of the assay will depend upon the tissue, cell line or surrogate tissue sample that is utilized e.g., for example Western Blot and ELISA may be used with any or all types of tissues, cell lines or surrogate tissues, whereas the IHC method would be more appropriate wherein the tissue utilized in the methods of the present invention was a tumor biopsy. FACs analysis would be most applicable to

samples that were single cell suspensions such as cell lines and isolated peripheral blood mononuclear cells. See for example, US 20070212736 "Functional Immunohistochemical Cell Cycle Analysis as a Prognostic Indicator for Cancer". Alternatively, molecular genetic testing may be used for determination of retinoblastoma gene status. Molecular genetic testing for retinoblastoma includes the following as described in Lohmann and Gallie "Retinoblastoma. Gene Reviews" (2010): "A comprehensive, sensitive and economical approach for the detection of mutations in the RB1 gene in retinoblastoma" Journal of Genetics, 88(4), 517-527 (2009).

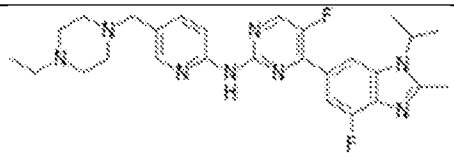
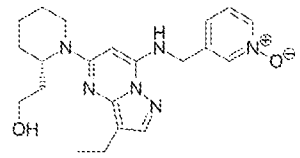
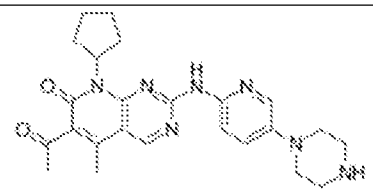
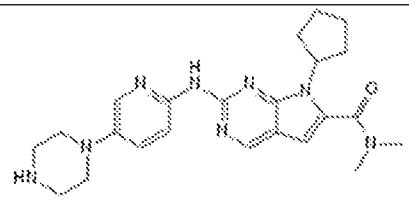
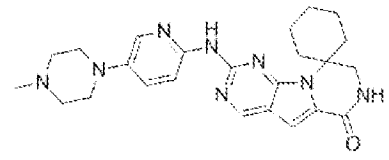
In some embodiments, the cancer to be treated is a solid tumor cancer or a leukemia. Solid tumor cancers include, but are not limited to, non-small-cell lung carcinoma (NSCLC), lung cancer, breast cancer, ewing sarcoma, central nervous system neoplasm, skin cancer, head and neck cancer, ovarian cancer, colon cancer, anal cancer, stomach cancer, gastrointestinal cancer, fallopian tube cancer, endometrial cancer, cervical cancer, vaginal cancer, vulvar cancer, esophageal cancer, endocrine cancer, thyroid cancer, parathyroid cancer, adrenal cancer, soft tissue sarcoma, urethral cancer, penile cancer, testicular cancer, brain stem glioma, pituitary cancer, adrenocortical cancer, gallbladder cancer, multiple myeloma, cholangiocarcinoma, fibrosarcoma, lymphoma, liver cancer, kidney cancer, bone cancer, bladder cancer, colorectal cancer, endometrial cancer, renal cell cancer, pancreatic cancer, prostate cancer, thyroid cancer, mesothelioma, neuroblastoma, retinoblastoma, and melanoma. Leukemia includes, but is not limited to, acute myeloid leukemia (AML) and T-cell acute lymphocytic leukemia (T-ALL), Mantle Cell Lymphoma (MCL), and Multiple Myeloma (MM).

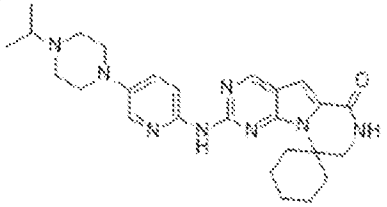
4. CDK4/6 inhibitors and CDK4/6-directed PROTACs

CDK4/6 inhibitors and CDK4/6-directed PROTACs may be collectively referred to as CDK4-6-directed therapies in this disclosure. The CDK4/6 inhibitors suitable for use in the methods described herein are compounds which are capable of interfering with the enzymatic activity of CDK4, CDK6, or both CDK4 and CDK6. As used herein, an "inhibitor" refers to an agent that restrains, retards, or otherwise causes inhibition of a physiological, chemical or enzymatic action or function. An inhibitor can cause an at

least 5% decrease in enzyme activity. An inhibitor can also or alternately refer to a drug, compound, or agent that prevents or reduces the expression, transcription, or translation of a gene or protein. An inhibitor can reduce or prevent the function of a protein, e.g., by binding to or activating/inactivating another protein or receptor. Examplary CDK4/6 inhibitors are disclosed in International Patent Application Publications WO2016040858, WO2011130232, WO2011101409, WO2016025650, and WO2013006532, incorporated by reference in their entirety. CDK4/6 inhibitors suitable for use include, but are not limited to the following compounds and pharmaceutically acceptable derivatives and prodrugs thereof:

Table 1: CDK4/6 Inhibitors and their structures

CDK4/6 inhibitor	Structure
abemaciclib (Verzenio)	
dinaciclib	
palbociclib (Ibrance)	
ribociclib (Kisqali)	
trilaciclib (G1T28)	

G1T38	

The CDK4/6-directed PROTACs suitable for use in the methods described herein are heterobifunctional small molecules which selectively degrade CDK4, CDK6, or both CDK4 and CDK6 ("PROteolysis TArgeting Chimeras" or "PROTACs") are useful in the treatment of CDK4/6- mediated cancers. The CDK4/6-directed PROTACs (also known as CDK4/6 degraders/disruptors) useful in the methods of this disclosure include, but are not limited to MS140 (**FIG. 4A**) and other molecules disclosed in International Patent Application Publication WO2018106870PCT, disclosed herein in its entirety. These PROTACs comprise a CDK4/6 ligand (or targeting moiety) conjugated to a degradation tag. Linkage of the CDK4/6 ligand to the degradation tag can be direct, or indirect via a linker. The CDK4/6 ligand or targeting moiety can be a CDK4/6 inhibitor (e.g., abemaciclib, palbociclib, ribociclib, trilaciclib (G1T28), G1T38, SHR6390, and analogs thereof. The degradation/disruption tags of the present disclosure include, e.g., thalidomide, pomalidomide, lenalidomide, VHL-1, adamantane, 1 -((4,4,5,5,5-pentafluoropentyl)sulfinyl)nonane, nutlin-3a, RG7112, RG7338, AMG 232, AA-115, bestatin, MV-1, LCL161, and/or analogs thereof.

The CDK4/6-directed PROTACs that are suitable for use in the methods described herein include the PROTACs shown in **FIGs. 14-18**. These PROTACs are described in Brand M. et al., (2019) Cell Chem. Biol Feb 21;26(2):300-306; Jiang B, et al. Angew Chem Int Ed Engl. 2019 May 6;58(19):6321-6326. doi: 10.1002/anie.201901336. Epub 2019 Mar 29; Rana S. et al, Bioorganic & Medicinal Chemistry Letters, 2019; 29(11): 1375-1379; Anderson NA, et al. Bioorg Med Chem Lett. 2020 May 1;30(9):127106. doi: 10.1016/j.bmcl.2020.127106. Epub 2020 Mar 10; and De Dominici M, et al. Blood. 2020 Apr 30;135(18):1560-1573.

5. Predicting response to a cancer therapy

This disclosure features methods of predicting the response of a subject with cancer (e.g., a Rb-proficient cancer) to any particular CDK4/6 therapy and predicting the prognosis of a subject with cancer. The method involves measuring the CDK6 and/or CDK4 levels obtained from a biological sample from the subject (e.g., a tumor). Based on the levels of CDK6 and/or the CDK4/CDK6 ratio, the probability of response to the cancer therapy, a future risk of cancer progression in the subject is determined in the subject and/or an indication of whether the cancer will be successfully treated with a particular cancer therapy (e.g., a CDK4/6 inhibitor). For instance, based on the levels of CDK6 and/or CDK4, a specific tumor phenotype in a subject with cancer can be categorized as "CDK4-high/CDK6-low" as determined by assessing RNA and/or protein levels of CDK6 and CDK4 in the tumor as described elsewhere. Such a tumor is classified as a CDK4 dependent tumor and is likely to be more sensitive to CDK4/6-directed PROTACs, than to CDK4/6 inhibitors. In another instance, a tumor phenotype in a subject with cancer can be categorized as "CDK4-low/CDK6-high" as determined by assessing RNA and/or protein levels of CDK6 and CDK4 in the tumor as described elsewhere. Such a tumor is classified as a CDK6 dependent tumor and is likely to be more sensitive to CDK4/6-directed PROTACs, than to CDK4/6 inhibitors. Without being bound by theory, it is postulated that a CDK6 dependent tumor can be sensitive to CDK4/6 driven therapy (including CDK4/6 inhibitors and CDK4/6-directed PROTACs) if CDK6 binds strongly to heat shock protein (Hsp90) and Cell Division Cycle 37 (HSP90/CDC37) as shown in **FIG. 7**. Alternatively, a CDK6 dependent tumor can be resistant to CDK4/6 driven therapy if CDK6 binds weakly to HSP90/CDC37 as shown in **FIGS. 6-7**.

6. Methods of Treatment

The methods disclosed herein enable the characterization of a tumor based on biomarker levels (e.g., CDK6 and/or CDK4) in a subject's biological sample, followed by treatment of said subject with an appropriate cancer therapy. The methods disclosed herein enable the assessment whether or not a subject having or suspected of having

cancer is likely to respond to a cancer therapy. A subject having or suspected of having cancer who is likely to respond to the cancer therapy can be administered the cancer therapy. Conversely, a subject having or suspected of having cancer who is not likely to respond to a cancer therapy can be administered a different cancer therapy that is suitable for treatment of cancer.

The methods of this disclosure also enable the stratification of cancerous tumors into tumors that are more likely to benefit, and tumors that are less likely to benefit, from treatment comprising a particular cancer therapy. The ability to select one or more tumors being considered for treatment with a cancer therapy is beneficial for administering an effective treatment to the subject with the one or more tumors.

The subjects who are considered for treatment comprising a cancer therapy include, but are not limited to, subjects having, or suspected of having, cancer. In one embodiment, the subject to be treated with a cancer therapy has, is suspected of having, or is likely to develop one or more tumors.

If the subject having cancer is more likely to respond to a cancer therapy (based on levels of CDK6 and/or CDK4 biomarkers, and/or the ratio of CDK4 to CDK6), the subject can then be administered an effective amount of the cancer therapy. An effective amount of the compound can suitably be determined by a health care practitioner taking into account, for example, the characteristics of the patient (age, sex, weight, race, etc.), the progression of the disease, and prior exposure to the drug. If the subject is less likely to respond to one cancer therapy, the subject can then be optionally administered a different cancer therapy.

Subjects of all ages can be affected by cancer. Therefore, a biological sample used in a method described herein can be obtained from a human subject of any age, including a fetus, an infant, a child, an adolescent, or an adult, such as an adult having, or suspected of having, cancer.

After stratifying or selecting a tumor based on whether the subject will be more likely or less likely to respond to a cancer therapy, a medical practitioner (e.g., a doctor) can administer the appropriate therapeutic modality to the subject. Methods of administering cancer therapies are known in the art.

It is understood that any therapy described herein can include one or more additional therapeutic agents. That is, any therapy described herein can be co-administered (administered in combination) with one or more additional therapeutic agents such as, but not limited to, other cancer therapies described herein. Furthermore, any therapy described herein can include one or more agents for treating one or more side-effects of a therapy comprising the cancer therapy. Combination therapies (e.g., co-administration of a cancer therapy and one or more additional cancer therapies or additional therapeutic agents) can be, e.g., simultaneous or successive. For example, a cancer therapy and the additional therapeutic agent(s) can be administered at the same time or at different times. In some embodiments, the one or more additional therapeutic agents can be administered first in time and the cancer therapy can be administered second in time. The one or more additional cancer therapies include but are not limited to surgery, chemotherapy, radiation therapy, hormone therapy, targeted therapy, and immunotherapy.

In cases where the subject having cancer and predicted to respond to a cancer therapy has been previously administered the cancer therapy, the therapy can replace or augment a previously or currently administered therapy. For example, upon treating with a CDK4/6 inhibitor, administration of a non-CDK4/6 inhibitor therapy can cease or diminish, e.g., be administered at lower levels. Administration of the previous therapy can be maintained while the therapy comprising CDK4/6 inhibitor is administered. In some embodiments, a previous therapy can be maintained until the level of CDK4/6 inhibitor reaches a level sufficient to provide a therapeutic effect.

7. Combination therapies

The methods disclosed herein describe the use of cancer therapy (CDK4/6-driven therapies for Rb-proficient cancers) in combination with one or more additional therapeutic regimens including, but not limited to surgery, chemotherapy, radiation therapy, hormone therapy, targeted therapy, and immunotherapy. Such therapeutic regiments are well-known in the art. See e.g., Chessum N et al, Prog Med Chem 2015, Lee YT et al; Eur J Pharmacol 2018; Chin HM et al, Journal of Immunotherapy and

Precision Oncology (2019) 2 (1): 10–16. Targeted therapy regimens include the use of agents such as MEK inhibitors, ERK inhibitors, hormonal therapy, and RAS(G12C) inhibitors. MEK inhibitors include, but are not limited to, trametinib, cobimetinib, binimetinib, selumetinib, PD-325901, CI-1040, PD035901, and TAK-733. ERK inhibitors include, but are not limited to, ulixertinib, BVD-523, CC-90003, GDC-0994, and MK-8533. RAS(G12C) inhibitors include but are not limited to AMG 510 and MRTX849.

8. Kits

This disclosure also provides kits. In certain embodiments, the kit can include an antibody or antibodies that can be used to detect one or more of the biomarkers disclosed herein or their concentration or expression levels. For example, the kit can include an antibody that specifically binds CDK6 and/or CDK4. The antibodies in the kit may be monoclonal or polyclonal and can be further conjugated with a detectable label. In some embodiments, the kit includes probes that can be used to identify or detect CDK6 and/or CDK4. In some embodiments, the kit includes any of the nucleic acid arrays. In some embodiments, the kit includes probes and antibodies that can be used to identify or detect CDK6 and/or CDK4 or their expression or expression levels. The kits can, optionally, contain instructions for detecting and/or measuring the concentration of one or more proteins or the levels of mRNA in a biological sample (e.g., a tumor sample).

The kits can optionally include, e.g., a control (e.g., a concentration standard for the protein being assessed) or control labeled-amplicon set containing known amounts of one or more amplicons recognized by nucleic acid probes of the array. In some instances, the control can be an insert (e.g., a paper insert or electronic medium such as a CD, DVD, or floppy disk) containing an expression level or expression level ranges of one or more proteins or RNAs (e.g., of CDK6/CDK4).

In some embodiments, the kits can include one or more reagents for processing a biological sample (e.g., calibration reagents, buffers, diluents, color reagents, reagents to stop a reaction). For example, a kit can include reagents for isolating a protein from a tumor sample and/or reagents for detecting the presence and/or amount of a protein in a

tumor sample (e.g., an antibody that binds to the CDK6 that is the subject of the detection assay and/or an antibody that binds the antibody that binds to the CDK6).

In certain embodiments, the kit includes at least one microplate (e.g., a 96 well plate; i.e., 12 strips of 8 wells). The microplate can be provided with its corresponding plate cover. The microplate can be polystyrene or of any other suitable material. The microplate can have the antibody that is used to identify the presence of a particular biomarker (e.g., CDK6) coated inside each well. The antibody may be conjugated to a detectable label.

In some embodiments, the kits can include a software package for analyzing the results of, e.g., expression profile or a microarray analysis.

The kits can also include one or more antibodies for detecting the protein expression of the genes described herein (e.g., CDK6/CDK4). For example, a kit can include (or in some cases consist of) one or a plurality of antibodies capable of specifically binding to one or more proteins encoded by any of the genes described herein and optionally, instructions for detecting and/or measuring the concentration of one or more proteins and/or a detection antibody comprising a detectably-labeled antibody that is capable of binding to at least one antibody of the plurality. In some embodiments, the kits can include antibodies that recognize CDK6, CDK4, or both. In some embodiments, the kits can include antibodies that recognize CDK6. In some embodiments, the kits can include antibodies that recognize CDK4. In certain embodiments, the kit can also optionally include one or more unit doses of a cancer therapy.

The kits described herein can also, optionally, include instructions for administering a cancer therapy, where the concentration of one or more proteins or expression level of one or more RNAs predicts that a subject having or suspected of having a cancerous tumor will respond to a cancer therapy.

In a specific embodiment, the kit comprises one or more of the following:

(i) a microplate (e.g., a 96 well plate). The microplate can be coated with an anti-CDK6 antibody that is conjugated with a detectable label. The anti-CDK6 antibody may monoclonal or polyclonal. The antibody can be e.g., from mouse, rabbit, rat, or guinea pig. The detectable label can be e.g., horse radish peroxidase, biotin, a fluorescent moiety,

a radioactive moiety, a histidine tag, or a peptide tag. The microplate can be provided with a cover.

(ii) a vial containing anti-CDK6 conjugated with a detectable label. The detectable label can be e.g., horse radish peroxidase, biotin, a fluorescent moiety, a histidine tag, a peptide tag. The vial can also include a preservative.

(iii) a vial containing a CDK6 standard of known concentration. The CDK6 can be a recombinant human CDK6.

(iv) a vial containing an assay diluent.

(v) a vial containing a calibrator diluent.

(vi) a vial containing wash buffer. The buffer may be provided as a concentrate.

(vii) one or more vials containing color reagents.

(viii) a vial containing a stop solution to stop the colorimetric reaction.

The following examples are provided to better illustrate the claimed invention and are not to be interpreted as limiting the scope of the invention. To the extent that specific materials are mentioned, it is merely for purposes of illustration and is not intended to limit the invention. One skilled in the art can develop equivalent means or reactants without the exercise of inventive capacity and without departing from the scope of the invention.

EXAMPLES

The following examples are provided to better illustrate the claimed invention and are not to be interpreted as limiting the scope of the invention. To the extent that specific materials are mentioned, it is merely for purposes of illustration and is not intended to limit the invention. One skilled in the art can develop equivalent means without the exercise of inventive capacity and without departing from the scope of the invention.

The following materials and methods were used for the Examples set forth in this disclosure.

Compounds

Commercially available compounds Palbociclib, Ganetespib, Luminespib and Bortezomib were obtained from Selleckchem. Abemaciclib, Ribociclib and MLN4924 were obtained from Medchem Express. Blasticidin S, cycloheximide and doxycycline were purchased from Sigma. Chemical compounds were dissolved either in DMSO or water and stored at -20°C.

Cell culture

Cell lines HEK293T, HEK293FT, Huh-7, U87MG, A375 and A673 were maintained in DMEM supplemented with 10% fetal bovine serum, 2 mM glutamine, and penicillin/streptomycin, MCF7, T47D, ZR-75-1, CAMA-1, NCI-H358, Colo205, SK-MEL-1, IGROV-1, TC-71, Mino, JeKo-1, Granta 519, Z-138, REC-1, KMS-12-PE, MV4-11, MOLM-14, Pfeiffer, SK-MEL-2, A549, Calu-6, NCI-H1792, NCI-H2087, NCI-H2291, NCI-H1915, NCI-H1666, NCI-H1395, HCC827, PC9, HCT15, HCT116, RKO, LoVo, HT-29, SW620 and BT549 were maintained in RPMI1640 supplemented with 10% fetal bovine serum, 2 mM glutamine, and penicillin/streptomycin (ThermoFisher Scientific). MCF7, T47D, ZR-75-1, CAMA-1, NCI-H358, Colo205, A375, A549, SK-MEL-2, Calu-6, NCIH1792, NCI-H2087, NCI-H2291, NCI-H1915, NCI-H1666, NCI-H1395, HCC827, PC9, RKO, HCT15, HCT116, SW620, LoVo, HT-29 and HEK293T were purchased from ATCC. U87MG, IGROV-1 and BT549 were provided by Dr. Ramon Parsons. Huh-7 cells were provided by Dr. Amaia Lujambio. Z-138 cells were provided by Dr. E. Premkumar Reddy. REC-1, KMS-12-PE and Pfeiffer were gifts from Dr. Samir Parekh. MV-4-11 and MOLM-14 cells were provided by Dr. Iannis Aifantis (New York Univeristy). Mino, JeKo-1, Granta 519 were gifts from Dr. Shannon M. Buckley (University of Nebraska). A673 and TC-71 were provided by Dr. Christine A. Pratilas (Johns Hopkins). HEK293FT cells were a kind gift from Dr. William Kaelin Jr (Harvard Medical School).

Plasmids

Tet-pLKO-puro (item #21915) was purchased from Addgene. shCDK4 (**SEQ ID NO: 5**; TRCN0000018364: 5' - CCG GGA TGA CTG GCC TCG AGA TGT ACT CGA GTA CAT CTC GAG GCC AGT CAT CTT TTT G -3' and **SEQ ID NO: 6**; TRCN0000010520: 5' - CCG GAC AGT TCG TGA GGT GGC TTT ACT CGA GTA AAG CCA CCT CAC GAA CTG TTT TTT G -3'), shCDK6 (**SEQ ID NO: 7**; TRCN0000010081: 5'- CCG GGT CTC ACC CAT ACT TCC AGG ACT CGA GTC CTG GAA GTA TGG GTG AGA CTT TTT G-3' and **SEQ ID NO: 8**; TRCN0000000488: 5'- CCG GCA GAC AGA GAA ACC AAA CTA ACT CGA GTT AGT TTG GTT TCT CTG TCT GTT TTT G -3') were subcloned into Tet-pLKO plasmid. Human CDK6 coding sequences were amplified from HEK293H cDNA using a SuperScript III First-Strand kit (Thermo Fisher). C-terminal V5-tagged CDK6 were subcloned using pcDNA3 as a backbone. shRNA-resistant CDK6 and S178P CDK6 were generated with a QuikChange II XL Site-Directed Mutagenesis Kit (Agilent).

siRNA knockdown

A673, TC-71 and NSCLC cell lines were transfected with SMARTpool ON-TARGETplus Non-targeting pool, or SMARTpool ON-TARGETplus CDK4-siRNA (L-003238-00-0005), or SMARTpool ON-TARGETplus CDK6-siRNA (L-003240-00-0005) (Dharmacon) using Lipofectamine RNAiMAX (Thermo Fisher).

Western Blot and immunoprecipitation

Cells were washed with PBS and lysed on ice for 10 min in NP40 buffer (50mM Tris pH 7.5, 1% NP40, 150mM NaCl, 10% Glycerol, 1mM EDTA) supplemented with protease and phosphatase inhibitors (Roche). Lysates were centrifuged at 15,000 rpm for 10 min and the protein concentration was quantified using BCA (Thermo Fisher). Protein G agarose (Thermo Fisher) was used for immunoprecipitations. The following antibodies were used: phospho-Rb (Ser807/811), Rb (4H1), PLK1 (208G4), cyclin D2 (D52F9), cyclin E2, Akt1 (2H10), JAK2 (D2E12), DHFR and ß-Actin (13E5) (Cell Signaling), CDK4 (H-22), CDK6 (C-21; DCS-83), CDK2 (D-12), CDK7 (C-19), CDK9 (D-7), p15/16 (C-7), p16 (C-20), p18 (118.2), p57 (KP39), cyclin A (H-432; B-8), cyclin D1 (M-

20; A-12), cyclin D3 (D-7), cyclin E1 (HE12), CDC37 (H-271; E-4), Hsp70 (W27) and Hsp90α/ß (F-8) (Santa Cruz), p27 and c-Raf (BD Transduction Laboratories), p21 and phospho-CDK4-T172 (ABclonal),CRBN (Novus Biologicals) and V5 (Thermo Fisher).

In vitro CDK4 and CDK6 kinase assay

Palbociclib, MS140 and ribociclib were assayed by Reaction Biology Corporation for *in vitro* kinase activity. Briefly, skinase substrates were added to base reaction buffer [20 mM Hepes (pH 7.5), 10 mM MgCl2, 1 mM EGTA, 0.02% Brij35, 0.02 mg/ml BSA, 0.1 mM Na3VO4, 2 mM DTT, 1% DMSO]. Differential CDK4/6 complexed with cyclin D1 were then diluted in the substrate solution. A range concentrations of compounds were incubated with the kinase reaction mixture by acoustic technology (Echo550; nanoliter range) and incubated for 20 minutes at room temperature followed by the addition of 10 μ Ci/μL 33P-ATP. Reactions were incubated for 2 hours at room temperature and radioactivity was detected by filter binding methods. Kinase activity data were presented as % kinase activity in samples relative to DMSO control. IC50 values were calculated using GraphPad Prism 5.

Lentivirus transduction and stable cell lines

Lentivirus was produced by transfecting HEK293FT cells with a lentiviral transfer vector, psPAX2 and pMD.G at a 5:4:1 ratio using Lipofectamine 2000 (Thermo Fisher). The viral supernatant was collected 72 h after transfection and filtered through a 0.45μm filter unit (Millipore). For Dox-inducible stable CDK4/6 knockdown cell lines, cells were transduced with shCDK4 or shCDK6 lentivirus in the presence of 8 μg/ml polybrene (EMD Millipore) and selected with 2 μg/ml puromycin (Thermo Fisher) for 5-7 days.

Cellular thermal shift assay

For basal CDK6 thermal stability assay, cells resuspended in 1 ml PBS containing protease inhibitor cocktail (Roche) were aliquoted with 100 μl cell suspension each into nine PCR tubes (~ 3 x 106 cells). Heat the PCR tubes at different temperature endpoints

(35-59 oC) for 3 min in the Veriti 96-well thermal cycler (Thermo Fisher). Remove and incubate the tubes at room temperature for 3 min. Snap-freeze the samples immediately in liquid nitrogen. Freeze and thaw the cells three times using liquid nitrogen and a thermal cycler at 25°C. Cell lysates were centrifuged at 20,000 x g for 15 min at 4 oC. Supernatants were collected for Western blots. For PB or MS140-ve-induced thermal shift assay, cells were treated with 1 μM PB or 15 μM MS140-ve for 2 hr. Cells resuspended in PBS containing protease inhibitor cocktail (Roche) were aliquoted with 100 μl cell suspension each into nine PCR tubes. Samples were heated at different temperature endpoints for 3 min using the Veriti 96-well thermal cycler (Thermo Fisher), followed by incubation at room temperature for 3 min and snap-freezing in liquid nitrogen. After three freeze and thaw cycles, samples were centrifuged at 20,000 x g for 15 min at 4°C. Supernatants were collected for Western blot analysis.

ATP-biotin competition assay

Cell lysates were prepared and labeled according to the manual instructions for the Pierce Kinase Enrichment Kits and ActivX Probes (Thermo Scientific). Briefly, cells were pre-treated with PB for 2 hr, lysed and centrifuged at 16,000 x g for 10 min at 4 oC. The supernatant was desalted through Zeba Spin Desalting Columns. 1 mg of total cell lysates in 500 μl were used for ATP competition reaction with a final concentration of 5 μM desthiobiotin-ADP probe for 10 min at room temperature. Samples were mixed with 500 μl 8 M urea and 50 μl streptavidin agarose for 1 hr at room temperature on a rotator. Beads were washed with 4 M Urea/lysis buffer and collected by centrifugation at 1000 x g for 1 min. Proteins were eluted with 2 x sample buffer at 95°C for 5 min. Samples were analysed by immunoblotting.

Generation of CRBN-/- ZR-75-1 cell line by CRISPR/Cas9

Human *CRBN* gRNA (5'-CACCGTAAACAGACATGGCCGGCGA-3') was designed using the following website: http://crispr.mit.edu/ and cloned into *Bsm*BI-digested lentiCRISPRv2 (Addgene, #52961). ZR-75-1 cells were transduced with sgCRBN lentivirus in the presence of 8 μg/ml polybrene followed by selecting with 2

μg/ml puromycin for 5 days. Cells were plated at 0.3 cells/well in a 96-well plate. After 2-3 weeks, individual clones were expanded. *CRBN* homozygous knockout clones were validated by genotyping with primers (F : 5'- AAG TCA TGC TAA GGG CTG GAA C - 3', R: 5'- GGA TGG GTT TCC TGT TCT TAA TAG -3') and Western Blotting.

Sample preparation for quantitative mass spectrometry analysis

Colo205 cells were treated with 0.3 μM MS140 or MS140-ve for 5 hr in duplicate. Cell pellets were lysed in lysis buffer containing 8 M urea, 50 mM Tris, pH 8.0, 75 mM NaCl, 1 mM MgCl2, and 500 units Benzonase. Proteins were reduced with DTT and alkylated with iodoacetamide. After precipitation, proteins were first digested with LysC for 4 hr at 37°C. The solution was diluted 4-fold with 25 mM Tris, pH 8.0, 1 mM CaCl2 and further digested with trypsin (Promega) for 12 hr at 37 °C. Peptides were desalted on Sep-Pak Light C18 cartridges (Waters, Milford, MA) and dissolved in 30% acetonitrile, 0.1% TFA before loading on a 300-m Source 15S (GE Healthcare, Pittsburgh, PA) column for basic reversed phase chromatography (bRPLC). A linear LC gradient was performed by increasing buffer B from 0 to 70% within 60 min, where buffer A was aqueous 10 mM ammonium formate, and buffer B was 90% AcCN (Acetonitrile) in 10 mM ammonium formate. A total of 30 fractions were collected for each of the basal (WHIM2) and luminal (WHIM16) samples and non-contiguously recombined to five fractions per sample. The fractions were dried and desalted using a stop-and-go-extraction tip (StageTip) protocol containing 4 1-mm C18 extraction disk (3 M).

Liquid Chromatography-Tandem Mass Spectrometry

Samples were desalted using PepClean C18 spin columns (Pierce) according to the manufacturer's directions and resuspended in aqueous 0.1% formic acid. Sample analysis was performed via reversed phase LC-MS/MS using a Proxeon 1000 nano-LC system coupled to a Q Exactive mass spectrometer (Thermo Scientific, San Jose, CA). The Proxeon system was configured to trap peptides using a 3-cm long, 100-m inner diameter C18 column at 5 l/min liquid flow that was diverted from the analytical column via a vent valve, whereas elution was performed by switching the valve to place the trap

column in line with a 15-cm long, 75-m inner diameter, 3.5-m, 300-Å particle C18 analytical column. Analytical separation of all the tryptic peptides was achieved with a linear gradient of 2–30% buffer B over 240 min at a 250 nl/min flow rate, where buffer A was aqueous 0.1% formic acid, and buffer B was acetonitrile in 0.1% formic acid. LC-MS experiments were also performed in a data-dependent mode with full MS (externally calibrated to a mass accuracy of 5 ppm and a resolution of 70,000 at m/z 200) followed by high energy collision-activated dissociation-MS/MS of the top 20 most intense ions. High energy collision-activated dissociation-MS/MS was used to dissociate peptides at a normalized collision energy of 27 eV in the presence of nitrogen bath gas atoms. All five bRPLC fractions were derived from three process technical replicates of each tumor sample and were subjected to two independent LC-MS runs resulting in the production of 20 LC-MS runs for global peptide analysis. Mass spectra were processed, and peptide identification was performed using the Andromeda search engine found in MaxQuant software version 1.3.0.5 (Max Planck Institute, Germany). All protein database searches were performed against the UniProt human and mouse protein sequence database downloaded from the Clinical Proteomic Tumor Analysis Consortium Data Portal. This database contains 105,001 annotated proteins, and the sequences were derived from the UniProt December 2012 assembly. Peptides were identified with a target decoy approach using a combined database consisting of reverse protein sequences of the UniProt human, mouse, and common repository of adventitious proteins. The common repository of adventitious proteins database was obtained from the Global Proteome Machine. Peptide inference was made with a false discovery rate (FDR) of 1%, and peptides were assigned to proteins with a protein FDR of 5%. A precursor ion mass tolerance of 20 ppm was used for the first search that allowed for m/z retention time recalibration of precursor ions that were then subjected to a main search using a precursor ion mass tolerance of 6 ppm and a product ion mass tolerance 0.5 Da. Search parameters included up to two missed cleavages at Lys/Arg on the sequence, oxidation of methionine, and protein N-terminal acetylation as a dynamic modification. Carbamidomethylation of cysteine residues was considered as a static modification. Peptide identifications are reported by

filtering of reverse and contaminant entries and assigning to their leading razor protein. All of the mass spectrometry data on PDX tumor samples were deposited at the CPTAC Data Coordinating Center as raw and mzML files for public access.

Peptide and Protein Quantitation

LFQ was performed based on peak area. The measured area under the curve of m/z and the retention time-aligned extracted ion chromatogram of a peptide were performed via the label-free quantitation module found in MaxQuant version 1.3.0.5 (30). All replicates for each PDX were included in the LFQ experimental design with peptide-level quantitation performed using unique and razor peptide features corresponding to identifications filtered with a posterior error probability of 0.06, peptide FDR of 0.01, and protein FDR of 0.05. The MaxQuant peptide and protein groups files were processed and stored in an Oracle database, and statistical analysis, model building, and visualization of a majority of data were performed based on Statistical Analysis Software code and R script that was developed in-house.

Mass spectrometry to identify CDK6-interacting proteins: Preparation of Samples for Mass Spectrometry

The affinity purified proteins were reduced, alkylated, and digested with trypsin directly on the beads. Briefly, the beads were resuspended in 100uL 100mM ammonium bicarbonate. Proteins were reduced with 2µl of 0.2M dithiothreitol (Sigma) for one hour at 57ºC at pH 7.5, alkylated with 2µl of 0.5M iodoacetamide (Sigma) for 45 minutes at room temperature in the dark, and digested using 200ng sequencing grade trypsin (Promega) overnight at room temperature with gentle shaking. The solution was transferred to a new tube and the digestion stopped by adding 100 ul of a 5% formic acid and 0.2% trifluoroacetic acid (TFA) R2 50 µm Poros (Applied Biosystems) beads slurry in water. The samples were allowed to shake at 4ºC for 3 hour. The beads were loaded onto C18 ziptips (Millipore), equilibrated with 0.1% TFA, using a microcentrifuge for 30 seconds at 6,000 rpm. The beads were washed with 0.5% acetic acid. Peptides were eluted with 40% acetonitrile in 0.5% acetic acid followed by 80% acetonitrile in 0.5%

acetic acid. The organic solvent was removed using a SpeedVac concentrator and the sample reconstituted in 0.5% acetic acid.

Mass Spectrometry Analysis

1/10th of each sample was loaded onto an Acclaim PepMap trap column (2 cm x 75 μm) in line with an EASY-Spray analytical column (50 cm x 75 μm ID PepMap C18, 2 μm bead size) using the auto sampler of an EASY-nLC 1200 HPLC (Thermo Fisher Scientific) with solvent A consisting of 2% acetonitrile in 0.5% acetic acid and solvent B consisting of 80% acetonitrile in 0.5% acetic acid. The peptides were gradient eluted into a Thermo Fisher Scientific Q Exactive HF-X Mass Spectrometer using the following gradient: 5 - 35% in 60 min, 35 - 45% in 10 min, followed by 45 - 100% in 10 min. High resolution full MS spectra were recorded with a resolution of 45,000, an AGC target of 3e6, with a maximum ion time of 45ms, and a scan range from 400 to 1500m/z. The MS/MS spectra were collected using a resolution of 15,000, an AGC target of 1e5, maximum ion time of 120ms, one microscan, 2 m/z isolation window, and Normalized Collision Energy (NCE) of 27.

Data Processing

The MS/MS spectra were searched against the Uniprot human reference proteome database containing common contaminant proteins using Sequest within Proteome Discoverer 2.3. The search parameters were as follows: precursor mass tolerance ±10 ppm, fragment mass tolerance ± 0.02 Da, digestion parameters trypsin allowing two missed cleavages, fixed modification of carbamidomethyl on cysteine, variable modification of oxidation on methionine, variable modification of deamidation on glutamine and asparagine, and a 1% peptide and protein FDR searched against a decoy database. The results were filtered to only include proteins identified by at least two unique peptides.

Cell viability assay

Cells were seeded at 3-10 x 103 cells/well in 96-well plates. 24 hr after seeding, cells were treated with palbociclib and MS140 at a range of concentrations for 72 hr. 10 µl of 0.1 mg/ml resazurin (Sigma-Aldrich) was added to cells and incubated for 2-3 hr at 37°C. Cell viability was determined by measuring the fluorescence at 560 nm excitation wavelength and 590 nm emission wavelength using a Molecular Devices Spectramax M5 plate reader. IC_{50} values were calculated using log-transformed, normalized data in GraphPad Prism 5.0.

Crustal violet cell growth assay

Cells were seeded at 1-10 x 103 cells/well in six-well plates. The next day, cells were treated with the increasing concentrations of palbociclib and MS140 for 10-15 days. Cell culture medium was replaced every 2 days in the presence or absence of inhibitors. Cells were fixed with 10% formalin solution (Sigma-Aldrich) for 5 min at RT followed by 0.05% crystal violet for 25 min. cells were de-stained with tap water and air-dried.

Quantitative Real-time PCR

Total RNA was extracted using Trizol Reagent (Thermo Fisher). Complementary DNA was synthesized with a SuperScript IV First-Strand kit (Thermo Fisher). Quantitative real-time PCR was performed using a Fast SYBR Green Master Mix kit (Thermo Fisher) with a 7500 Fast realtime PCR system (Applied Biosystems). PCR primers are as follows: h*GAPDH* forward: 5'-ACA ACT TTG GTA TCG TGG AAG G-3', reverse: 5'- GCC ATC ACG CCA CAG TTT C-3'; h*PLK1* forward: 5'- CAC CAG CAC GTC GTA GGA TTC-3', reverse: 5'- CCG TAG GTA GTA TCG GGC CTC-3'; h*CCNA2* forward: 5'- CGC TGG CGG TAC TGA AGT C-3', reverse: 5'- CGC TGG CGG TAC TGA AGT C- 3'; h*AURKB* forward: 5'- CAG AAG AGC TGC ACA TTT GAC G -3', reverse: 5'- CCT TGA GCC CTA AGA GCA GAT TT -3'; h*CDC45* forward: 5'- CTT GAA GTT CCC GCC TAT GAA G -3', reverse: 5'- GCA TGG TTT GCT CCA CTA TCT C -3'; h*PCNA* forward: 5'- CCT GCT GGG ATA TTA GCT CCA -3', reverse: 5'- CAG CGG TAG GTG TCG AAG C -3'; h*RAD51* forward: 5'- CGA GCG TTC AAC ACA GAC CA - 3', reverse: 5'- GTG GCA CTG TCT ACA ATA AGC A -3';

h*RRM2* forward: 5'- GTG GAG CGA TTT AGC CAA GAA -3', reverse: 5'- CAC AAG GCA TCG TTT CAA TGG -3'. m*GAPDH* forward: 5'- AGG TCG GTG TGA ACG GAT TTG -3', reverse: 5'- TGT AGA CCA TGT AGT TGA GGT CA -3'; m*AURKB* forward: 5'- CAG AAG GAG AAC GCC TAC CC 3', reverse: 5'- GAG AGC AAG CGC AGA TGT C -3'; m*CCNA2* forward: 5'- GCC TTC ACC ATT CAT GTG GAT -3', reverse: 5'- TTG CTC CGG GTA AAG AGA CAG -3'; m*E2F1* forward: 5'- CTC GAC TCC TCG CAG ATC G -3', reverse: 5'- GAT CCA GCC TCC GTT TCA CC -3'; m*PCNA* forward: 5'- TTT GAG GCA CGC CTG ATC C, reverse: 5'- GGA GAC GTG AGA CGA GTC CAT -3'; m*PLK1* forward: 5'- CTT CGC CAA ATG CTT CGA GAT -3', reverse: 5'- TAG GCT GCG GTG AAT TGA GAT -3'; m*RAD51* forward: 5'- AAG TTT TGG TCC ACA GCC TAT TT -3', reverse: 5'- CGG TGC ATA AGC AAC AGC C -3'; m*CDC45* forward: 5'- GAT TTC CGC AAG GAG TTC TAC G -3', reverse: 5'- TAC TGG ACG TGG TCA CAC TGA -3'; m*RRM2* forward: 5'- TGG CTG ACA AGG AGA ACA CG -3', reverse: 5'- AGG CGC TTT ACT TTC CAG CTC -3'. Differences in expression were calculated by the ΔΔCt method.

Genetic dependency data and genomics data

CRISPR dependency data were obtained from the 20Q1 public Avana dataset containing genome-scale CRISPR knockout screens for 18,333 genes in 739 cell lines. The gene dependencies were estimated for each gene and cell lines by the CERES algorithm (Meyers RM et al, 2017). RNA interference (RNAi) dependency data were derived from combination of the Broad Institute Project Achilles, Novartis Project Drive, and Marcotte et al. database (Robert McDonald 3rd et al. (2017), Cell Jul 27;170(3):577-592.e10; Tsherniak A. et al., Cell. 2017 Jul 27;170(3):564-576; Richard Marcotte R. et al. (2016) Cell Jan 14;164(1-2):293-309). The genetic dependencies were estimated using the DEMETER2 model across 712 unique cancer cell lines (McFarland J. M. (2018) Nat Commun Nov 2;9(1):4610). Cancer cell line mRNA expression were taken from the DepMap 20Q1 data release. Cancer cell line encyclopedia (CCLE) proteomics data were obtained by quantitative profiling of proteins by mass spectrometry across 375 cell lines (Kamerling J. P. et al., (1988) Dec 5;241(1-2):246-50). The normalized protein

quantitation data was obtained from the portal https://gygi.med.harvard.edu/publications/ccle. All cell line omics data can be downloaded at DepMap depmap.org/portal/.

Animal experiments

To determine in vivo degradation efficacy, 5-7 week-old female athymic Nude-*Foxn1nu* mice obtained from Envigo Laboratories were injected subcutaneously with 1 x 107 JeKo-1 cells in 1:1 PBS/ Matrigel GFR membrane Matrix (Corning) or 5 x 106 Colo205 cells in PBS. Mice were treated with vehicle (5% DMSO and 95% PEG 300) or MS140 (25 or 30 mg/kg) intraperitoneally, twice daily, or palbociclib (50 or 60 mg/kg) orally once daily for 3 days when tumors reach around 100 mm3. 5 h after the last dose, tumors were collected for further analysis. Liver and kidney were collected for qPCR analysis. For efficacy of MS140 on tumor xenograft, 5 x 10^6 Colo205 cells in PBS or 10 x 10^6 JeKo-1 cells in 1:1 PBS/ Matrigel GFR membrane Matrix were injected subcutaneously on the left flank in 6-week old female athymic Nude-*Foxn1nu* mice. Tumors were allowed to reach 100 mm^3 in size before the animals were randomized in two groups of 5-8 mice per group. Mice were treated with vehicle (5% DMSO and 95% PEG 300) or MS140 (25 or 30 mg/kg) intraperitoneally, twice daily, or palbociclib (50 or 60 mg/kg) orally once daily for 3 weeks. Tumor size was measured using caliper every 3 days and tumor volume was calculated as the following formula: (Length xWidth2)/2. Tumor samples and organs were collected at the end of treatment for further analysis. All experiments were conducted under a protocol approved by Mount Sinai School of Medicine Institutional Animal Care and Use Committee (IACUC).

Complete blood count

5-7 week-old female C57B/6 mice (Envigo Laboratories) were dosed intraperitoneally with 25 mg/kg MS140 twice daily or 50 mg/kg PB once daily via oral gavage for 3 weeks. Mouse blood samples were collected in K3EDTA tubes before treatment and last treatment. Complete blood counts were performed with Coulter Ac•T 5diff Hematology Analyzer (BECKMAN).

Chemical synthesis: Chemistry General Procedures

HPLC spectra for all compounds were acquired using an Agilent 1200 Series system with DAD detector. Chromatography was performed on a 2.1×150 mm Zorbax 300SB-C18 5 μm column with water containing 0.1% formic acid as solvent A and acetonitrile containing 0.1% formic acid as solvent B at a flow rate of 0.4 mL/min. The gradient program was as follows: 1% B (0−1 min), 1−99% B (1−4 min), and 99% B (4−8 min). High resolution mass spectra (HRMS) data were acquired in positive ion mode using an Agilent G1969A API-TOF with an electrospray ionization (ESI) source. Nuclear Magnetic Resonance (NMR) spectra were acquired on a Bruker DRX-600 spectrometer (600 MHz 1H, 150 MHz 13C) or a Varian Mercury spectrometer (400 MHz 1H, 100 MHz 13C). Chemical shifts are reported in ppm (δ). Preparative HPLC was performed on Agilent Prep 1200 series with UV detector set to 254 nm. Samples were injected into a Phenomenex Luna 75 x 30 mm, 5 μm, C18 column at room temperature. The flow rate was 40 mL/min. A linear gradient was used with 10% (or 50%) of MeOH (A) in H2O (with 0.1 % TFA) (B) to 100% of MeOH (A). HPLC was used to establish the purity of target compounds. All final compounds had > 95% purity using the HPLC methods described above.

(2-(2,6-Dioxopiperidin-3-yl)-1,3-dioxoisoindolin-4-yl)glycine

A solution of 2-(2,6- dioxopiperidin-3-yl)-4-fluoroisoindoline-1,3-dione (1.38 g, 5.0 mmol), *tert*-butyl glycinate (0.66g, 5.0 mmol), and *N,N*-diisopropylethylamine (1.31 mL, 7.5 mmol) in DMF (10 mL) was heated to 85 °C in a microwave reactor for 40 min. After cooling to RT, the reaction was diluted with water and extracted with ethyl acetate (3x). Combined organic phase was dried over anhydroussodium sulfate and concentrated under reduced pressure. The resulting residue was purified by silica gel chromatography to give the desired tBu ester intermediate as oil. This intermediate was treated with a solution of hydrogen chloride in dioxane (10 mL, 4.0 M) for 16 h. The reaction was concentrated under reduced pressure to give desired acid product (0.24 g, 14%). 1H NMR

(600 MHz, CD3OD) δ 7.57 (dd, *J* = 8.5, 7.1 Hz, 1H), 7.11 (d, *J* = 7.1 Hz, 1H), 6.95 (d, *J* = 8.5 Hz, 1H), 5.07 (dd, *J* = 12.6, 5.5 Hz, 1H), 4.12 (s, 2H), 2.86 (ddd, *J* = 18.0, 14.4, 5.4 Hz, 1H), 2.74 – 2.67 (m, 2H), 2.15 – 2.08 (m, 1H). MS (ESI) *m/z* 332.1 [M+H]+.

4-((2-(4-(6-(((6-Acetyl-8-cyclopentyl-5-methyl-7-oxo-7,8-dihydropyrido[2,3-d]pyrimidin-2-yl)amino)pyridin-3-yl)piperazin-1-yl)-2-oxoethyl)amino)-2-(2,6-dioxopiperidin-3-yl)isoindoline-1,3-dione (MS140)

To a solution of (2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisoindolin-4-yl)glycine (0.017 g, 0.051 mmol) in DCM (10 mL) and DMSO (2 mL) were added palbociclib (0.023 g, 0.051 mmol), 4-methylmorpholine (0.020 g, 0.20 mmol), 1-hydroxy-7-azabenzotriazole (0.0090 g, 0.066 mmol) and *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (0.013 g, 0.066 mmol). The reaction mixture was stirred at room temperature for 16 h, before being concentrated under reduce pressure. The resulting residue was purified by reverse-phase prep-HPLC to yield the product (0.028 g, 72%) as yellow solid. 1H NMR (600 MHz, CD3OD) δ 9.08 (s, 1H), 8.21 (dd, *J* = 9.6, 2.8 Hz, 1H), 7.86 (d, *J* = 2.8 Hz, 1H), 7.55 – 7.50 (m, 2H), 7.03 (d, *J* = 7.0 Hz, 1H), 6.99 (d, *J* = 8.5 Hz, 1H), 6.04 – 5.97 (m, 1H), 5.08 (dd, *J* = 12.7, 5.5 Hz, 1H), 4.24 (s, 2H), 3.88 – 3.73 (m, 4H), 3.42 – 3.31 (m, 4H), 2.92 – 2.82 (m, 1H), 2.80 – 2.67 (m, 2H), 2.50 (s, 3H), 2.43 (s, 3H), 2.35 – 2.27 (m, 2H), 2.15 – 2.06 (m, 3H), 1.94 – 1.87 (m, 2H), 1.73 – 1.66 (m, 2H). HRMS (ESI-TOF) *m/z*: [M+H]+ calcd for C39H41N10O7, 761.3160; found: 761.3150

Example 1: Intrinsic resistance to CDK4/6i is associated with incomplete inhibition of Rb/E2F and expression of CDK6

To gain insight into mechanisms of intrinsic resistance or sensitivity to CDK4/6i, the concentration-dependent effects of PB on the growth of cancer cell lines derived from a variety of Rb-proficient tumor types are assessed. Large variations in cell line response to CDK4/6i are observed, consistent with previous reports (Gong X. et al., (2017) Cancer Cell 32, 761-776; Kim S. et al., (2018) Oncotarget 9, 35226-35240; Ruscetti M. et al., (2018). Science 362, 1416-1422). In the "CDK4/6i-sensitive" (CDK4/6i-S) group, PB

concentrations under 500nM and in many cases as low as 80 nM are sufficient for substantial (over 90%) growth inhibition. In contrast, in the "CDK4/6i-resistant" (CDK4/6i-R) group, PB concentrations even as high as 2μM have only modest effects on cell growth (**FIG. 1A** and **FIG. 8A**). The CDK4/6i-S group include ER+ positive breast cancer cell lines, as expected (Finn R.S. et al., (2009) Breast Cancer Res 11, R77), as well as cancer cell lines from several other tumor types. However, most Rb-proficient cell lines derived from common solid tumor types including non-small cell lung carcinoma (NSCLC), melanoma and colorectal carcinoma (CRC) showed a CDK4/6i-R phenotype (**FIG. 1A** and **FIG. 8A**).

Next, the association between the observed differences of tumor cell sensitivity to PB with the extent of suppression of Rb phosphorylation and E2F pathway inhibition by the drug is assessed. Suppression of both Rb phosphorylation and of downstream E2F target proteins, PLK1 cyclin A and DHFR upon PB treatment are found to be consistently of greater magnitude in CDK4/6i-S compared to CDK4/6i-R tumor cells (**FIG. 1B** and **FIGS. 8B-C**). Other FDA-approved CDK4/6i, including Ribociclib (8) and Abemaciclib (7), show a similar profile (**FIGS. 1C-1D**), indicating that incomplete inhibition of Rb/E2F signaling in CDK4/6i-R tumor cells is a general property of CDK4/6i. The expression of several known CDK4/6 signaling-related proteins, including members of the cyclin D and cyclin E, INK4 and CIP/KIP families, are not found to be different between the two groups (**FIG. 1E**). However, most CDK4/6i-S cell lines are found to express CDK6 at very low levels, whereas the CDK4/6i-R cell lines express both CDK4 and CDK6 (**FIG. 1E**).

Example 2: Tumors expressing both CDK4 and CDK6 depend selectively on CDK6

Overexpression of a target is a mechanism that can drive resistance to small-molecule inhibitors. Thus, shRNA-mediated knockdown of CDK4 or CDK6 is conducted to assess the sensitization of PB-unresponsive cells to CDK4/6i. As expected, in cell lines expressing predominantly CDK4 and low levels of CDK6, knockdown of CDK4, but not of CDK6, is found to reduce Rb/E2F signaling (**FIG. 9A**). In CDK4/6i-R tumor cells, inducible CDK6 knockdown is found to suppress phospho-Rb and downstream E2F

signaling (**FIG. 2A**), as well as cell growth (**FIG. 2B**), which is further suppressed by treatment with PB. Surprisingly, knock-down of CDK4 in these same cells has no effect on either pRb levels or sensitivity to PB (**FIGS. 2A-B**). CDK6 (but not CDK4) knockdown is also found to suppress Rb/E2F output as well as growth in additional CDK4/6i-R cell lines (**FIG. 2C**). Moreover, ectopic expression of an shRNA-resistant mutant of CDK6 is found to rescue cell growth and Rb/E2F output inhibition promoted by CDK6 shRNA-mediated knock down (**FIG. 2D**), thus establishing the specific role of CDK6 in driving Rb/E2F signaling and cell growth in these cells. Further, phosphorylation of CDK4-T172, an established marker of CDK4 activation (Kato, J.Y. et al., (1994) Mol Cell Biol 14, 2713-2721), is readily detected in the CDK4/6i-S cells, but is substantially lower in the CDK4/6i-R cells (**FIG. 1E**). All of these findings suggest that despite expression of both CDK4 and CDK6, Rb/E2F signaling is driven selectively by CDK6 in CDK4/6i-R tumor cells.

To assess the generality of these observations in an unbiased approach, the AVANA Dependency Map (DepMap) dataset was analyzed to look at the correlation between the expression and protein levels to the dependency profiles of CDK4 and CDK6 (Meyers R. M. et al., (2017). Nat Genet 49, 1779-1784; McFarland J. M. et al. (2018) Nat Commun 9, 4610; 18; Tsherniak A. et al., (2017) Cell 170, 564-576; Marcotte R. et al., (2016) Cell 164, 293-309 (2016); Nusinow D. P. et al., (2020) Cell 180, 387-402 e316). The AVANA Dependency map is an online research and analysis tool developed by the Broad Institute, based on data derived from profiling hundreds of cancer cell line models for genomic information and sensitivity to genetic and small molecule perturbations. First, similar to the panel of cell lines used in this study, CDK4 expression is found to be relatively uniform across cancer cell lines, however CDK6 expression is highly variable. Further, and consistent with the biochemical data, CDK6 expression and protein levels are found to be predictive of dependence on CDK6, with high levels of CDK6 correlating with dependence on CDK6 and low CDK6 levels correlating with dependence on CDK4, however CDK4 protein or mRNA expression levels are not predictive of dependence on CDK4 (**FIG. 2E** and **FIG. 9B**). The data are similar whether dependency was interrogated using shRNA or sgRNA mediated loss-of

function (McFarland J.M. et al., (2018) Nat Commun 9, 4610), further validating the observation. Thus, the analysis confirms that cells expressing CDK4 and low levels of CDK6 depend on CDK4, but cells expressing high levels of CDK6 depend on CDK6 applies broadly and across tumor lines derived from various cancer types.

Example 3: Low expression of CDK6 predicts for sensitivity to CDK4/6i in NSCLC

The observation that CDK4-dependent tumors with low CDK6 expression are sensitive to CDK4/6i, led to the assessment of whether this information can be used to identify subsets of sensitive cell lines within larger, predominantly Rb-proficient, tumor types. The focus of this study is on Non-small-cell lung carcinoma (NSCLC), in which previous studies have shown clinical activity of these drugs, but failed to show a significant increase in either Progression Free Survival (PFS) or Overall Survival (OS), over current standard of care (J. W. Goldman et al., (2018) Journal of Clinical Oncology 36, 9025-9025; J. Pacheco, E. Schenk, (2019) Oncotarget 10, 618-619). In addition to the NSCLC lines examined initially (**FIG. 1A** and **FIG. 8A**), five additional NSCLC lines with the highest score for CDK4 dependence (i.e. H1792, H2087, H2291, HCC827 and H1915, in addition to H358) are examined. For comparison, two additional NSCLC CDK6-dependent lines (PC9 and H1666, in addition to A549, Calu6) are examined. The dependence of this NSCLC panel on either CDK4 or CDK6 is first confirmed. Consistent with the reported large scale data, in the CDK4-dependent cells, siRNA-mediated knockdown of CDK4, but not CDK6 suppresses pRb signaling, whereas downregulation of CDK6, but not of CDK4 suppresses pRb and downstream signaling in CDK6-dependent cells (**FIG. 3A**). Further, and consistent with the biochemical data (**FIGS. 2A-D**) and DepMAP analysis (**FIG. 2E**), CDK4-dependent cells expresses low levels of CDK6 compared to CDK4 (**FIG. 3B**).

Next, whether the response to CDK4/6i correlates with low expression of CDK6 (and consequently CDK4-dependence) in NSCLC tumor cells is assessed. In all cases, significantly higher sensitivity of CDK6-low cells to CDK4/6i is observed, both in terms of cell signaling (**FIG. 3C** and **FIG. 10A**) and of cell growth (**FIG. 3D**) compared to

CDK4/6i-R cells with high levels of CDK6 (H358 and A549, Calu6 data are included in **FIGS. 1A-B**, and **FIG. 8A**, and a list of the NSCLC cell lines used is in **FIG. 10B**).

To assess the clinical relevance of these observations, RNA expression data is analyzed from tumors from the phase III JUNIPER trial (NCT02152631), in which abemaciclib is evaluated in KRASmutated, advanced NSCLC patients (21), alongside erlotinib, a tyrosine kinase inhibitor. In a retrospective assessment, patients with NSCLC tumors with low CDK6 expression show significantly longer PFS and OS, compared to patients with tumors expressing high or intermediate levels of CDK6 in the abemacicib arm (**FIGS. 3E-F**). These data suggest that low CDK6 expression is predictive for response to treatment with abemaciclib, and not associated with less aggressive tumors in general. Further, these data confirm in a large clinical data set a critical role of CDK6 expression in the tumor in determining outcome of cancer patients treated with CDK4/6i and suggest that CDK6 expression in the tumor might be used a biomarker to stratify NSCLC patients for CDK4/6i treatment.

Together, these preclinical and clinical data analysis indicate that low CDK6 expression can be a predictive biomarker for tumor cell response to CDK4/6i in many tumor types, including a substantial portion of NSCLC, for which there are currently no available targeted therapeutics.

Example 4: A CDK4/6-degrader (PROTAC) is more effective than CDK4/6i in CDK4/6i-S tumor cells

The inactivity of CDK4/6i in cells expressing both CDK4 and high levels of CDK6, led to the postulation that targeted degradation (Lai, A.C. M. Crews, (2017) Nat Rev Drug Discov 16, 101-114) of CDK4/6 might result in more potent inhibition of Rb/E2F output and growth suppression of CDK4/6i-R tumor cells. As an approach, hetero-bifunctional small molecules are developed (Gadd M.S., et al., (2017) Nat Chem Biol 13, 514-521) that both inhibit CDK4/6 kinase activity as well as target CDK4/6 proteins for degradation. Based on the crystal structure of CDK6 in complex with PB (PDB: 5L2I) (Chen P. et al., (2016) Mol Cancer Ther 15, 2273-2281), the solvent-exposed piperazine is used as the linker attachment point. Heterobifunctional potential

CDK4/6-directed PROTACs are synthesized by linking PB to pomalidomide, a moiety with high affinity for the E3 ligase cereblon (CRBN), a component of a cullin-RING ubiquitin ligase complex (Bondeson D.P. et al., (2015). Nat Chem Biol 11, 611-617; Winter G. E. et al., (2015) Science 348, 1376-1381; E. S. Fischer E.S. et al., (2014) Nature 512, 49-53; Ito T. et al., (2010) Science 327, 1345-1350; Chamberlain P. P. et al., (2014) Nat Struct Mol Biol 21, 803-809), using linkers of various lengths and types. By screening and optimizing synthesized compounds for their ability to degrade CDK4/6 and inhibit Rb/E2F signaling in cells, MS140 is identified (**FIG. 4A**), as a highly potent CDK4/6 kinase inhibitor in vitro (**FIG. 4B**) that markedly suppressed Rb/E2F signaling and reduces CDK4 and CDK6 protein levels in a concentration and time-dependent manner (**FIGS. 11A-B**). Degradation of CDK4/6 proteins by MS140 is specific, as shown by its abrogation upon pretreatment with excess PB, or pomalidomide (**FIG. 4C** and **FIG. 11C**). CDK4/6 protein degradation by MS140 is also confirmed to be specifically mediated by the proteasome by its abrogation upon pretreatment with the proteasome inhibitor bortezomib, or with the Nedd8-activating enzyme inhibitor MLN4924 (**FIG. 4C** and **FIG. 11C**). Further, MS140 fails to degrade CDK4 in cells in which CRBN had been knocked-out using CRISPR/Cas9 technology (**FIG. 4D**), confirming that target degradation by MS140 was specifically mediated by CRBN. As a negative control, MS140-ve (**FIG. 11D**), a methyl analog of MS140 is designed and synthesized, which is predicted not to bind CRBN (C. Zhang C. et al., (2018). Eur J Med Chem 151, 304-314; and Lebraud H. et al., (2016) ACS Cent Sci 2, 927-934). Treatment with MS140-ve are not found to reduce CDK4/6 protein levels (**FIG. 4E**), further confirming that MS140 degrades its targets by linking them to the ubiquitin-proteasome machinery. Although PB is reported to bind a number of kinases in addition to CDK4/6, including CDK9 N. J. Sumi, N.J. (2015) ACS Chem Biol 10, 2680-2686). MS140 is not found to degrade CDK7 or CDK9 (**FIG. 4E** and **FIG. 11A**). Finally, global analysis of protein degradation using mass spectrometry reveals an impressively selective protein downregulation profile of MS140, with very few hits other than CDK4 and CDK6 (**FIG. 4F**). Thus, MS140 potently and selectively inhibits and degrades CDK4/6 kinases in CDK4/6i-S tumor cells by targeting them to the CRL4-CRBN-E3 ubiquitin complex.

Treatment of various Rb-proficient tumor cell lines with MS140 is found to result in 3 to 30-fold greater suppression of both Rb/E2F signaling and cell growth, compared to PB, in the CDK4/6i-S cells identified in our first screen (**FIGS. 4G-H**, and **FIG. 11E**). A notable exception is H358, in which MS140 only modestly decreased CDK4 levels or inhibited Rb signaling as compared to PB (**FIG. 11F**). However, this cell line exhibits very low endogenous levels of CRBN (**FIG. 1E**), consistent with the increased effectiveness of MS140 being CRBN-dependent. Next, additional cell lines of hematopoetic origin for Rb/E2F signaling and growth inhibition by MS140 in comparison to PB are assayed. All Mantle Cell Lymphomas (MCL) tested show sensitivity to PB and higher sensitivity to MS140 (**FIG. 11G**), associated with more potent inhibition of Rb/E2F signaling by MS140 (**FIGS. 11H-I**), consistent with these cells being predominantly CDK4-driven and expressing low levels of CDK6.

Of note, a smaller group of cancer cell lines, mainly of hematopoetic origin and driven by CDK6 (Kim S. et al., (2018) Oncotarget 9, 35226-35240; Ghandi M. et al., (2019) Nature 569, 503-508), show a similar CDK4/6i-S phenotype (**FIGS. 4I-J**). These data on growth response of hematopoetic tumor lines to PB and MS140 are summarized in **FIG. 11J**. Thus, by combining inhibition and degradation of CDK4/6 proteins, MS140 is more effective than PB in inhibiting Rb/E2F signaling and cell growth of many CDK4/6i-S tumor cell lines. CDK4/6i-S cells are predominantly those expressing CDK4 and low CDK6, but a smaller subset of CDK6-driven tumor cells are identified, that are also sensitive to CDK4/6i and more sensitive to the CDK4/6 degrader.

The increased effectiveness of MS140, as compared to PB in CDK4/6i-S cells, led to the assessment of its biochemical and antitumor effects *in vivo*. Treatment of mice with MS140 promotes degradation of CDK4/6 kinases and suppression of Rb/E2F signaling in JeKo-1 (MCL) and Colo25 (CRC) tumor cells grown as xenografts *in vivo* (**FIG. 4K** and **FIG. 11K**), resulting in equivalent or greater suppression of tumor growth compared to PB administered at the same total daily concentration (**FIG. 4L** and **FIG. 11L**) without significant body weight loss (**FIG. 11M**), or other apparent toxicities.

Example 5: In CDK4/6-R cells, CDK4/6 degraders fail to degrade CDK6 due to weak binding of compound

Since MS140 both inhibits and degrades CDK4/6, we would expect it to show increased potency compared to the parent CDK4/6i in all cell lines. Surprisingly however, in all CDK4/6i-R tumor lines tested, treatment with MS140 suppressed Rb/E2F signaling and cell growth less effectively than PB (**FIGS. 5A-B**). This result was unexpected, since shRNA-mediated knockdown of CDK6 sensitized these same tumor cells to PB (**FIGS. 2A-B**) suggesting that MS140 may bind CDK6 with different potency in the two sets of cells lines. To understand the basis for this apparent discrepancy, we compared the extent of reduction of CDK6 protein expression in response to shRNA-mediated CDK6 knock-down or MS140 treatment. As shown in **FIGS. 5C-D**, MS140 was less potent in promoting CDK6 protein degradation and in suppressing cell growth, compared to shRNA-mediated CDK6 knockdown. These results prompted us to directly compare the extent of CDK4/6 degradation by MS140 in CDK4/6i-S and CDK4/6i-R cell lines. Treatment with MS140 resulted in potent degradation of CDK4/6 in both CDK4-driven and CDK6-driven CDK4/6i-S cells, but promoted only minimal CDK4/6 degradation in CDK6-driven CDK4/6i-R cells (**FIG. 5E**). To assess whether this difference applied more generally to CDK4/6 degraders, we tested two recently reported CDK4/6-directed PROTACs, YKL-06-102(35, 36) and BSJ-02-162 (Jiang B. et al., (2019) Angewandte Chemie 58, 6321-6326). We found that, similarly to MS140, treatment with either compound resulted in more potent degradation of CDK6 in CDK4/6i-S as compared to CDK4/6i-R cells (**FIGS. 12A-B**). Of note, one of these compounds has been reported to be a CDK6-selective inhibitor, compared to CDK4. We confirmed that, as reported, that compound was relatively more potent degrader of CDK6 than CDK4 in CDK4/6-S cells, compared to other CDK4/6 degraders (not shown). However, as shown in **FIGS. 12A-B**, YKL-06-102 was similarly ineffective to the rest of CDK4/6-degraders in degrading CDK6 in the CDK4/6-R cells. Thus, failure to degrade CDK4/6 in CDK4/6i-R cells is a general property of CDK4/6 degraders.

All MS140-R (and Rb-proficient) tumor cells analyzed express high levels of CRBN, excluding it as being responsible for the cell context dependence of CDK4/6

degradation in response to MS140 (**FIG. 1E**). We thus focused on potential differences in binding of MS140 to CDK4/6 in the two contexts. Small molecule inhibitors have been shown to bind and stabilize their targets in cells resulting in an increase in the Tm as determined by Cellular Thermal Shift Assay (CETSA) (Martinez Molina D. et al., (2013) Science 341, 84-87; Jafari R. et al., (2014) Nat Protoc 9, 2100-2122). To directly assess whether binding of CDK4/6i differs between the two CDK6 states, we compared the shift of the CDK6 Tm promoted by CDK4/6i in CDK4/6i-S versus CDK4/6i-R tumor cells (all driven by CDK6). Treatment of CDK4/6i-S cells with PB resulted in a shift in Tm, indicating direct binding of inhibitor to CDK6 in these cells (**FIG. 5F**). In contrast, treatment of CDK4/6i-R cells with PB failed to cause a detectable shift, indicating weak inhibitor binding to CDK6 (**FIG. 5F**). Similar results were obtained with MS140-ve, used instead of MS140 to make sure there is no CRBN-mediated protein degradation that could confound the experiment (**FIG. 5G**). To assess inhibitor binding to its target in cells using a second method, we employed a probe-based chemoproteomic method using biotinylated phosphates of ATP or ADP that irreversibly react with protein kinases on conserved lysine residues in the ATP binding pocket (Patricelli M.P. et al., (2011) Chem Biol 18, 699-710), followed by western blot analysis. Target engagement in cells is detected by assessing the extent to which treatment with inhibitor prevents binding of the probe. We found that treatment with PB of CDK4/6i-S cells resulted in loss of binding of the probe to CDK6, however in CDK4/6i-R tumor cells treated with PB, the probe effectively bound and pulled down most of CDK6 (**FIG. 5H**).

Together these data show that CDK6-dependent tumor cells can be divided in two groups. In most tumor cells that express CDK6, in which CDK4/6i bind weakly to CDK6 and these cells are therefore resistant to CDK4/6i (and consequently CDK4/6 degraders). In a smaller group of CDK6-dependent cells, CDK6 binds strongly to CDK4/6i (a higher than 1°C shift in Tm as measured by CETSA), and they are thus sensitive to CDK4/6i and degraders.

Example 6: CDK4/6i-resistant cells express CDK6 as a thermostable, weak HSP90 client protein

To gain mechanistic insight on the basis of the difference in binding of CDK6 to CDK4/6i in CDK4/6-S versus CDK4/6-R cells, immunoprecipitation of CDK6 was carried out followed by mass spectrometry analysis of interacting partners. To this end, CDK6 immunoprecipitated by a CDK4/6-S (KMS-12-PE) was compared to that from a CDK4/6i-R (Calu6) cell line (both expressing high levels of and dependent on CDK6). Association of CDK6 with known CDK6 interactors, including members of the cyclin, INK4 or CIP/KIP families correlated well with their relative basal expression in the two cell lines (**FIG. 6A** and **FIG. 13A**). A 3-5 fold higher interaction of CDK6 is seen with components of the HSP90/CDC37 chaperoning complex in CDK4/6i-S compared to CDK4/6i-R cells (**FIG. 6B**). The observation was further confirmed by direct co-immunoprecipitation experiments showing a much stronger interaction of CDK6 with HSP90 and CDC37 in CDK4/6i-S as compared to CDK4/6i-R tumor cells (**FIG. 6C**).

Association of a kinase with the HSP90/CDC37 complex has been shown to correlate with kinase dependence on HSP90 for its folding and stability (Taipale et al., (2012) Cell 150, 987-1001). In fact, treatment with the HSP90 inhibitor Ganetespib (GAN) results in much more potent CDK6 degradation in CDK4/6i-S cells, than in CDK4/6i-R cells (**FIG. 6D** and **FIG. 13B**). A structurally distinct HSP90 inhibitor, Luminespib, also showed more efficient CDK6 degradation in CDK4/6i-S cells compared to CDK4/6i-R cells (**FIG. 13C**). As expected, treatment of CDK4 only-expressing cells with GAN resulted in potent degradation of CDK4 (**FIG. 13D**), a known strong HSP90 client (Verba K.A., et al., (2016) Science 352, 1542-1547 (2016); Taipale M. et al., (2013) Nat Biotechnol 31, 630-637). Other known HSP90 clients were degraded to a similar degree upon GAN treatment (**FIG. 13E**), indicating that the HSP90/CDC37 chaperone system operates similarly in the two settings, but CDK6 in CDK4/6i-S cells is expressed as a strong HSP90-client, whereas in CDK4/6i-R cells it is predominantly expressed as a weak HSP90-client.

Strong HSP90 client kinases have been shown to be highly thermo-unstable (Taipale M. et al., (2012) Cell 150, 987-1001). The thermostability of CDK6 in CDK6-driven CDK4/6i-S is compared with CDK4/6i-R tumor cells using CETSA. Consistent with the relative association of the two CDK6 forms with HSP90, lower thermostability

(lower Tm) of CDK6 protein in CDK4/6i-S cells was observed as compared to that of CDK6 expressed in CDK4/6i-R cells (**FIG. 6E**). CDK6 in the two cellular settings also differed remarkably in its overall stability. Cycloheximide treatment resulted in significantly faster degradation of CDK6 in CDK4/6i-S compared to CDK4/6i-R cells (**FIG. 6F**).

Stronger association of a kinase with HSP90 has been reported to shift the conformational ensemble of different activation states towards a more active distribution (Taipale M. et al., (2013) Nat Biotechnol 31, 630-637; Boczek E.E. et al., (2015) Proc Natl Acad Sci U S A 112, E3189-3198). Crystallographic data show that CDK4/6i are Type I in that they bind the active conformation of CDK4/6 (αC-IN/DFG-IN))(Zhang, J. et al., (2009) Nat Rev Cancer 9, 28-39). The findings here support a model by which, in CDK6-driven and CDK4/6i-S tumor cells, the CDK6 protein population is enriched in highly active conformations that have strong binding affinity for current CDK4/6i (and consequently for CDK4/6 degraders). In contrast, in CDK4/6i-R cells, CDK6 is expressed as thermostable, weak HSP90-client, with lower affinity for the CDK4/6i. To further test this idea, a previously reported activating mutation was inserted in CDK6 (S178P) (Bockstaele, L. et al., (2009) Mol Cell Biol 29, 4188-4200) and it is found to activate CDK6 (**FIG. 13F**). Treatment of cells expressing either wild-type (WT) or mutationally activated CDK6 with MS140 results in significantly more potent degradation of CDK6(S178P) compared to CDK6(WT) (**FIG. 6G**), consistent with a highly active conformation of CDK6 being more sensitive to degradation by MS140. Finally, a previous report using biotinylated phosphates of ATP or ADP followed by mass spectrometry has shown that in Rb-null cells, PB does not bind CDK4/6 kinases (Nomanbhoy T.K. et al., (2016) Biochemistry 55, 5434-5441). Consistent with those results, in BT549, Rb-null cells, CDK6 is also found to be highly thermostable and resistant to degradation by MS140, similar to the CDK6 state in PB-resistant cells (**FIG. 13G** and not shown).

MS140 was found to be more potent than PB in CDK4/6i-S tumors, but less potent than PB in CDK4/6i-R tumors, because in the latter cells, CDK6 binds weakly to CDK4/6i. This raises the possibility that MS140 would also minimally affect normal

tissues expressing CDK6 with similar properties, and it would thus exhibit a higher therapeutic index than PB. To address this question, the effect of treatment of the same daily dose of either MS140 or PB was assessed on expression of known E2F targets in tumor and normal tissue (kidney and liver). It is found that treatment with MS140 results in more potent inhibition in the tumor than in normal tissue of several E2F-target transcripts, compared to PB (**FIG. 6H**), suggesting that combined inhibition and degradation of CDK4/6 may be a pharmacologic strategy with higher therapeutic index than current clinical CDK4/6i. Finally, as a known toxicity induced by CDK4/6i is a reduction on white blood cells and neutropenia (*4*), complete blood counts were performed in mice after three weeks of treatment with either PB or MS140 at the same total daily concentration. Consistent with the clinical experience (*4*) and previous studies in mice (Bisi J.E. et al., (2017) Oncotarget 8, 42343-42358), treatment with PB results in a severe (over 50%) reduction in neutrophil count (**Fig. 6I**). Total white blood cell count and lymphocytes are also reduced in response to PB treatment, whereas red blood cells remained unaffected (**FIG. 13H**). However, none of these blood components is decreased after treatment with MS140, suggesting that it can be better tolerated at doses that are comparably effective with PB. These results indicate that CDK4/6-directed degradation may be both a highly effective and well-tolerated therapeutic strategy for patients with CDK4-driven tumors (ER+ breast cancers, Ewing sarcomas, MCL, etc), as well as for patients with tumors dependent on the CDK4/6insensitive CDK6.

OTHER EMBODIMENTS

While the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

WHAT IS CLAIMED IS

1. A method for treating a cancer in a human subject, the method comprising:

(a) obtaining a tumor sample from the subject;

(b) determining a level of cyclin dependent kinase 6 (CDK6) in the tumor sample; and

(c) comparing the level of CDK6 as determined in (b) with a reference level in a control;

wherein when the level of CDK6 in the tumor is lower than the reference level of CDK6 in the control, the tumor is classified as a CDK4-dependent tumor and treated with a therapeutically effective amount of one or more of a CDK4/6 inhibitor and a CDK4/6-directed PROTAC.

2. A method for treating a cancer in a human subject, the method comprising:

(a) obtaining a tumor sample from the subject;

(b) determining levels of CDK6 and CDK4 in the tumor sample; and

(c) determining the ratio of CDK4 to CDK6 levels;

wherein (i) when the CDK6 level is below a threshold level and/or the CDK4 to CDK6 ratio is above a threshold ratio, the tumor is classified as a CDK4-dependent tumor and the subject is treated with a composition comprising a therapeutically effective amount of a CDK4/6-directed PROTAC; or

(ii) when the CDK6 level is above a threshold level and/or the CDK4 to CDK6 ratio is below a threshold ratio, the tumor is classified as a CDK6-dependent tumor and the subject is treated with a composition comprising a therapeutically effective amount of a CDK4/6 inhibitor.

3. The method of claim 1 or 2, wherein the CDK4/6 inhibitor is one or more agents selected from a group consisting of abemaciclib (Verzenio), dinaciclib. palbociclib (Ibrance), ribociclib (Kisqali), trilaciclib (G1T28), G1T38, and the group of CDK4/6

inhibitor compounds referred to in International Patent Application Publications WO2016040858, WO2011130232, WO2011101409, WO2016025650, and WO2013006532 and the CDK4/6-directed PROTAC is one or more agents selected from MS140, the PROTACs referred to in **FIGs. 14-18**, and bivalent compounds referred to in International Patent Application Publication WO2018106870.

4. The method of claim 3, wherein the CDK4/6 inhibitor and/or CDK4/6-directed PROTAC is administered in combination with one or more additional therapeutic regimens selected from the group consisting of surgery, chemotherapy, radiation therapy, hormone therapy, targeted therapy, and immunotherapy.

5. The method of claim 4, wherein the targeted therapy is with one or more agents selected from a group consisting of MEK inhibitors, ERK inhibitors, hormonal therapy, and RAS(G12C) inhibitors.

6. The method of any one of claims 1-5, wherein the cancer is a solid tumor cancer.

7. The method of any one of claims 1-6, wherein the cancer is a cancer expressing functional retinoblastoma protein (Rb).

8. A method of treating a cancer in a human subject in need thereof, comprising administering to the human subject a therapeutically effective amount of a cancer therapy, wherein the human subject has been previously determined to have, in a biological sample obtained from the human subject, at least one of

(a) a CDK6 level prior to initiation of the cancer therapy that is lower than a reference level in a control, and

(b) a ratio of CDK4 to CDK6 levels prior to initiation of the cancer therapy that is higher than a reference level in a control.

9. A method for treating a human subject with cancer, comprising

(a) measuring a level of CDK6 and optionally measuring a level of CDK4, and further, optionally determining a ratio of the CDK4 to CDK6 levels in a biological sample taken from the subject; and

(b) treating the subject with a therapeutically effective amount of a cancer therapy if the measured levels of CDK6 and optionally the ratio of CDK4 to CDK6 indicate that the subject is a candidate for receiving the cancer therapy.

10. The method of claim 5 or 6, wherein the cancer therapy is one or more agents selected from a group consisting of abemaciclib (Verzenio), palbociclib (Ibrance), ribociclib (Kisqali), trilaciclib (G1T28), G1T38, and the group of CDK4/6 inhibitor compounds referred to in International Patent Application Publications WO2016040858, WO2011130232, WO2011101409, WO2016025650, and WO2013006532, MS140, the PROTACs referred to in **FIGs. 14-18**, and the group of bivalent compounds referred to in International Patent Application Publication WO2018106870.

11. The method of claim 10, wherein the cancer therapy is administered in combination with one or more additional therapeutic regimens selected from the group consisting of surgery, chemotherapy, radiation therapy, hormone therapy, targeted therapy, and immunotherapy.

12. The method of claim 11, wherein the targeted therapy is with one or more agents selected from a group consisting of MEK inhibitors, ERK inhibitors, hormonal therapy, and RAS(G12C) inhibitors.

13. The method of any one of claims 8-12, wherein the cancer is a solid tumor cancer or a leukemia.

14. The method of any one of claims 8-13, wherein the cancer is a cancer expressing functional retinoblastoma protein (Rb).

15. The method of claim 6 or 13, wherein the solid tumor cancer is selected from a group consisting of non-small-cell lung carcinoma (NSCLC), lung cancer, breast cancer,

ewing sarcoma, central nervous system neoplasm, skin cancer, head and neck cancer, ovarian cancer, colon cancer, anal cancer, stomach cancer, gastrointestinal cancer, fallopian tube cancer, endometrial cancer, cervical cancer, vaginal cancer, vulvar cancer, esophageal cancer, endocrine cancer, thyroid cancer, parathyroid cancer, adrenal cancer, soft tissue sarcoma, urethral cancer, penile cancer, testicular cancer, brain stem glioma, pituitary cancer, adrenocortical cancer, gallbladder cancer, multiple myeloma, cholangiocarcinoma, fibrosarcoma, lymphoma, liver cancer, kidney cancer, bone cancer, bladder cancer, colorectal cancer, endometrial cancer, renal cell cancer, pancreatic cancer, prostate cancer, thyroid cancer, mesothelioma, neuroblastoma, retinoblastoma, and melanoma.

16. The method of claim 13, wherein the leukemia is acute myeloid leukemia (AML) and T-cell acute lymphocytic leukemia (T-ALL), Mantle Cell Lymphoma (MCL), and Multiple Myeloma (MM).

17. A method for predicting prognosis of a human subject with cancer, comprising

(a) obtaining a biological sample of the cancer from the subject;

(b) determining a level of CDK6 in the biological sample; and

(c) comparing the level of CDK6 as determined in (b) with a reference level of CDK6 in a control;

wherein a lower level of CDK6 in the biological sample compared to the CDK6 reference level in the control is an indication that the cancer in the subject will be successfully treated with a CDK4/6 inhibitor.

18. A method of predicting response to a cancer therapy or predicting disease progression in a human subject with cancer comprising:

(a) obtaining a biological sample from the subject;

(b) determining levels of CDK4 and CDK6 in the sample and obtaining the ratio of CDK4/CDK6;

(c) based on the determinations of step (b), determining a probability of response to the cancer therapy or a future risk of cancer progression in the subject.

19. The method of any one of claims 1-18, wherein the levels of CDK6 and/or CDK4 are measured by determining one or more of the mRNA levels, cDNA levels and protein levels of CDK6 and/or CDK4.

20. A method of identifying a compound capable of treating cancer, or identifying a compound capable of reducing the risk of developing cancer, or identifying a compound capable of reducing the risk of cancer recurrence or development of metastatic cancer, comprising:

(a) providing a cell expressing CDK6;

(b) contacting the cell with a candidate compound; and

(c) determining whether the candidate compound reduces the expression or activity of CDK6;

wherein the reduction observed in the presence of the compound indicates that the compound is capable of treating cancer, or reducing the risk of developing cancer, or reducing the risk of cancer recurrence or development of metastatic cancer.

21. A kit for performing a method according to any one of the preceding claims, wherein the kit comprises means for quantifying the levels of CDK6 and/or CDK4.

22. A kit for measuring the levels of CDK6 and/or CDK4 in a biological sample, wherein the kit comprises reagents for specifically measuring the levels of CDK6 and/or CDK4.

23. The kit of claim 22, wherein the reagents are nucleic acid molecules or antibodies.

24. The method of claim 2, wherein the CDK6 threshold level in the biological sample is below about 5.64 CPM, as determined by RNA sequencing.

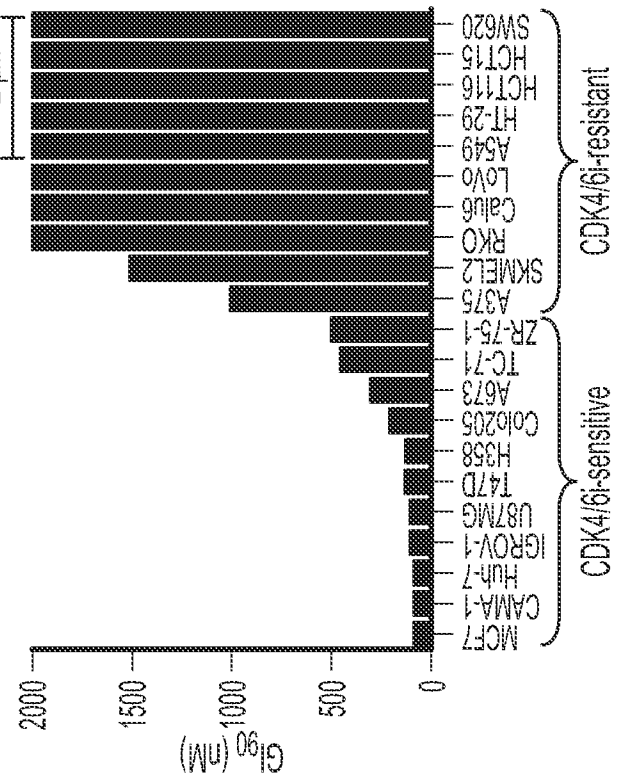


FIG. 1A

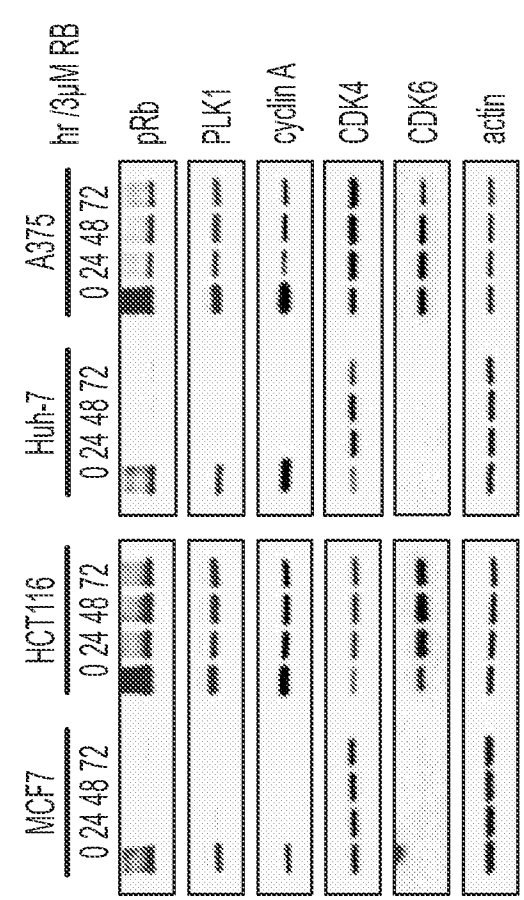


FIG. 1C

FIG. 1B

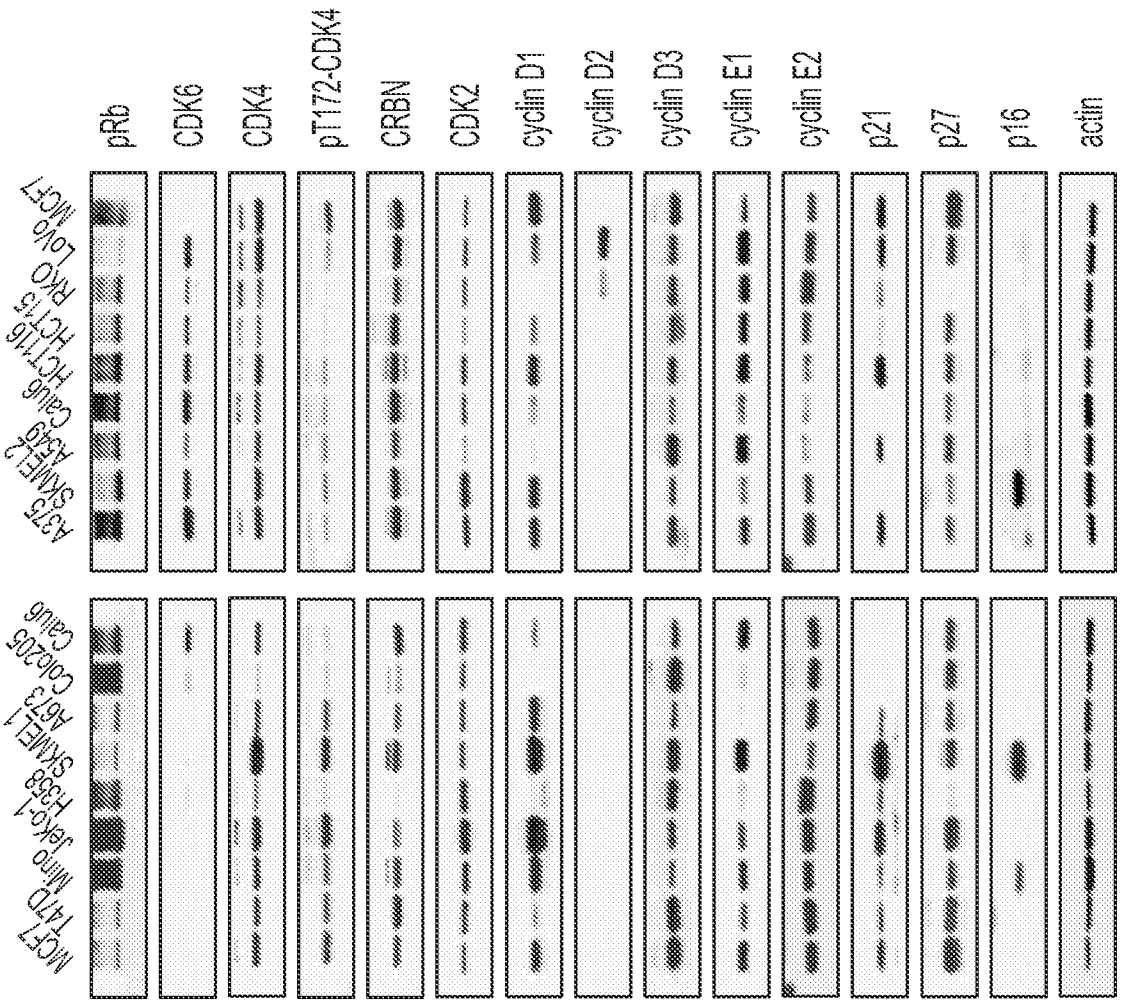

MCF7
T47D
Mino
Jak-1
H358
SkMel1
A673
Colo205
Calu6
A375
SkMel2
A549
Calu6
HCT116
HCT15
RKO
LoVo
MCF7
pRb
CDK6
CDK4
pT172-CDK4
CRBN
CDK2
cyclin D1
cyclin D2
cyclin D3
cyclin E1
cyclin E2
p21
p27
p16
actin

FIG. 1E

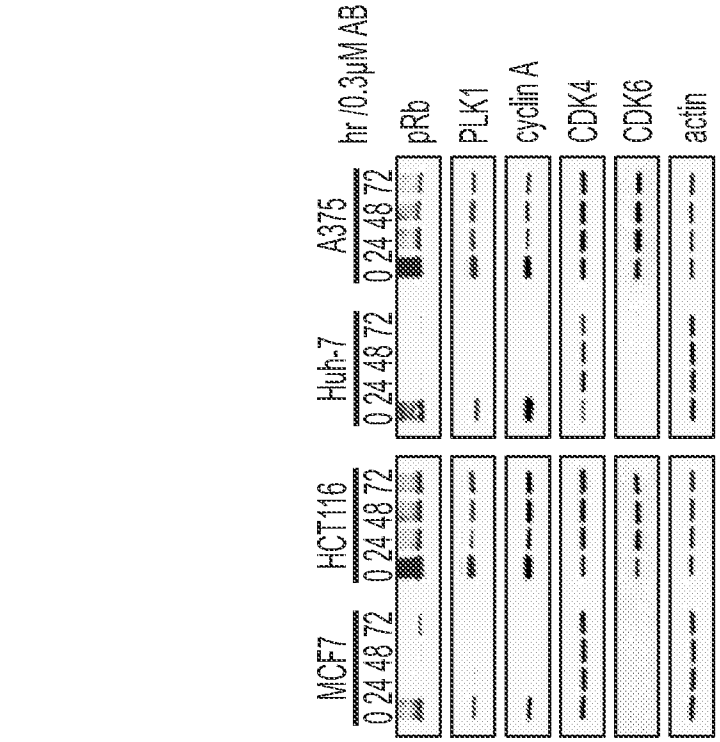

MCF7
HCT116
Huh-7
A375
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hr /0.3μM AB
pRb
PLK1
cyclin A
CDK4
CDK6
actin

FIG. 1D

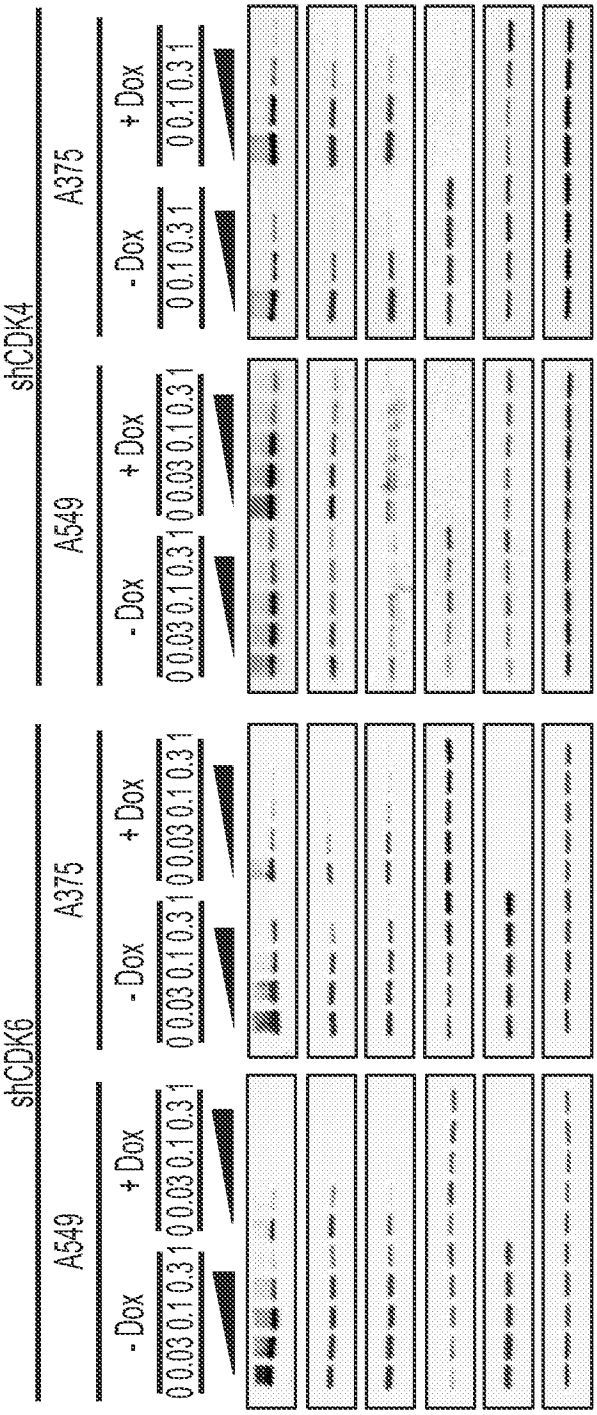

shCDK6
shCDK4
A549
A375
- Dox
+ Dox
0 0.03 0.1 0.3 1 0 0.03 0.1 0.3 1
μM PB
pRb
PLK1
cyclin A
CDK4
CDK6
actin

FIG. 2A

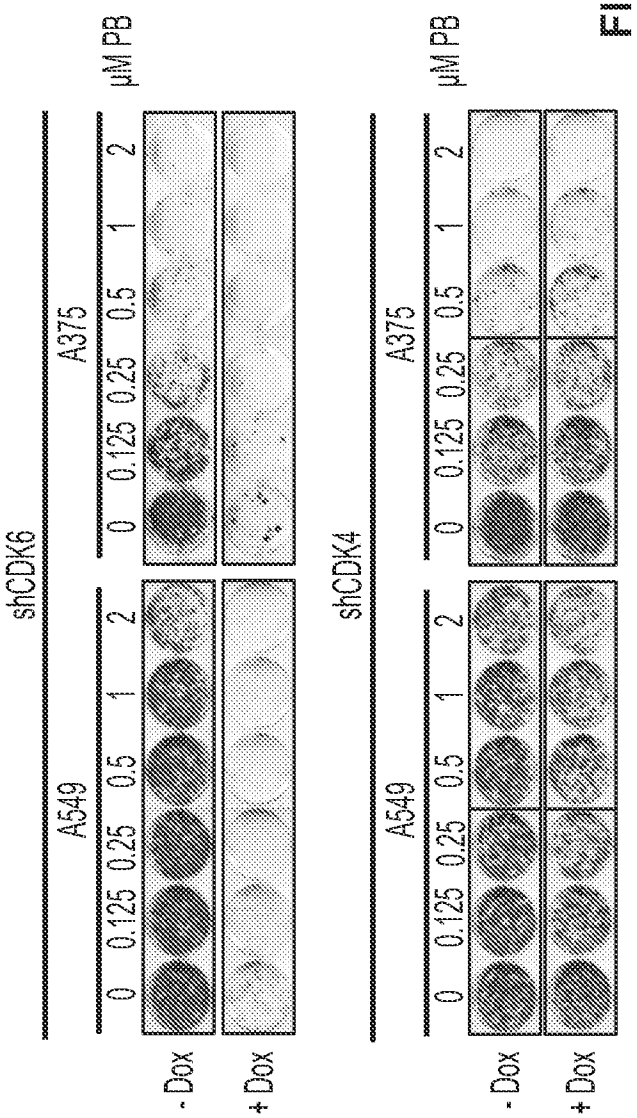

shCDK6
shCDK4
A549
A375
0 0.125 0.25 0.5 1 2
μM PB
- Dox
+ Dox

FIG. 2B

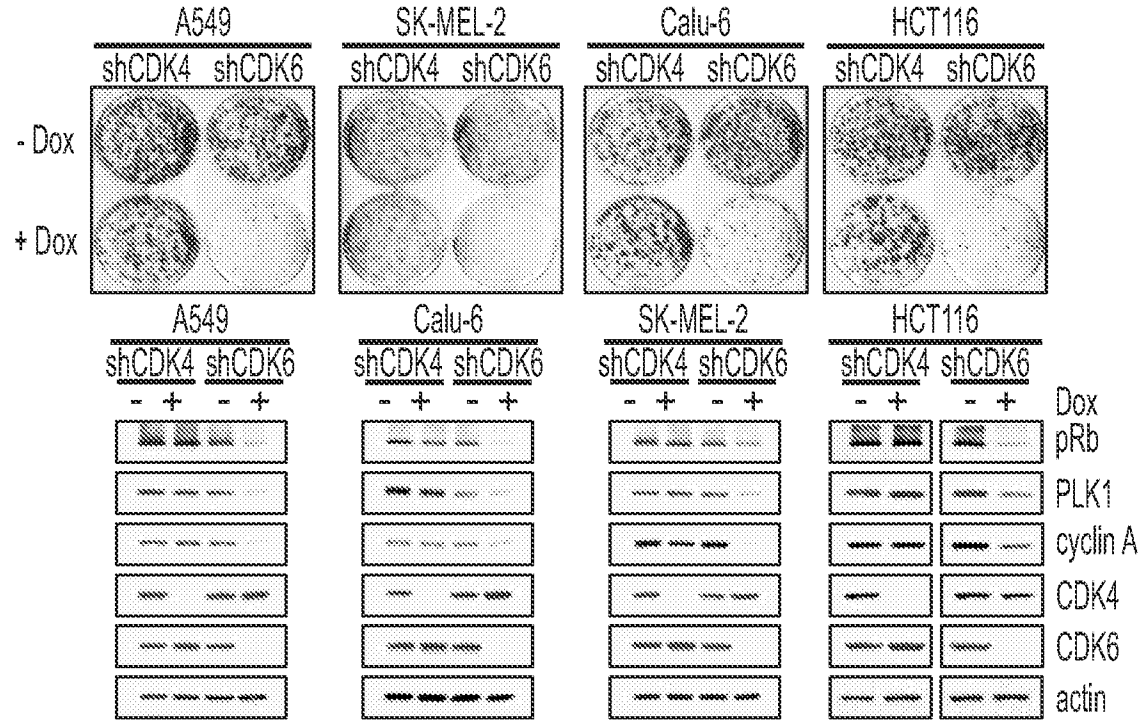

A549
SK-MEL-2
Calu-6
HCT116
shCDK4 shCDK6
- Dox
+ Dox
A549
Calu-6
SK-MEL-2
HCT116
shCDK4 shCDK6
- + - +
Dox
pRb
PLK1
cyclin A
CDK4
CDK6
actin

FIG. 2C

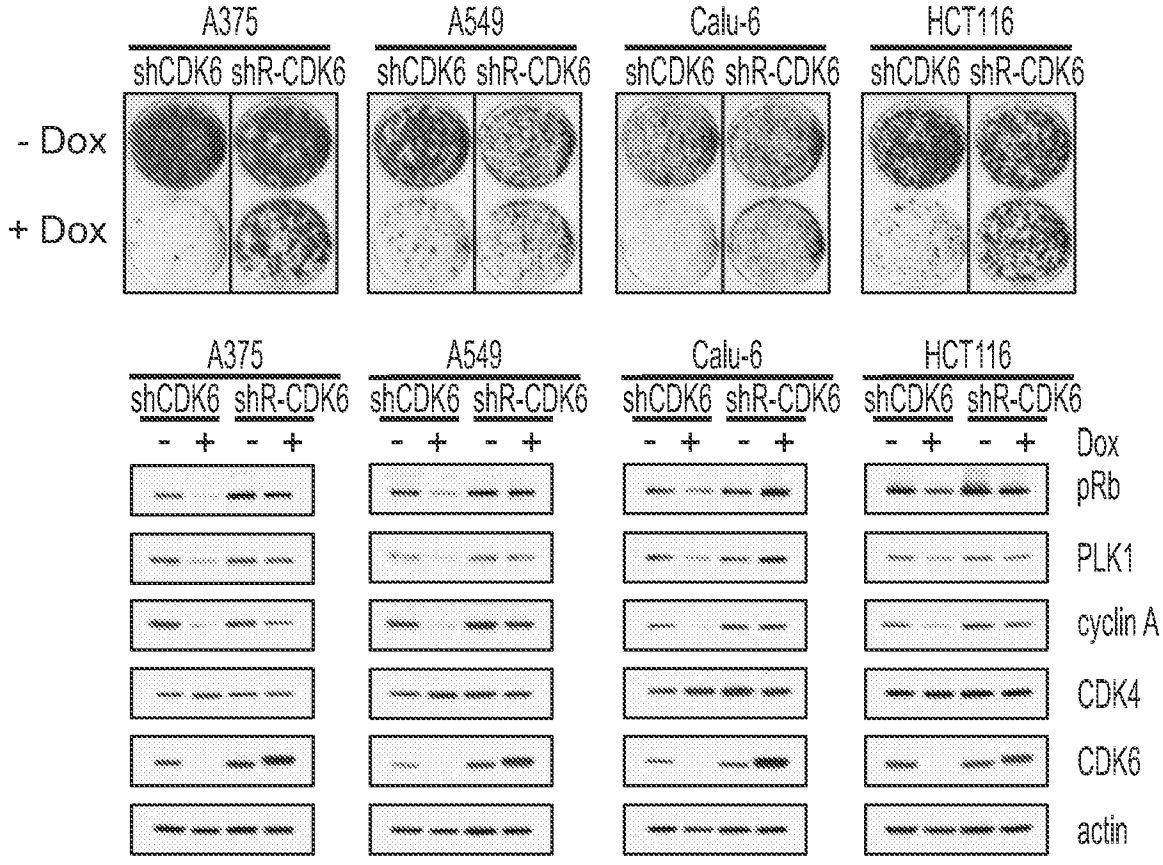

A375
A549
Calu-6
HCT116
shCDK6 shR-CDK6
- Dox
+ Dox
A375
A549
Calu-6
HCT116
shCDK6 shR-CDK6
- + - +
Dox
pRb
PLK1
cyclin A
CDK4
CDK6
actin

FIG. 2D

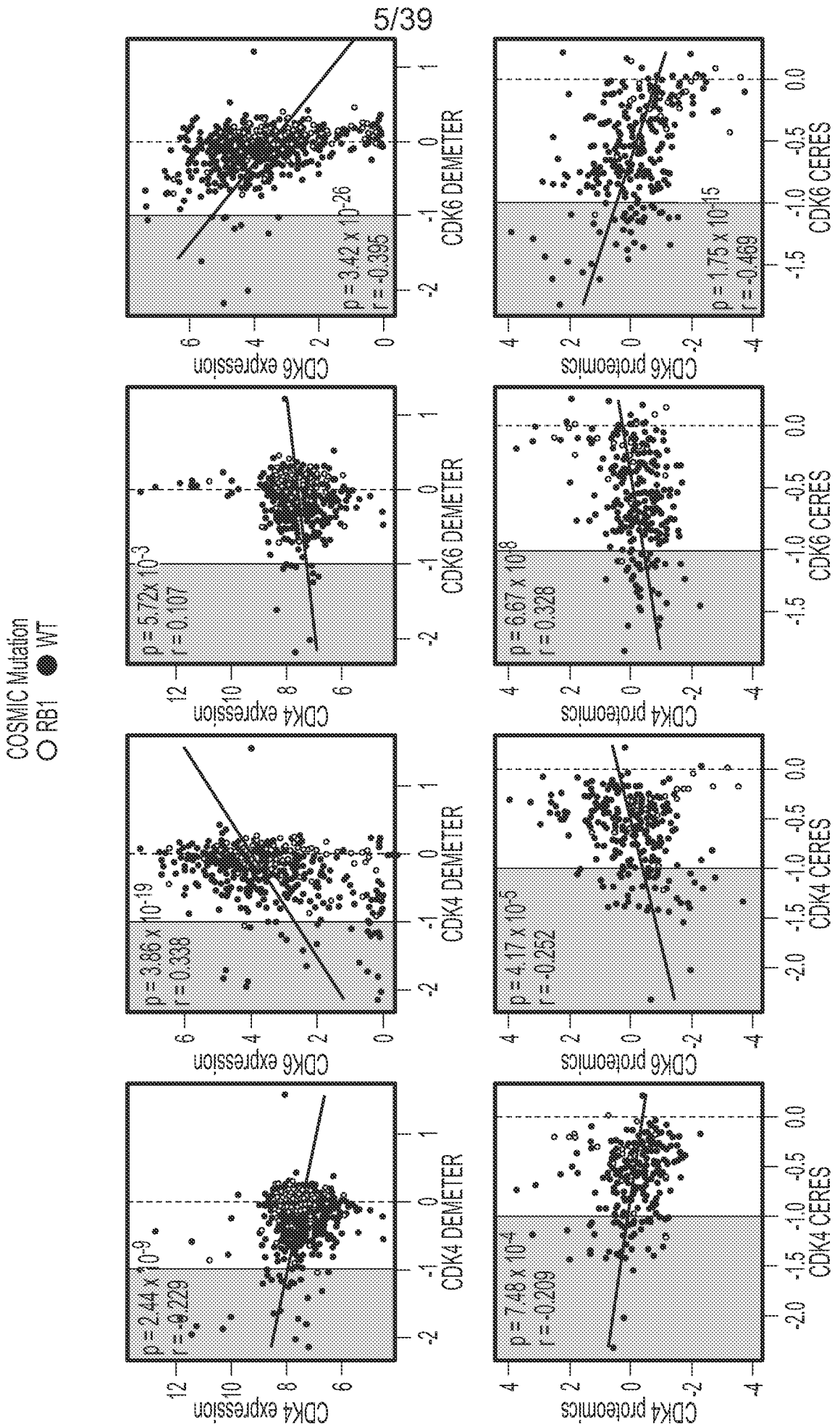

COSMIC Mutation
RB1
WT
CDK6 expression
CDK6 DEMETER
p = 3.42 x 10-26
r = -0.395
CDK6 proteomics
CDK6 CERES
p = 1.75 x 10-15
r = -0.469
CDK4 expression
CDK4 DEMETER
p = 5.72x 10-3
r = 0.107
CDK4 proteomics
CDK4 CERES
p = 6.67 x 10-8
r = 0.328
CDK6 expression
CDK6 DEMETER
p = 3.86 x 10-19
r = 0.338
CDK6 proteomics
CDK6 CERES
p = 4.17 x 10-5
r = -0.252
CDK4 expression
CDK4 DEMETER
p = 2.44 x 10-9
r = -0.229
CDK4 proteomics
CDK4 CERES
p = 7.48 x 10-4
r = -0.209

FIG. 2E

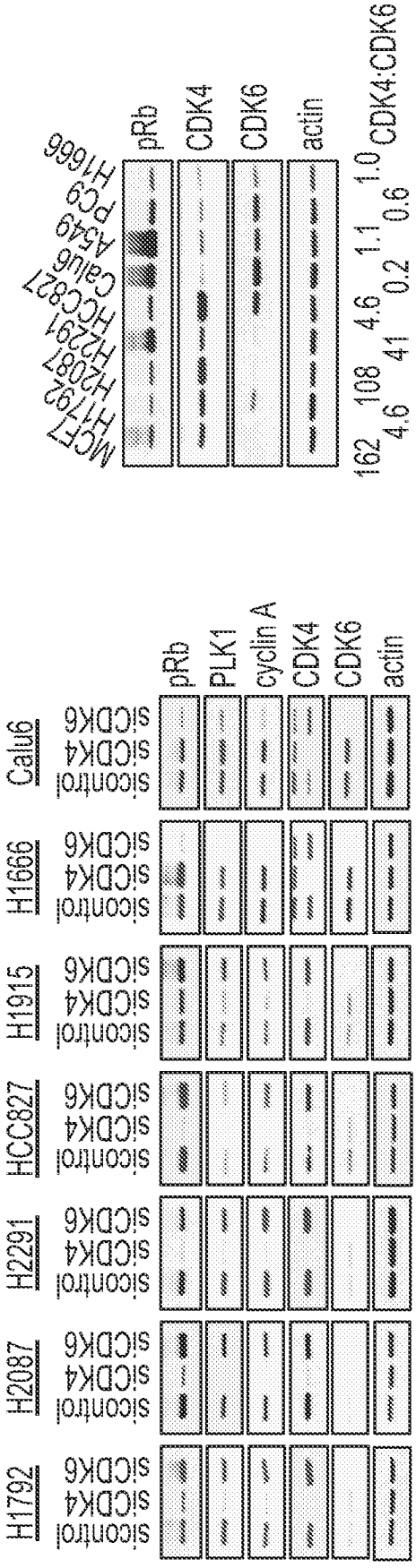


FIG. 3A

FIG. 3B

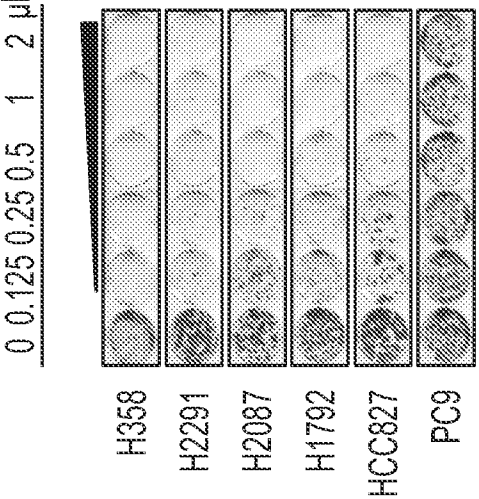


FIG. 3D

FIG. 3C

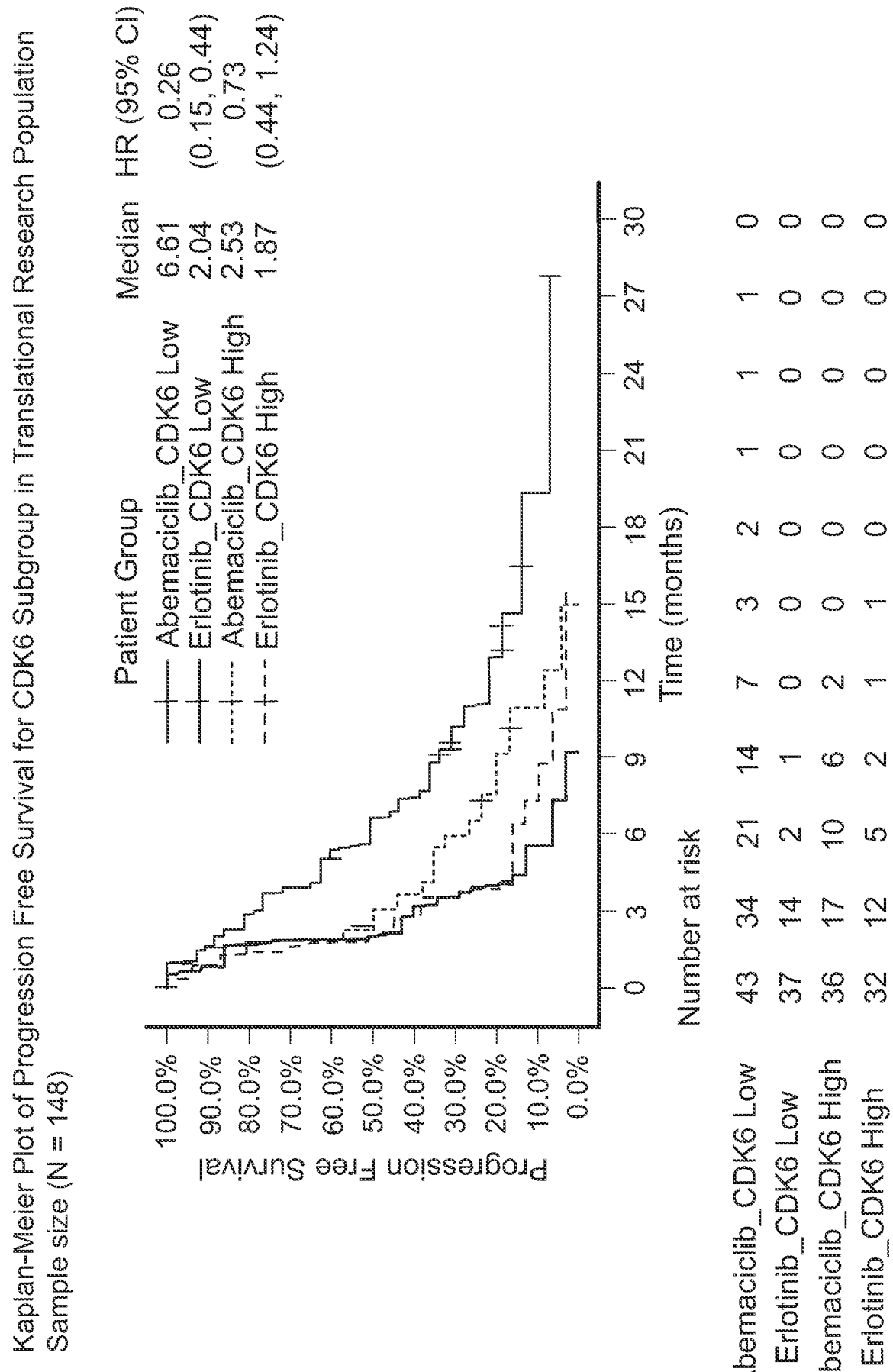

Kaplan-Meier Plot of Progression Free Survival for CDK6 Subgroup in Translational Research Population
Sample size (N = 148)
Patient Group
Median
HR (95% CI)
Abemaciclib_CDK6 Low 6.61
Erlotinib_CDK6 Low 2.04 0.26 (0.15, 0.44)
Abemaciclib_CDK6 High 2.53 0.73 (0.44, 1.24)
Erlotinib_CDK6 High 1.87
Progression Free Survival
100.0%
90.0%
80.0%
70.0%
60.0%
50.0%
40.0%
30.0%
20.0%
10.0%
0.0%
0 3 6 9 12 15 18 21 24 27 30
Time (months)
Number at risk
Abemaciclib_CDK6 Low 43 34 21 14 7 3 2 1 1 1 0
Erlotinib_CDK6 Low 37 14 2 1 0 0 0 0 0 0 0
Abemaciclib_CDK6 High 36 17 10 6 2 0 0 0 0 0 0
Erlotinib_CDK6 High 32 12 5 2 1 1 0 0 0 0 0

FIG. 3E

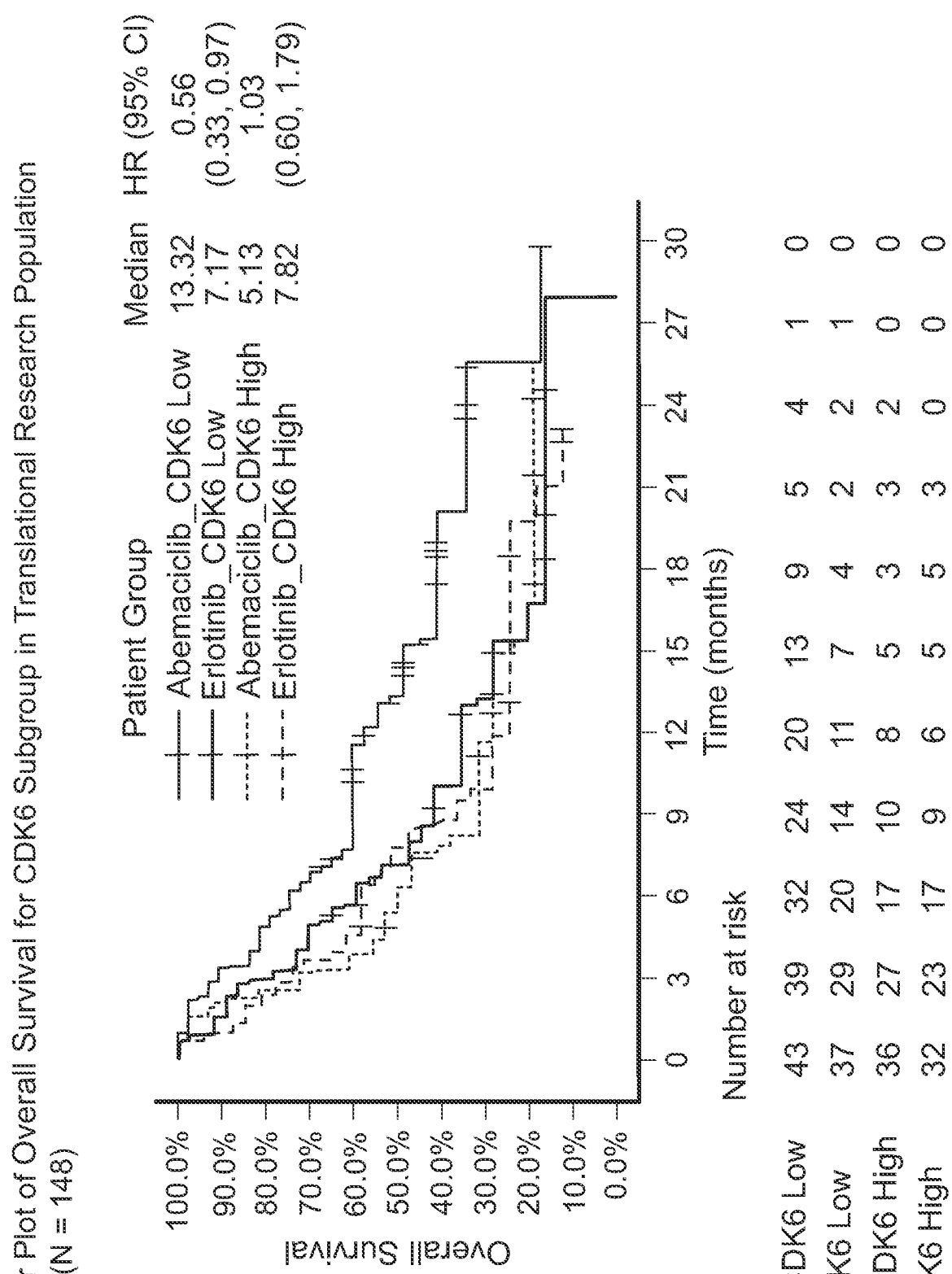

Kaplan-Meier Plot of Overall Survival for CDK6 Subgroup in Translational Research Population
Sample size (N = 148)
Patient Group
Median
HR (95% CI)
Abemaciclib_CDK6 Low 13.32
Erlotinib_CDK6 Low 7.17 0.56 (0.33, 0.97)
Abemaciclib_CDK6 High 5.13 1.03 (0.60, 1.79)
Erlotinib_CDK6 High 7.82
Overall Survival
100.0%
90.0%
80.0%
70.0%
60.0%
50.0%
40.0%
30.0%
20.0%
10.0%
0.0%
0 3 6 9 12 15 18 21 24 27 30
Time (months)
Number at risk
Abemaciclib_CDK6 Low 43 39 32 24 20 13 9 5 4 1 0
Erlotinib_CDK6 Low 37 29 20 14 11 7 4 2 2 1 0
Abemaciclib_CDK6 High 36 27 17 10 8 5 3 3 2 0 0
Erlotinib_CDK6 High 32 23 17 9 6 5 5 3 0 0 0

FIG. 3F

MS140

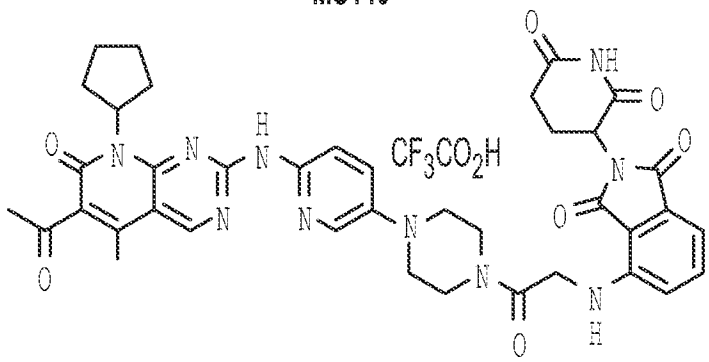


FIG. 4A

Kinases	PB (nM)	MS140 (nM)
CDK4/cyclin D1	0.29	0.38
CDK6/cyclin D1	0.14	0.28

FIG. 4B

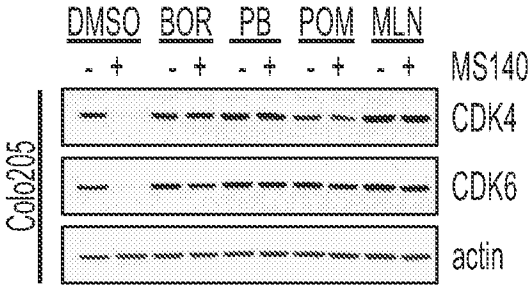


FIG. 4C

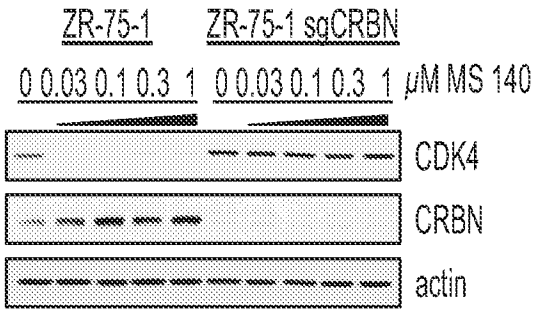


FIG. 4D

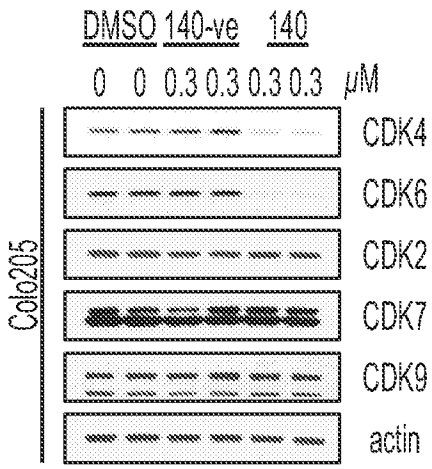


FIG. 4E

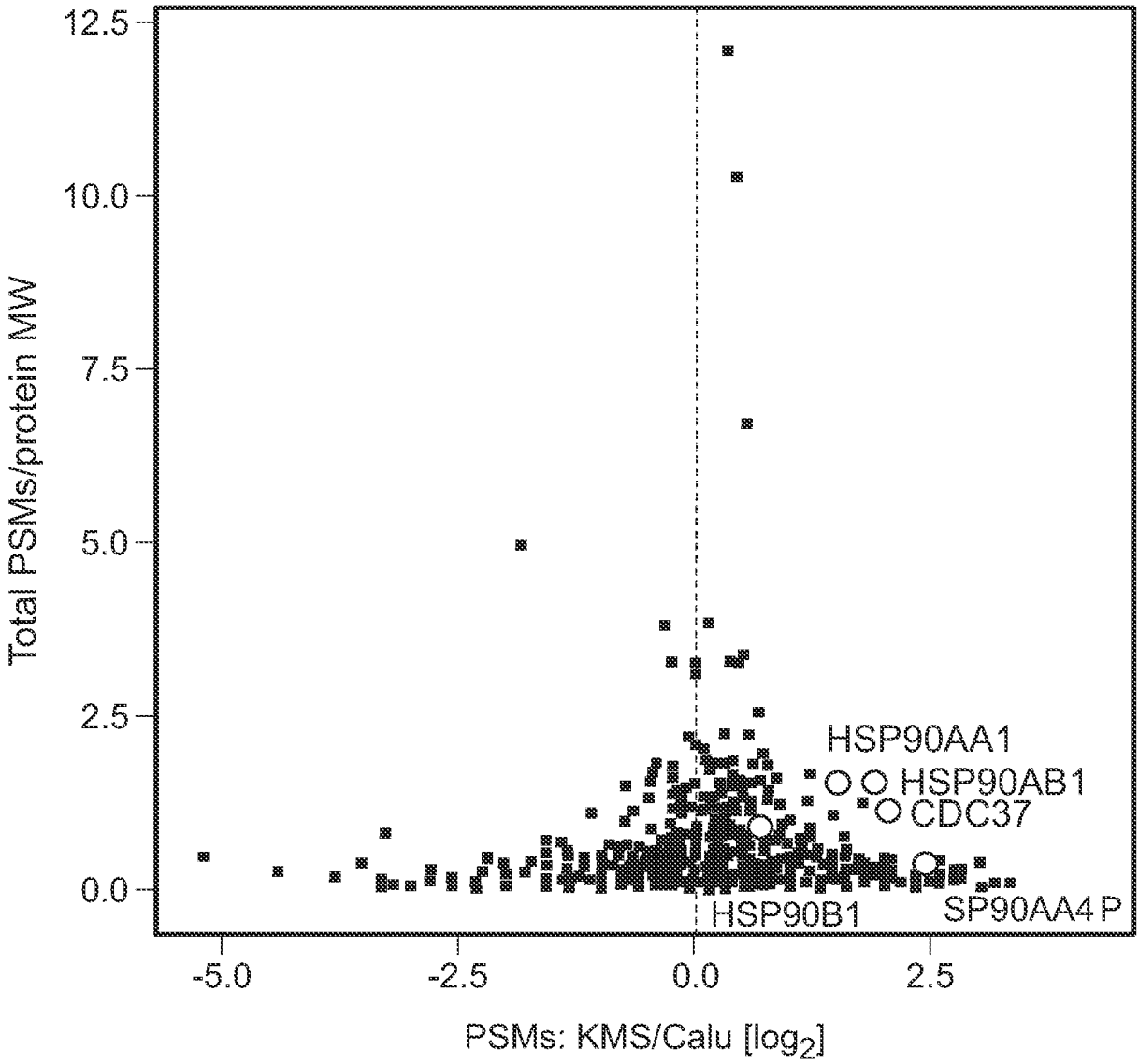

12.5
10.0
7.5
5.0
2.5
0.0
Total PSMs/protein MW
HSP90AA1
HSP90AB1
CDC37
HSP90B1
SP90AA4P
-5.0
-2.5
0.0
2.5
PSMs: KMS/Calu [log2]

FIG. 4F

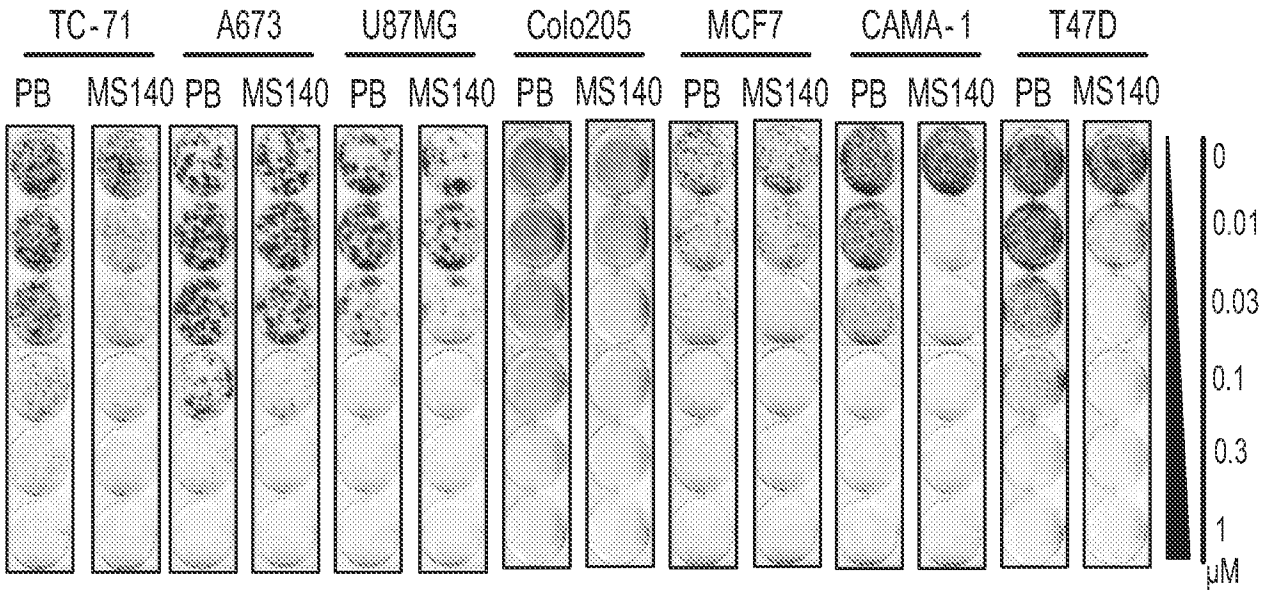

TC-71
A673
U87MG
Colo205
MCF7
CAMA-1
T47D
PB
MS140
0
0.01
0.03
0.1
0.3
1
μM

FIG. 4G

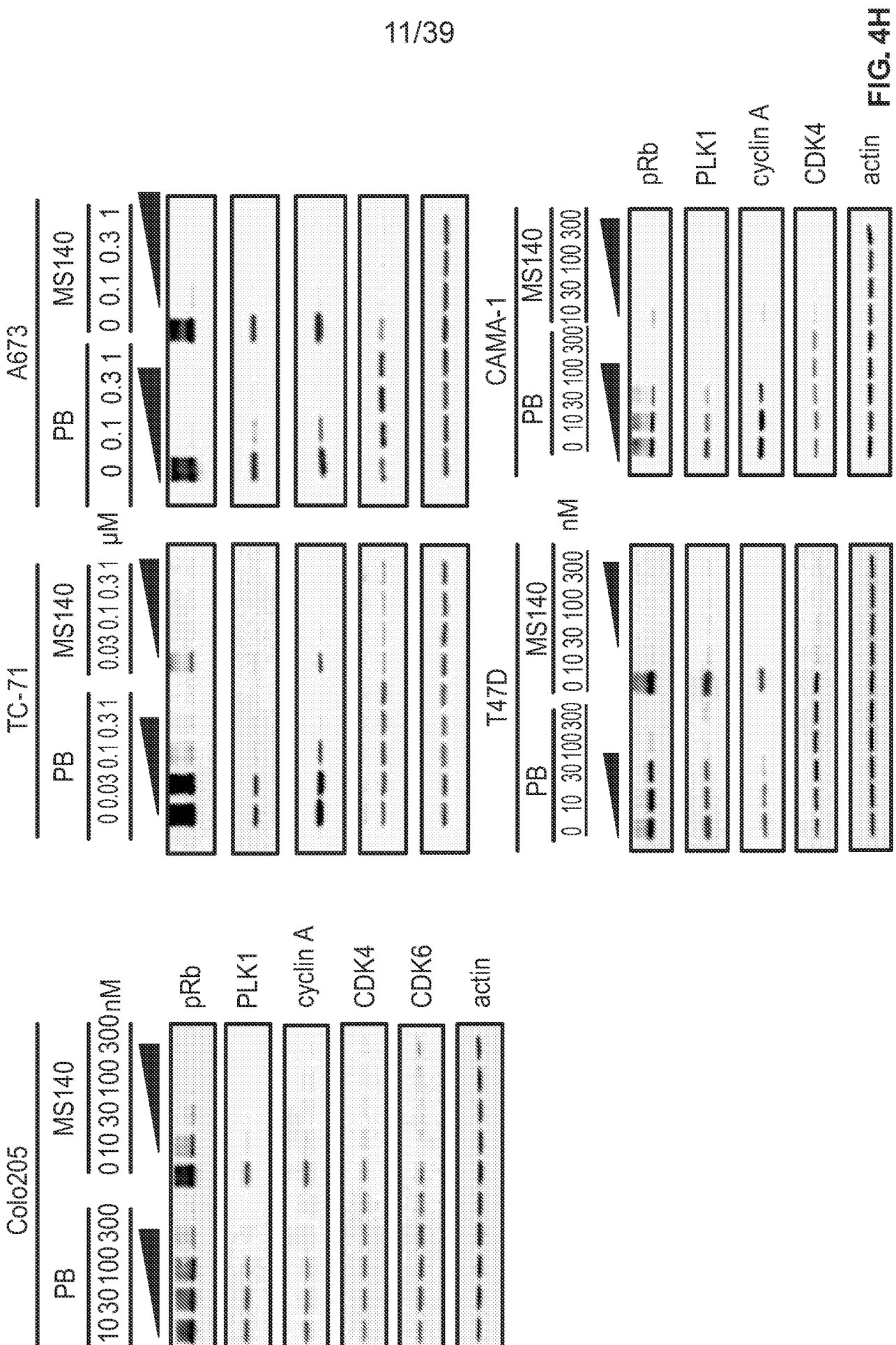
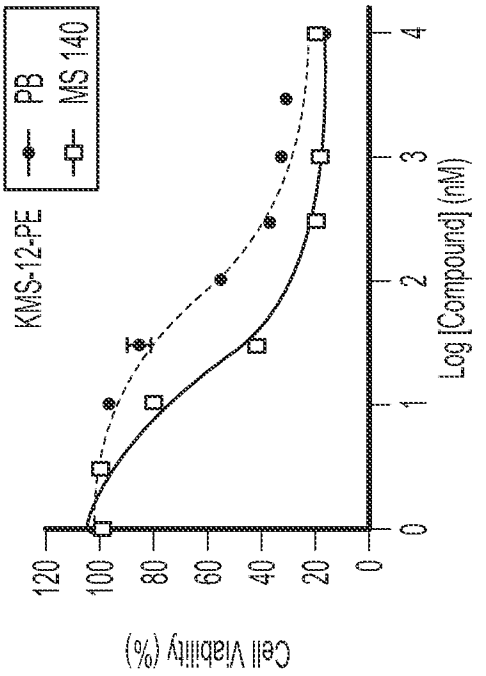

Colo205
PB
MS140
0 10 30 100 300
0 10 30 100 300 nM
pRb
PLK1
cyclin A
CDK4
CDK6
actin
TC-71
PB
MS140
0 0.03 0.1 0.3 1
0 0.03 0.1 0.3 1 μM
T47D
PB
MS140
0 10 30 100 300
0 10 30 100 300 nM
A673
PB
MS140
0 0.1 0.3 1
0 0.1 0.3 1
CAMA-1
PB
MS140
0 10 30 100 300
0 10 30 100 300
pRb
PLK1
cyclin A
CDK4
actin

FIG. 4H



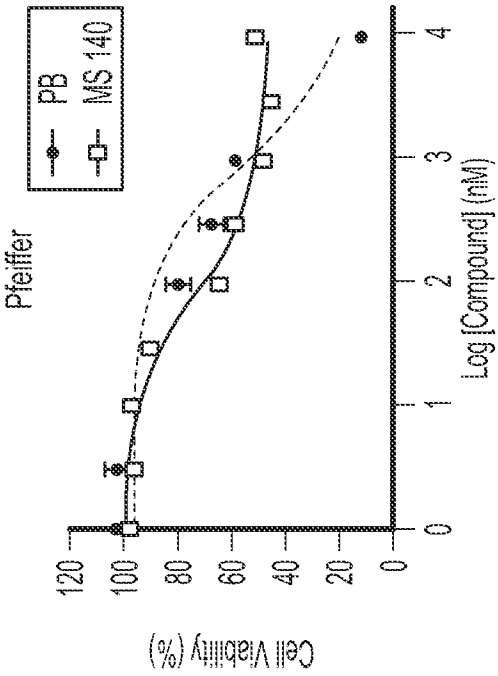


FIG. 4J

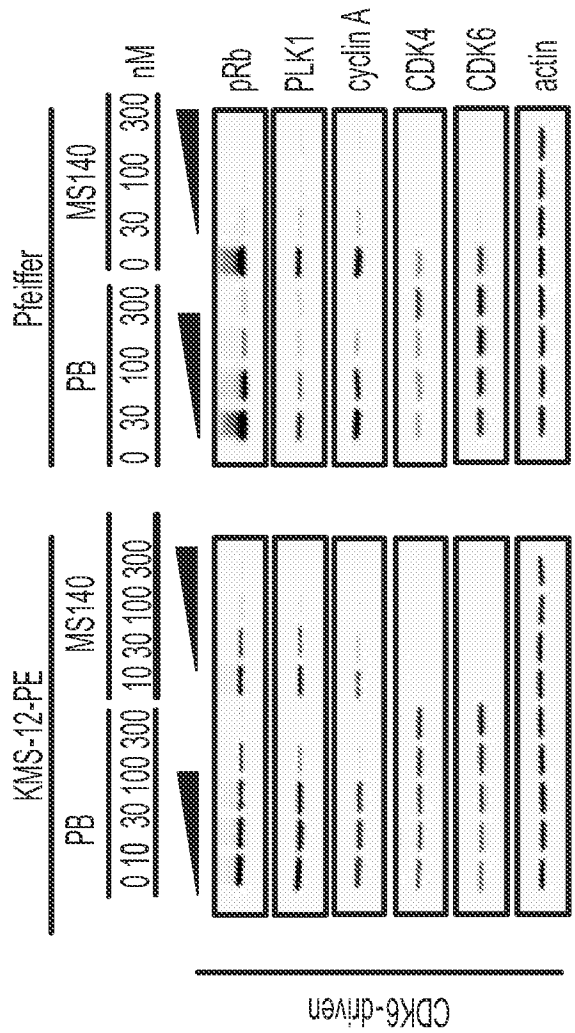


FIG. 4I

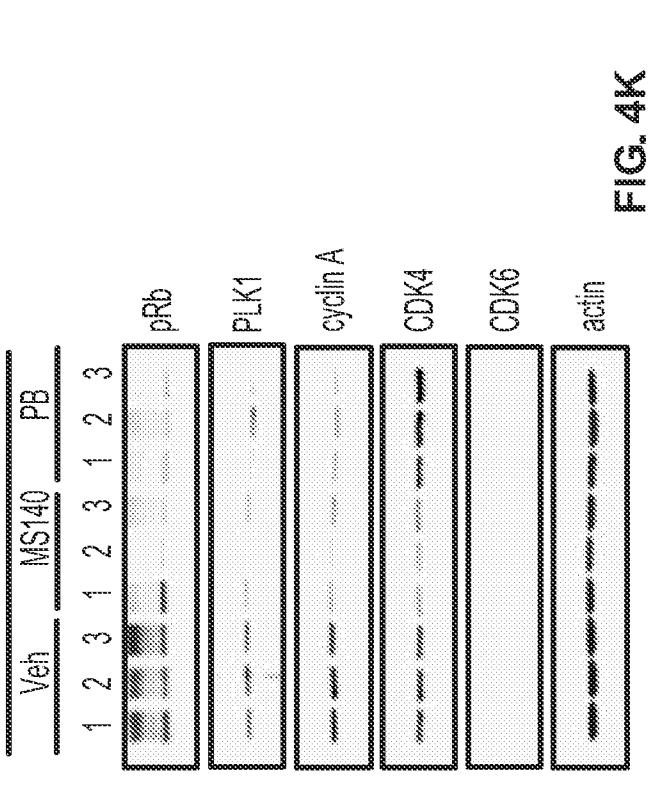


FIG. 4K

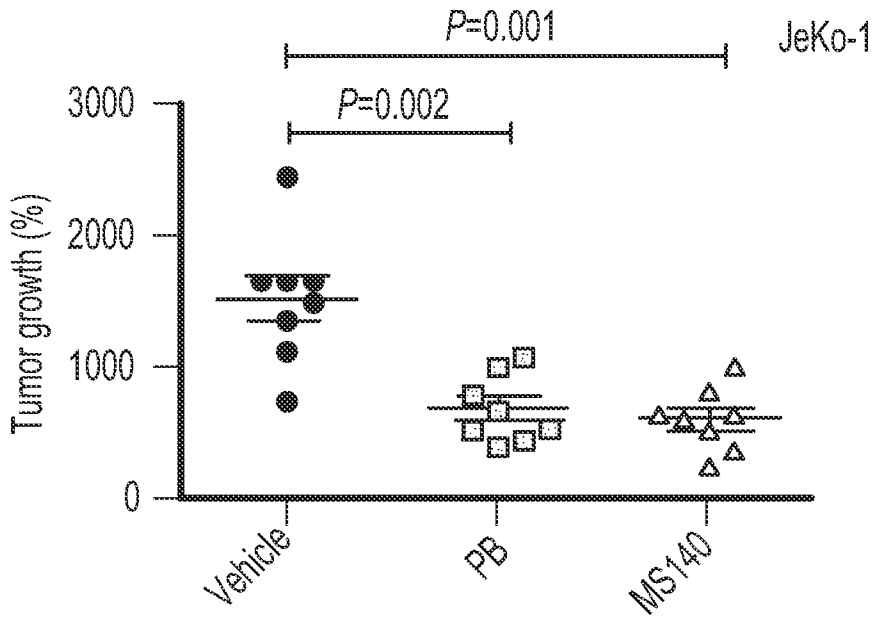

P=0.001
JeKo-1
P=0.002
3000
2000
1000
0
Tumor growth (%)
Vehicle
PB
MS140

FIG. 4L

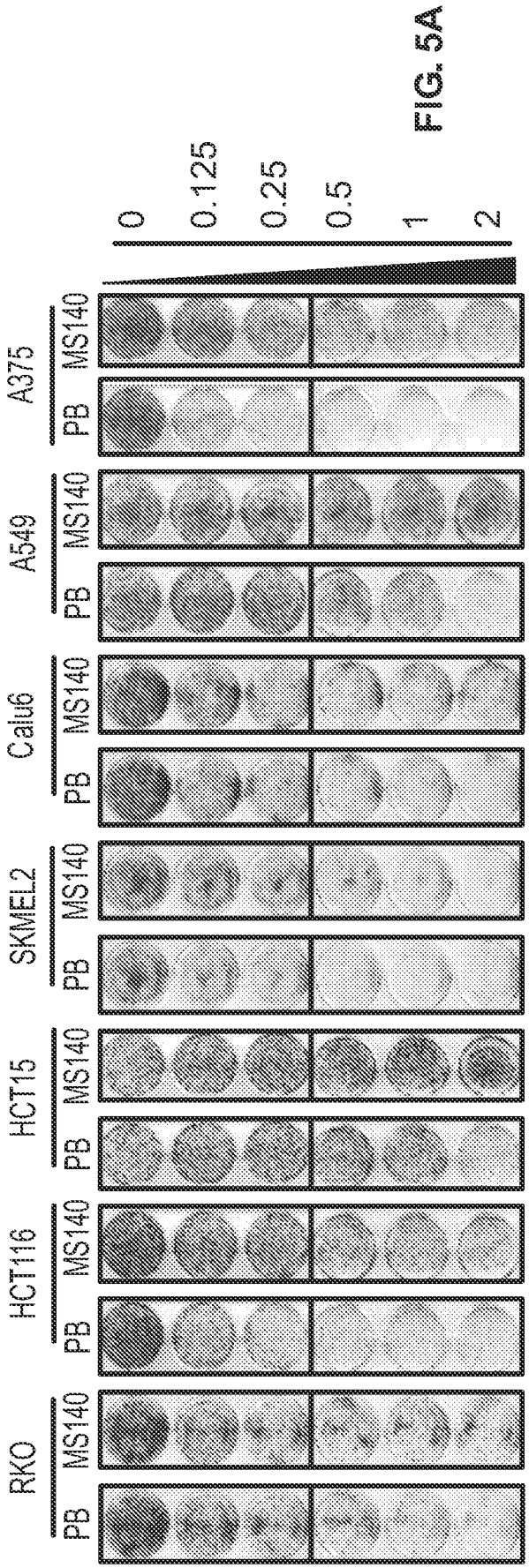

RKO
HCT116
HCT15
SKMEL2
Calu6
A549
A375
PB
MS140
0
0.125
0.25
0.5
1
2
μM

FIG. 5A

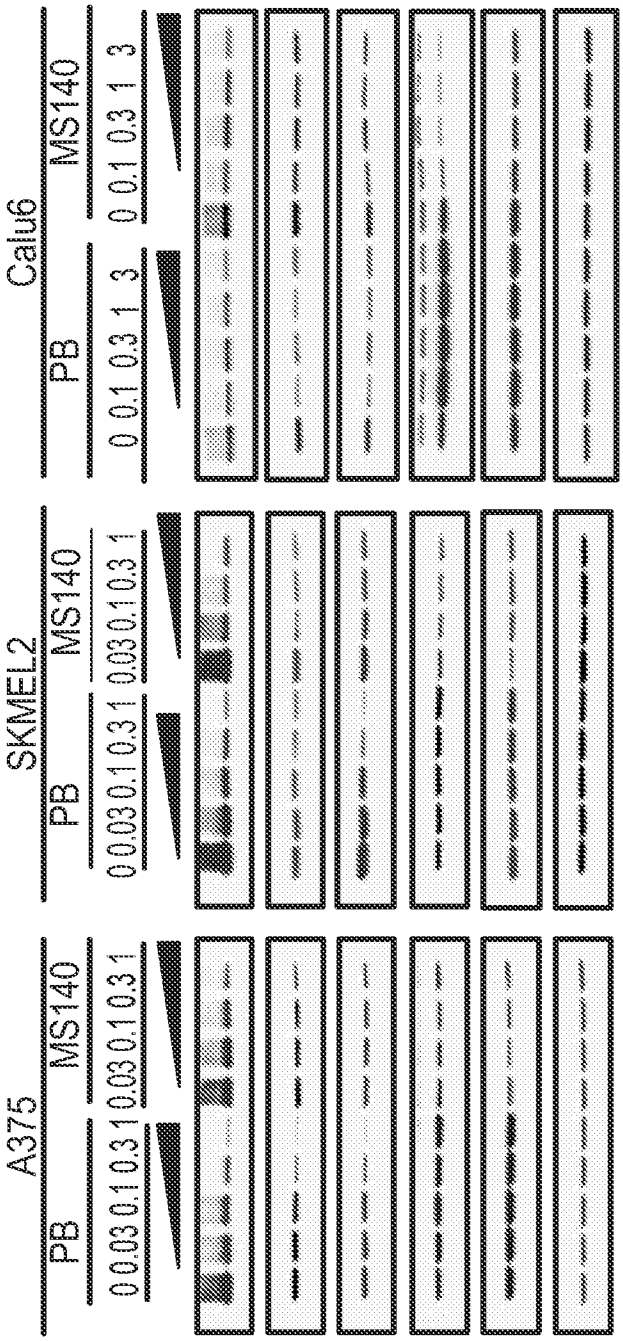

A375
SKMEL2
Calu6
PB
MS140
0 0.03 0.1 0.3 1
0 0.03 0.1 0.3 1
0 0.1 0.3 1 3
μM
pRb
PLK1
cyclin A
CDK4
CDK6
actin

FIG. 5B

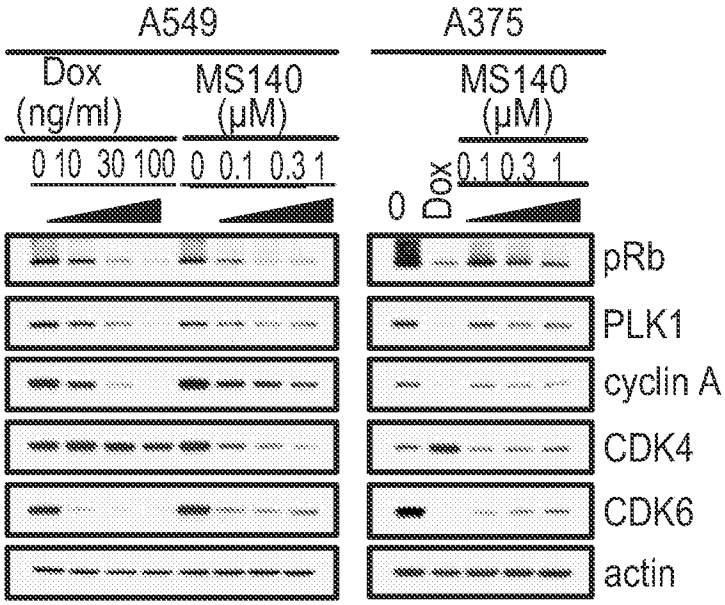

A549
A375
Dox (ng/ml)
MS140 (μM)
0 10 30 100
0 0.1 0.3 1
MS140 (μM)
0 Dox
0.1 0.3 1
pRb
PLK1
cyclin A
CDK4
CDK6
actin

FIG. 5C

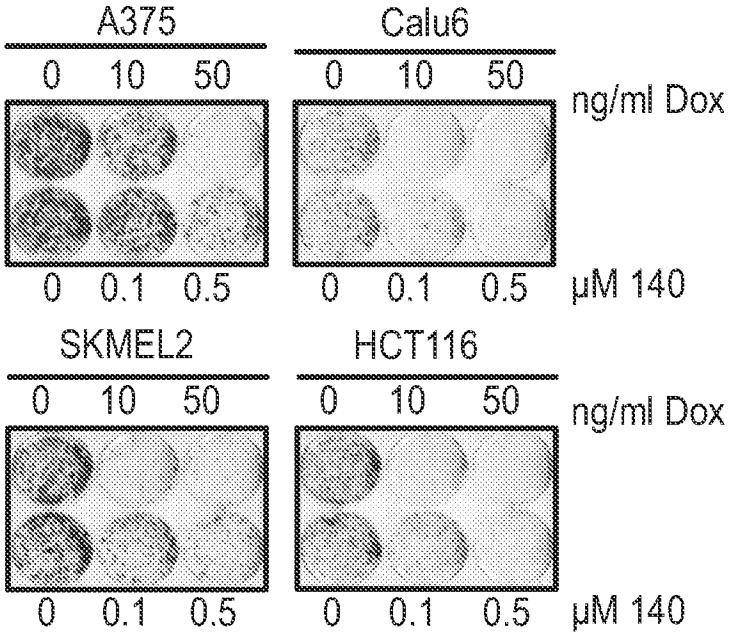

A375
Calu6
0 10 50
ng/ml Dox
0 0.1 0.5
μM 140
SKMEL2
HCT116
0 10 50
ng/ml Dox
0 0.1 0.5
μM 140

FIG. 5D

CDK4/6i-sensitive
CDK4-dependent
T47D
0 6 12 24
JeKo-1
0 6 12 24
U87MG
0 6 12 24
CDK4/6i-sensitive
CDK6-dependent
KMS12PE
0 6 12 24
MV4-11
0 6 12 24
Colo205
0 6 12 24
hr/MS140
CDK4
CDK6
actin
CDK4/6i-resistant
CDK6-dependent
HCT15
0 6 12 24
HCT116
0 6 12 24
Calu6
0 6 12 24
SKMEL2
0 6 12 24
A375
0 6 12 24
A549
0 6 12 24
hr/MS140
CDK4
CDK6
actin

FIG. 5E

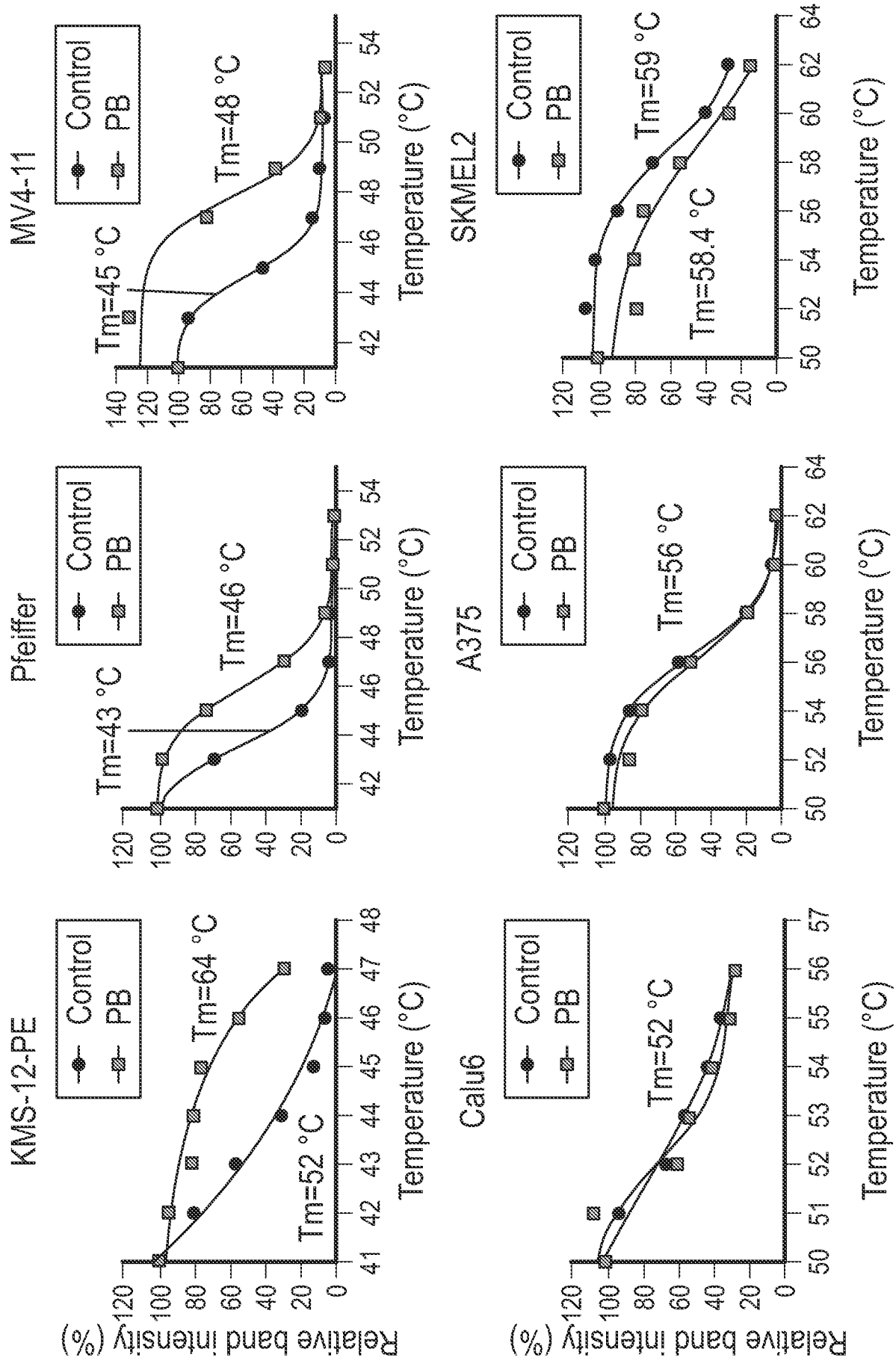

MV4-11
Control
PB
Tm=45 °C
Tm=48 °C
Pfeiffer
Tm=43 °C
Tm=46 °C
SKMEL2
Tm=58.4 °C
Tm=59 °C
KMS-12-PE
Tm=52 °C
Tm=64 °C
A375
Tm=56 °C
Calu6
Tm=52 °C
Relative band intensity (%)
Temperature (°C)

FIG. 5F

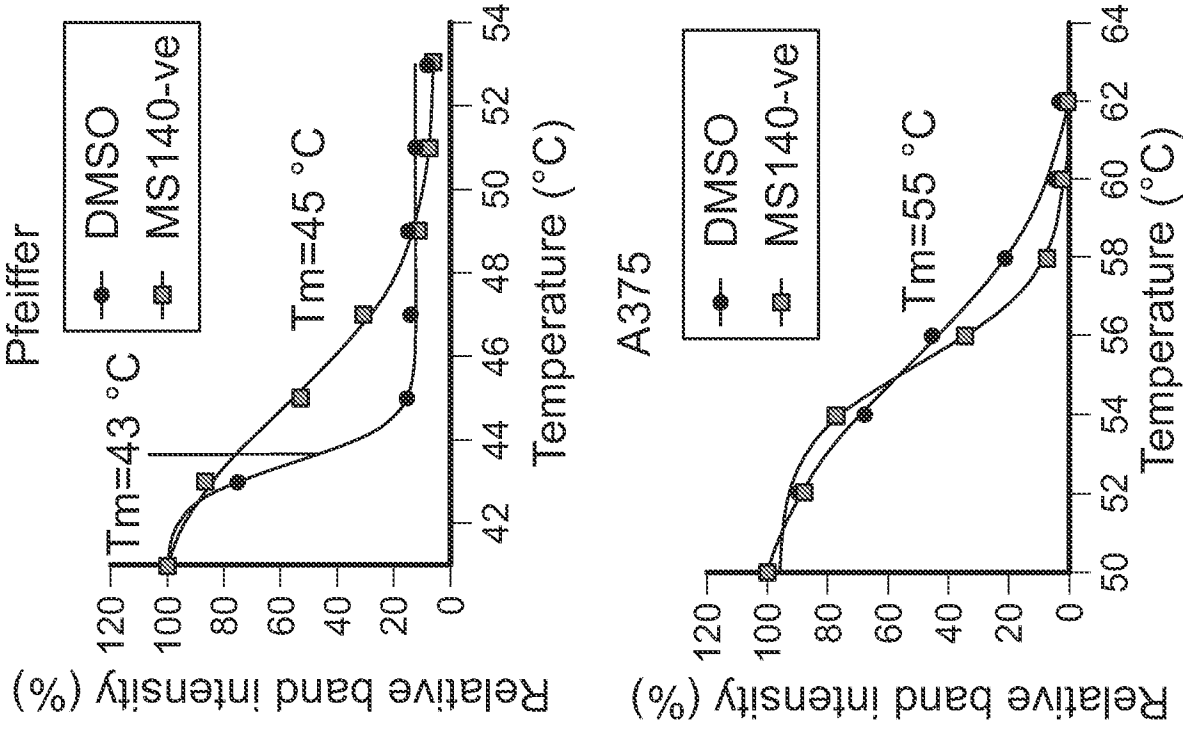

Pfeiffer
DMSO
MS140-ve
Tm=43 °C
Tm=45 °C
Relative band intensity (%)
Temperature (°C)
A375
DMSO
MS140-ve
Tm=55 °C
Relative band intensity (%)
Temperature (°C)

FIG. 5G

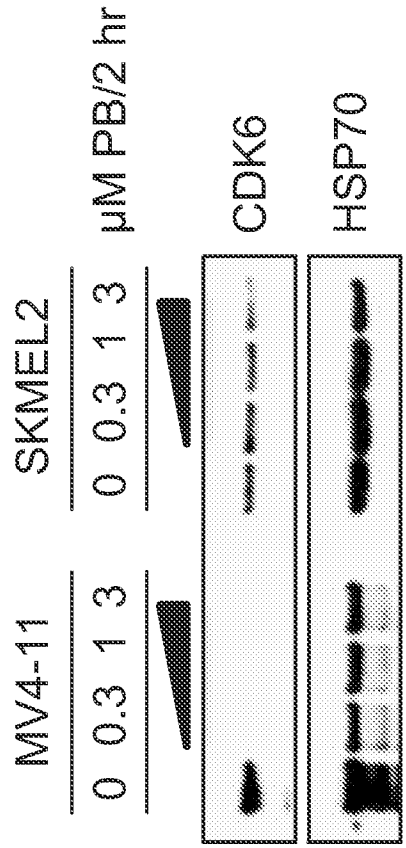
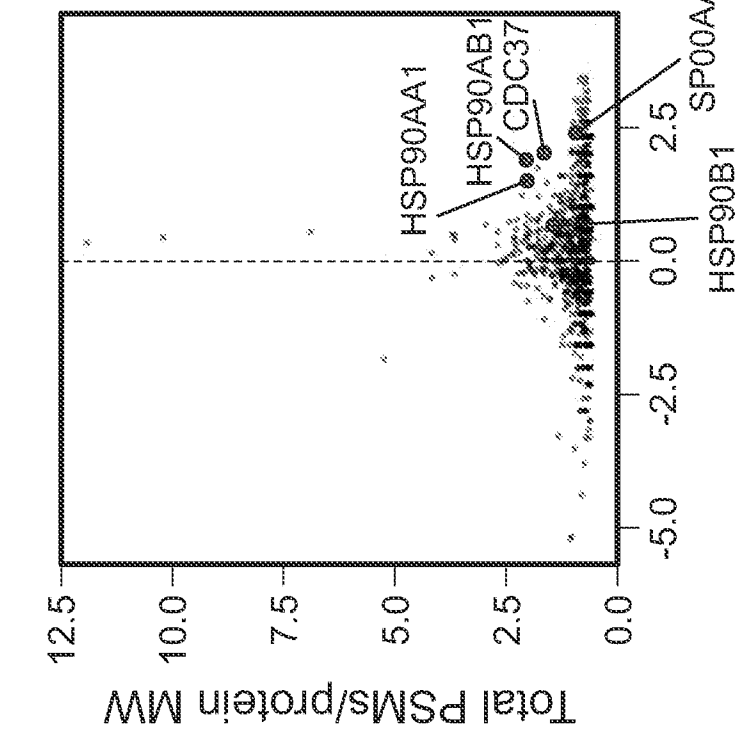

MV4-11
SKMEL2
0 0.3 1 3
0 0.3 1 3
μM PB/2 hr
CDK6
HSP70

FIG. 5H



Protein	# PSMs KMS12PE	# PSMs Calu6
HSP90AA1	97	34
HSP90AB1	103	28
HSP90B1	53	33
HSP90AA4P	16	3
CDC37	41	10

FIG. 6B

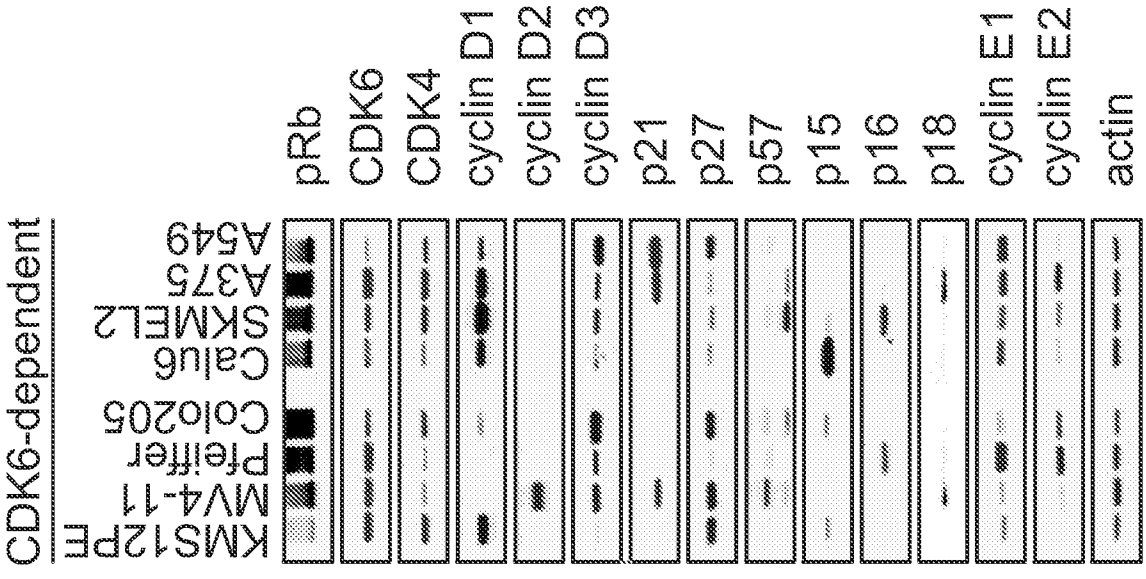


FIG. 6A

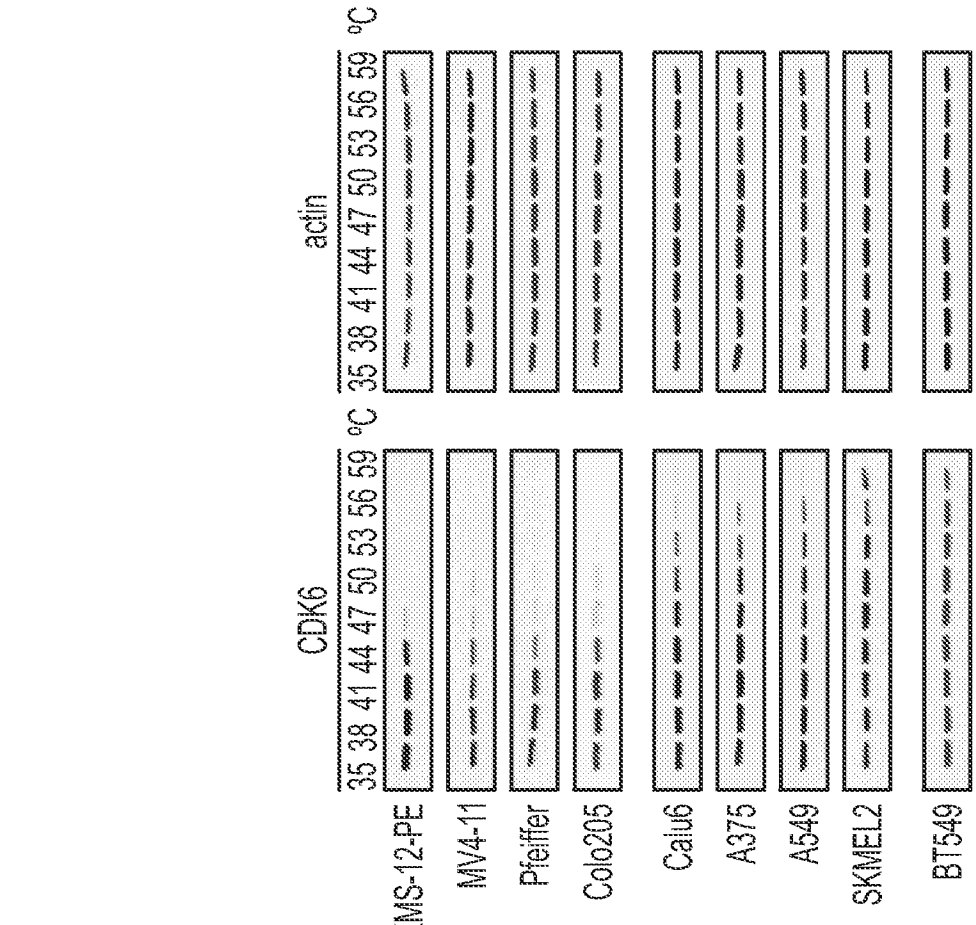


FIG. 6E

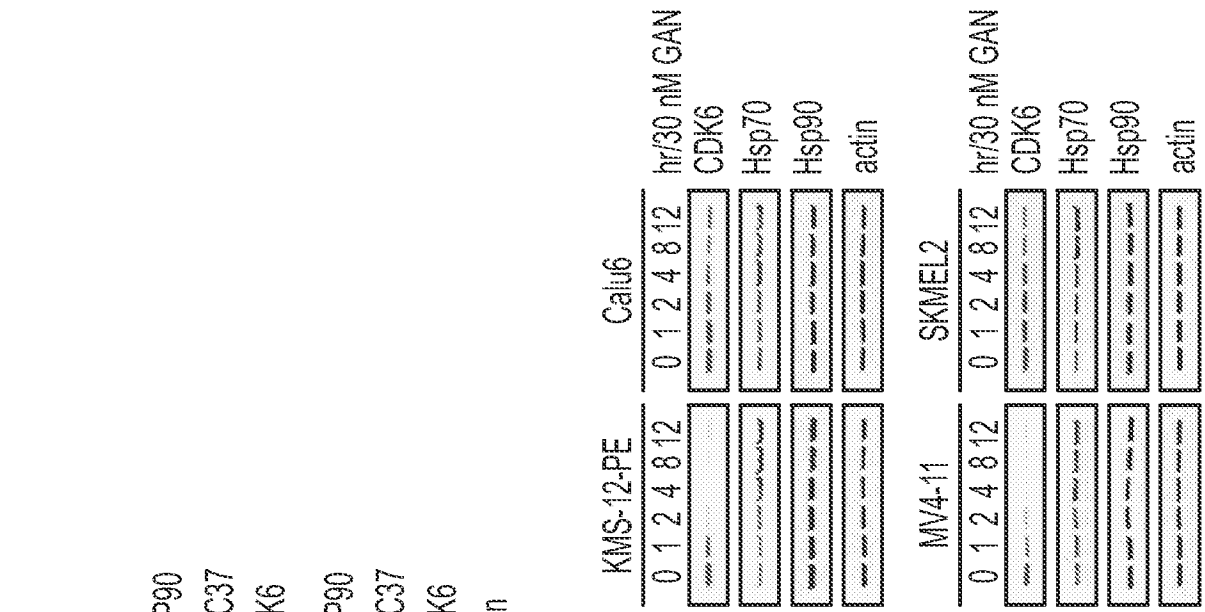


FIG. 6D

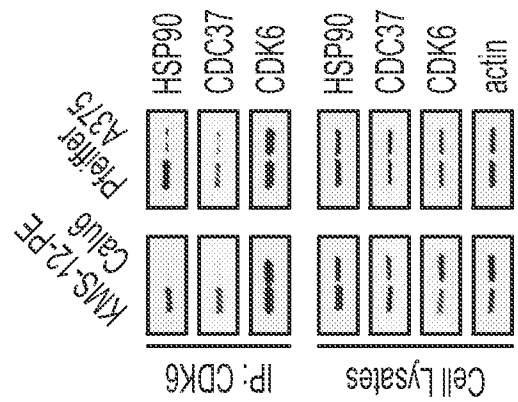


FIG. 6C

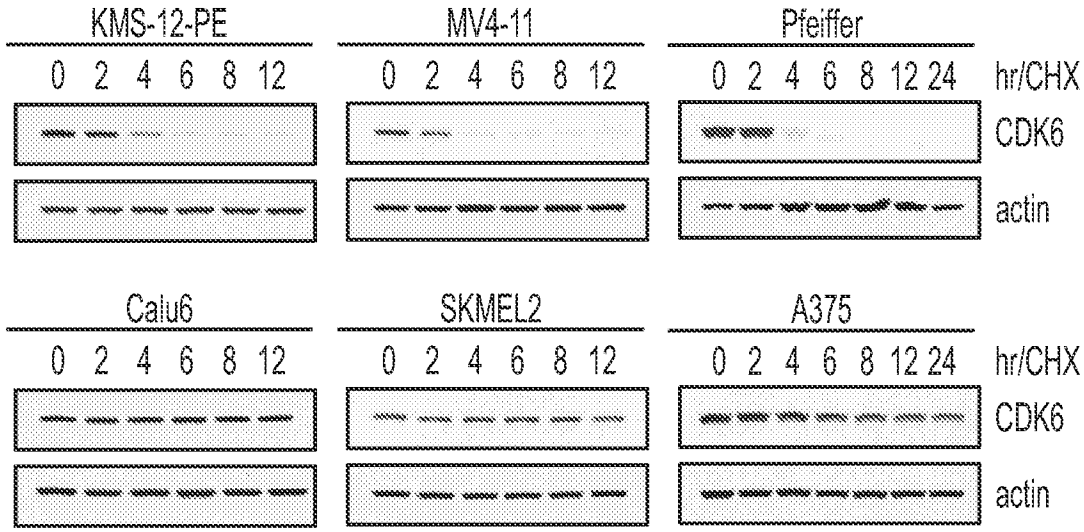

KMS-12-PE
MV4-11
Pfeiffer
0 2 4 6 8 12
0 2 4 6 8 12
0 2 4 6 8 12 24
hr/CHX
CDK6
actin
Calu6
SKMEL2
A375
0 2 4 6 8 12
0 2 4 6 8 12
0 2 4 6 8 12 24
hr/CHX
CDK6
actin

FIG. 6F

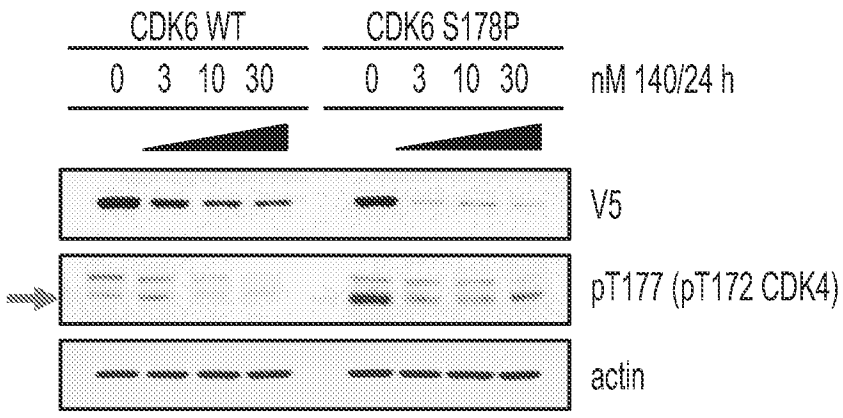

CDK6 WT
CDK6 S178P
0 3 10 30
0 3 10 30
nM 140/24 h
V5
pT177 (pT172 CDK4)
actin

FIG. 6G

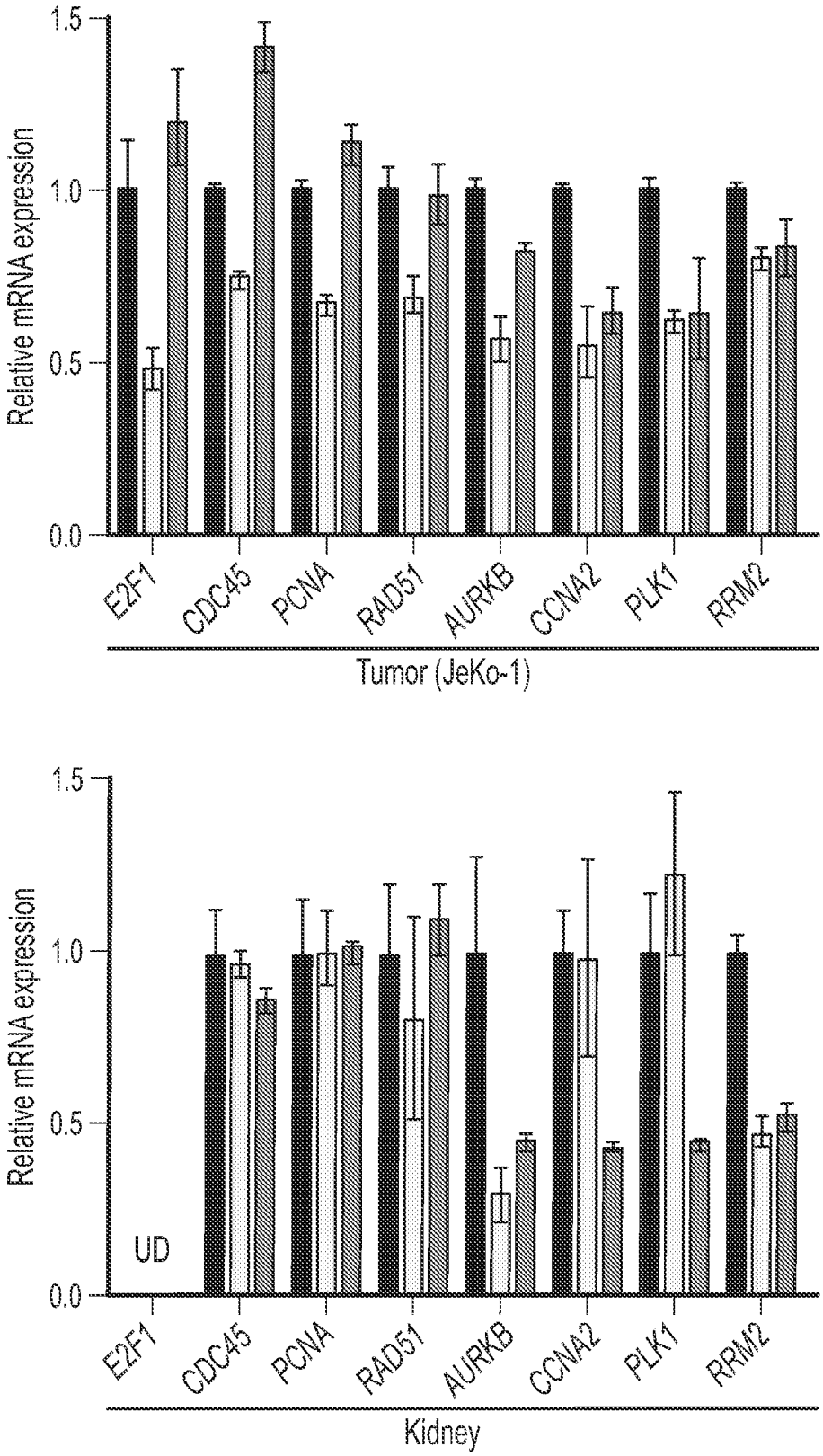

1.5
1.0
0.5
0.0
Relative mRNA expression
E2F1
CDC45
PCNA
RAD51
AURKB
CCNA2
PLK1
RRM2
Tumor (JeKo-1)
1.5
1.0
0.5
0.0
Relative mRNA expression
UD
E2F1
CDC45
PCNA
RAD51
AURKB
CCNA2
PLK1
RRM2
Kidney

FIG. 6H

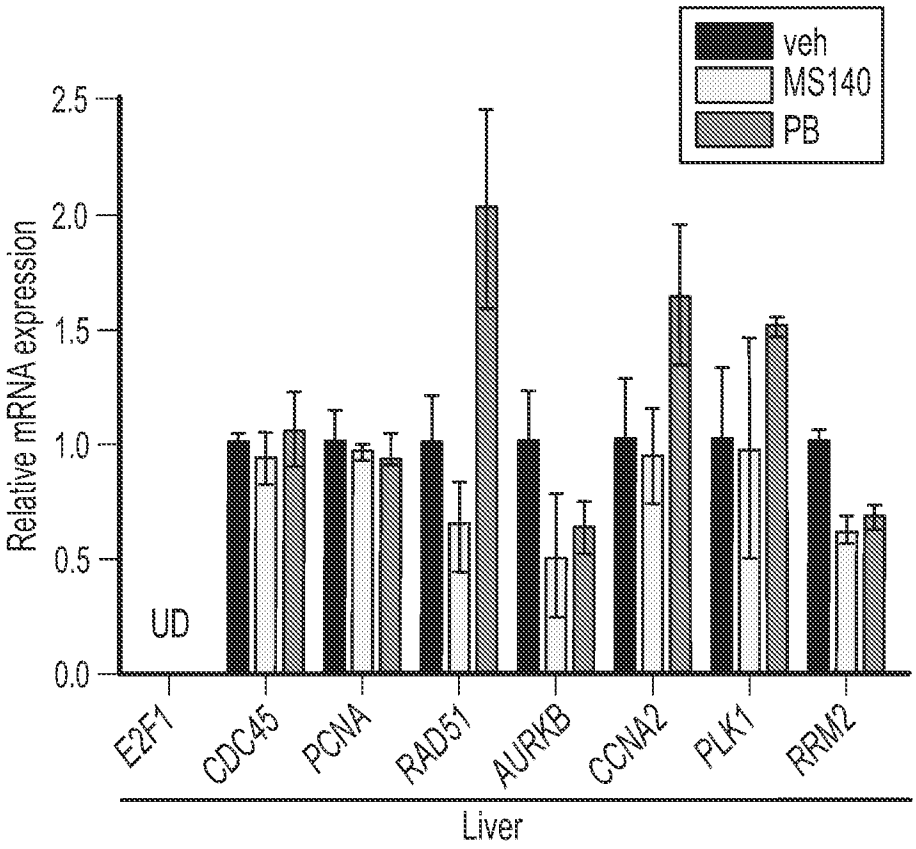


FIG. 6H (Cont.)

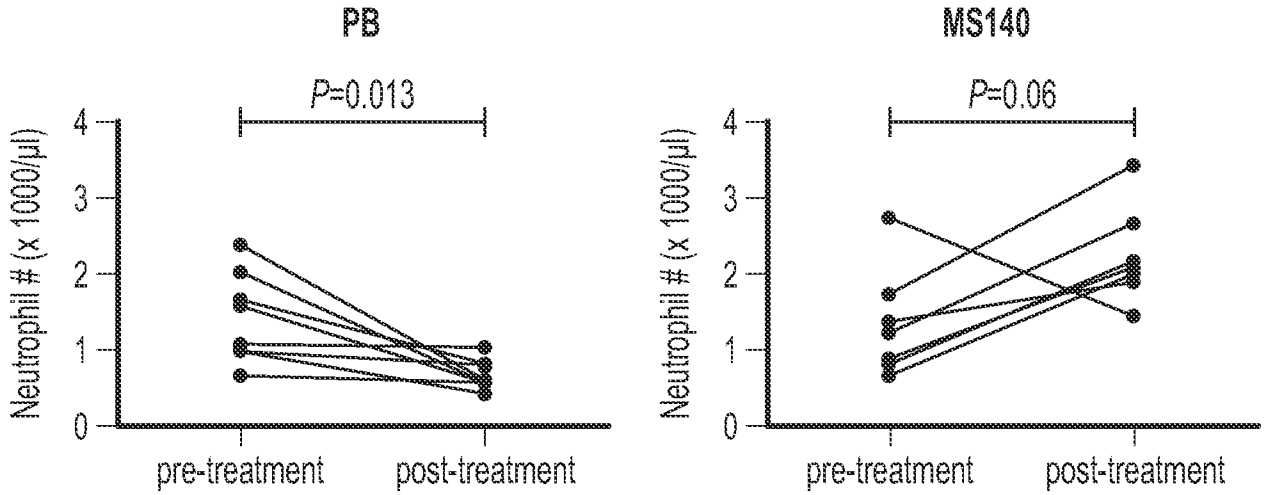


FIG. 6I

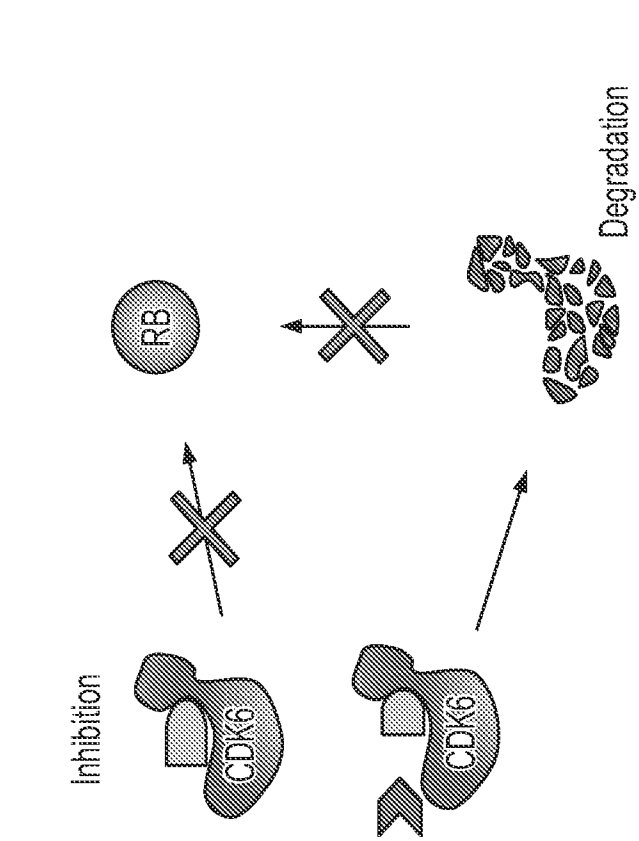

CDK4/6i
or
CDK4/6 degrader
Inhibition
CDK6
RB
CDK6
Degradation

FIG. 7A

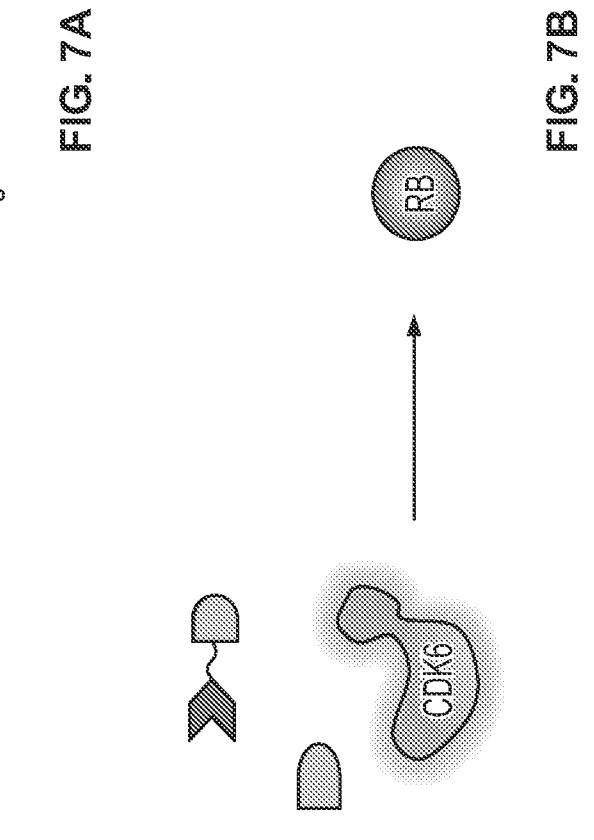
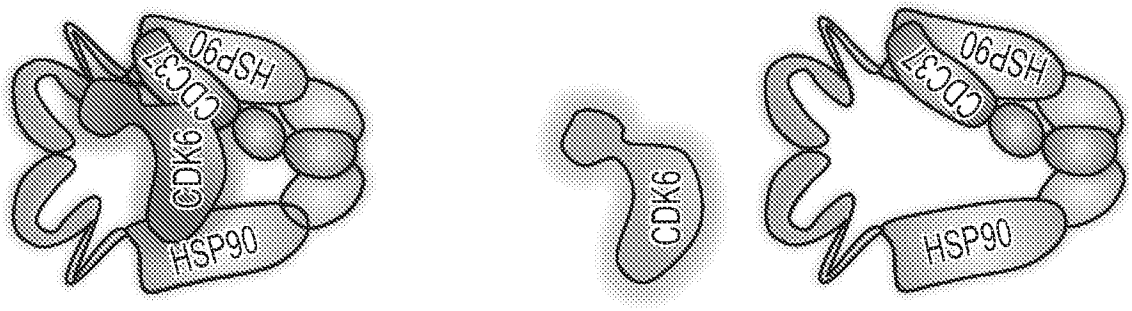

CDK4/6i
or
CDK4/6 degrader
CDK6
RB

FIG. 7B


HSP90
CDC37
CDK6
HSP90
CDK6
HSP90
CDC37

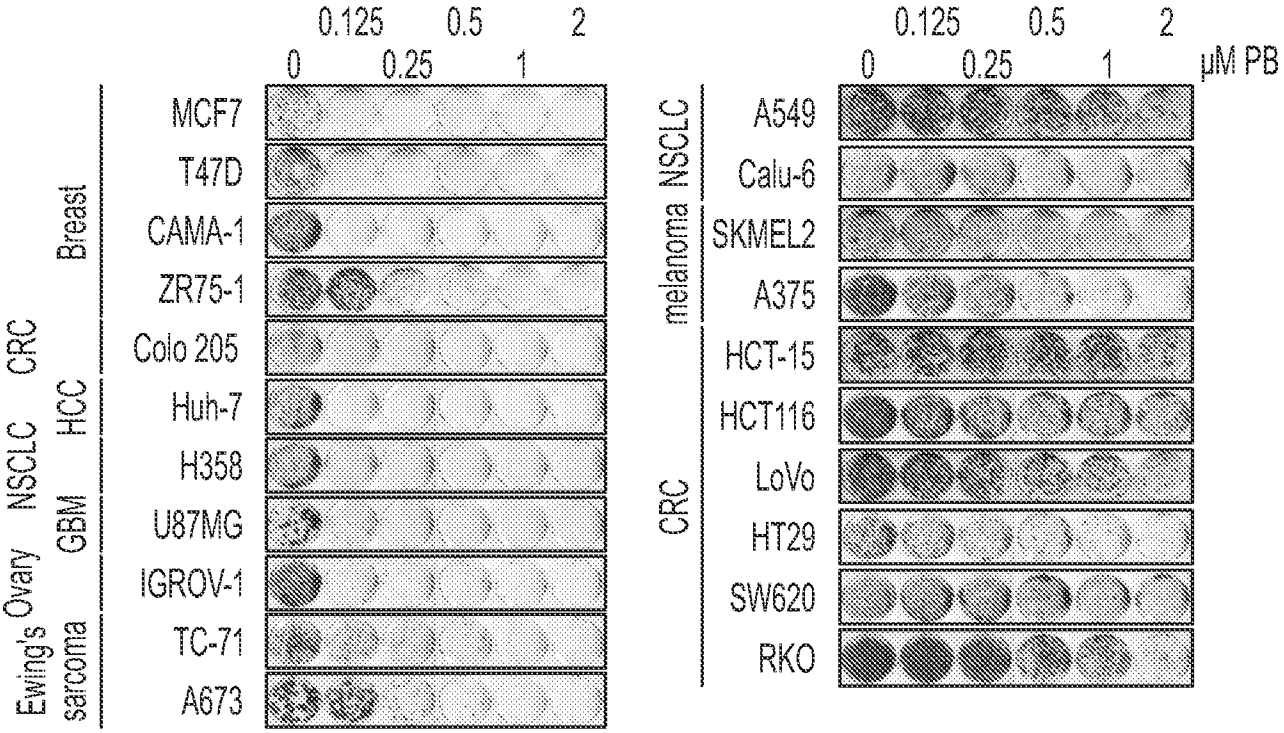


FIG. 8A

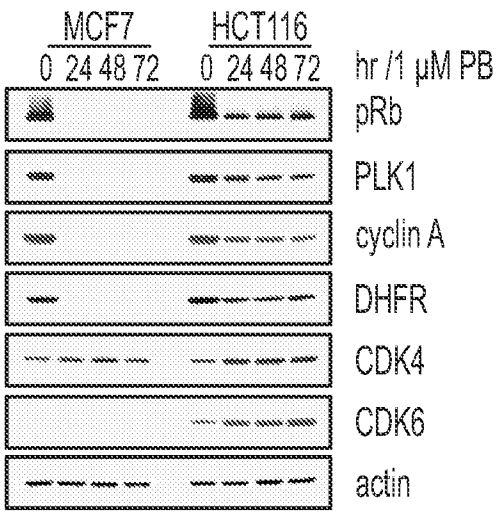


FIG. 8B

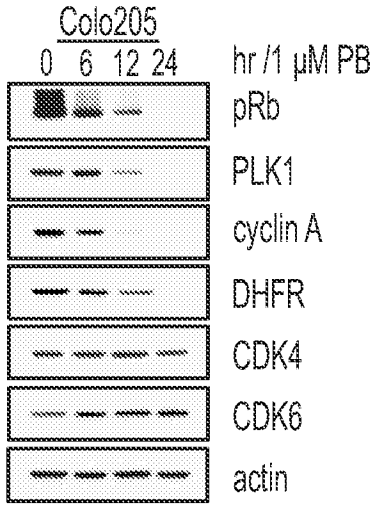


FIG. 8C

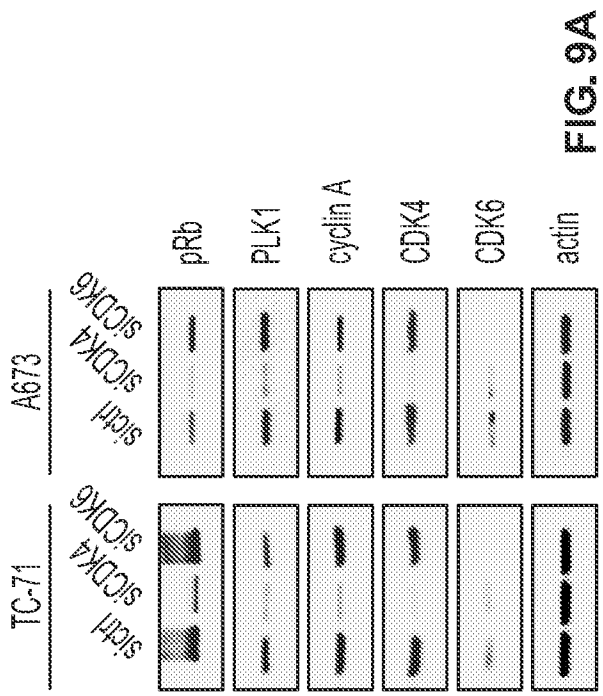

TC-71
A673
siCtrl
siCDK4
siCDK6
pRb
PLK1
cyclin A
CDK4
CDK6
actin

FIG. 9A

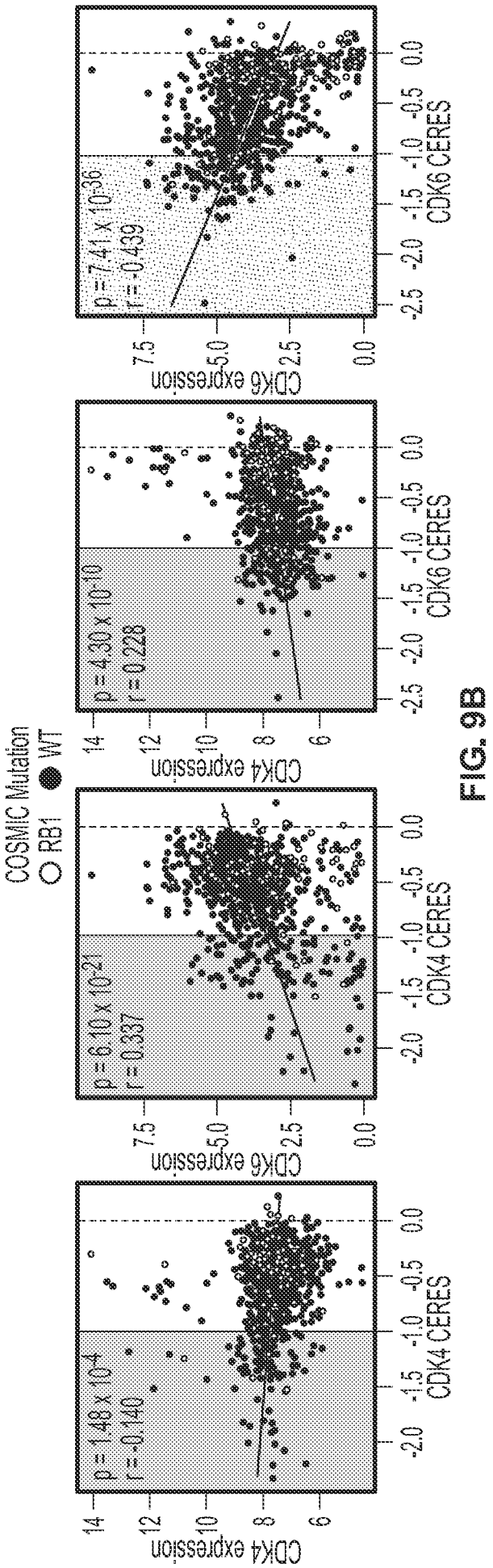

COSMIC Mutation
RB1
WT
p = 1.48 x 10-4
r = -0.140
p = 6.10 x 10-21
r = 0.337
p = 4.30 x 10-10
r = 0.228
p = 7.41 x 10-36
r = -0.439
CDK4 expression
CDK6 expression
CDK4 CERES
CDK6 CERES

FIG. 9B

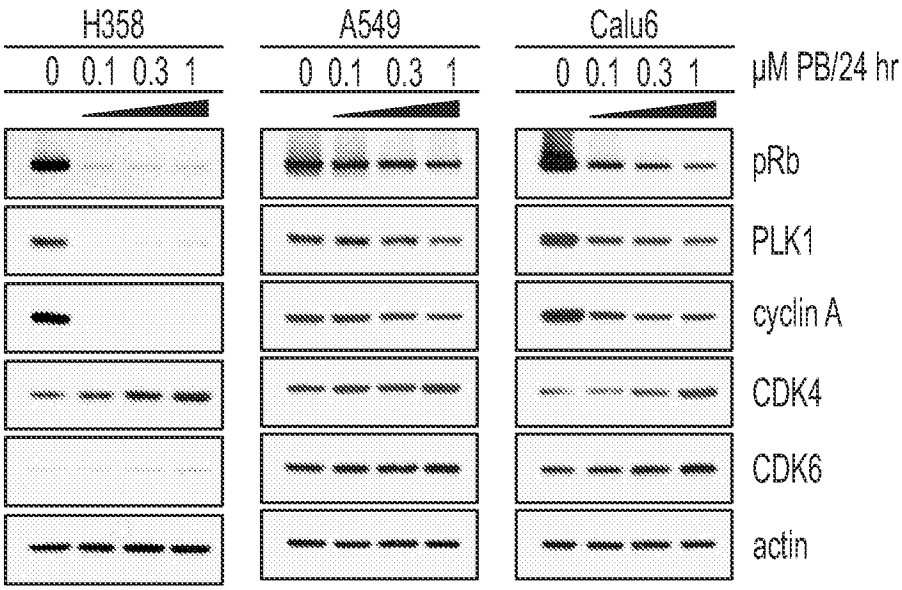


FIG. 10A

Tumor type	Cell lines	GI_{90} (nM PB)	CDK4/6 dependency
NSCLC	NCIH358	125	CDK4
NSCLC	NCIH2087	300	CDK4
NSCLC	NCIH1792	500	CDK4
NSCLC	NCIH2291	125	CDK4
NSCLC	HCC827	400	CDK4
NSCLC	Calu6	2000	CDK6
NSCLC	A549	>2000	CDK6
NSCLC	H1666	>2000	CDK6
NSCLC	PC9	>2000	CDK4/6

FIG. 10B

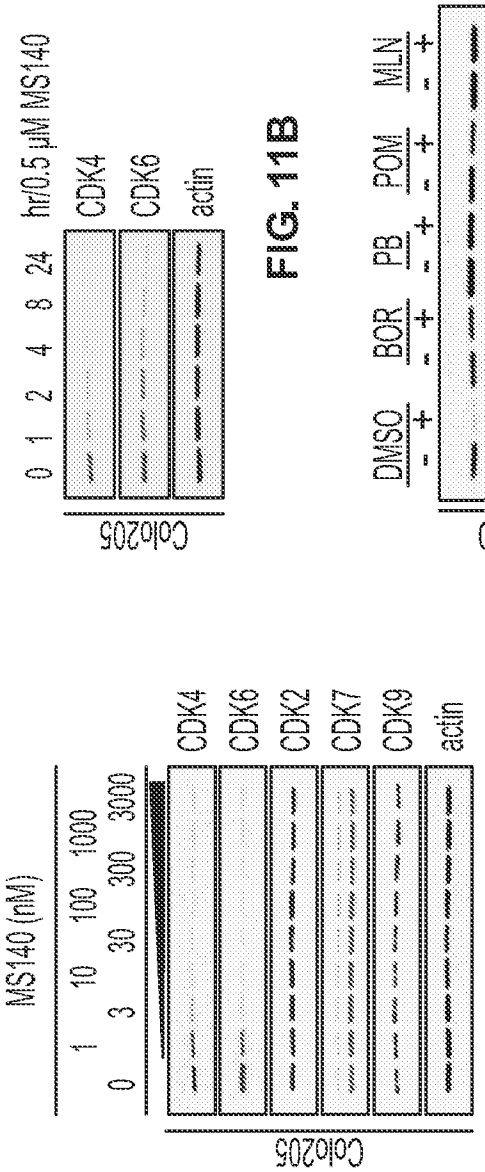


FIG. 11A

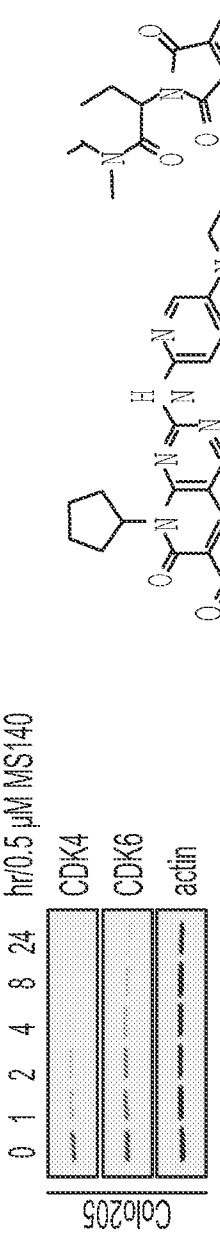


FIG. 11B

FIG. 11D

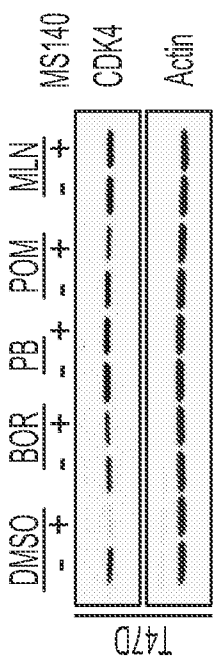


FIG. 11C

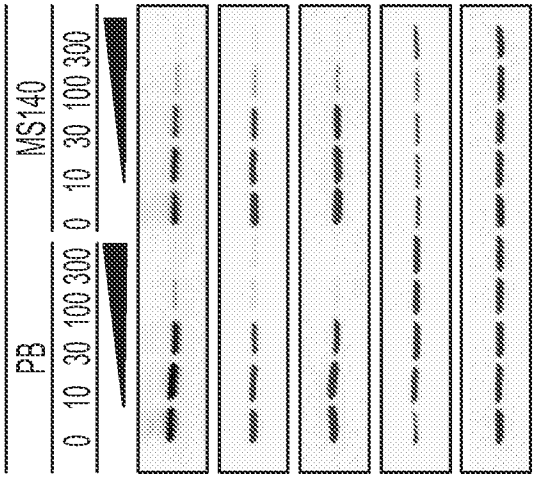


FIG. 11F

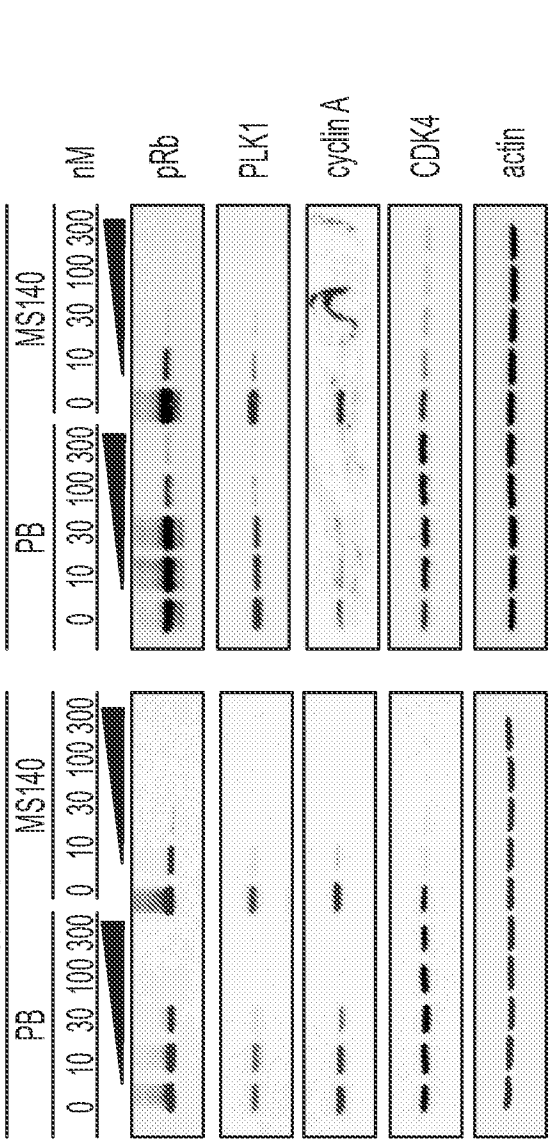


FIG. 11E

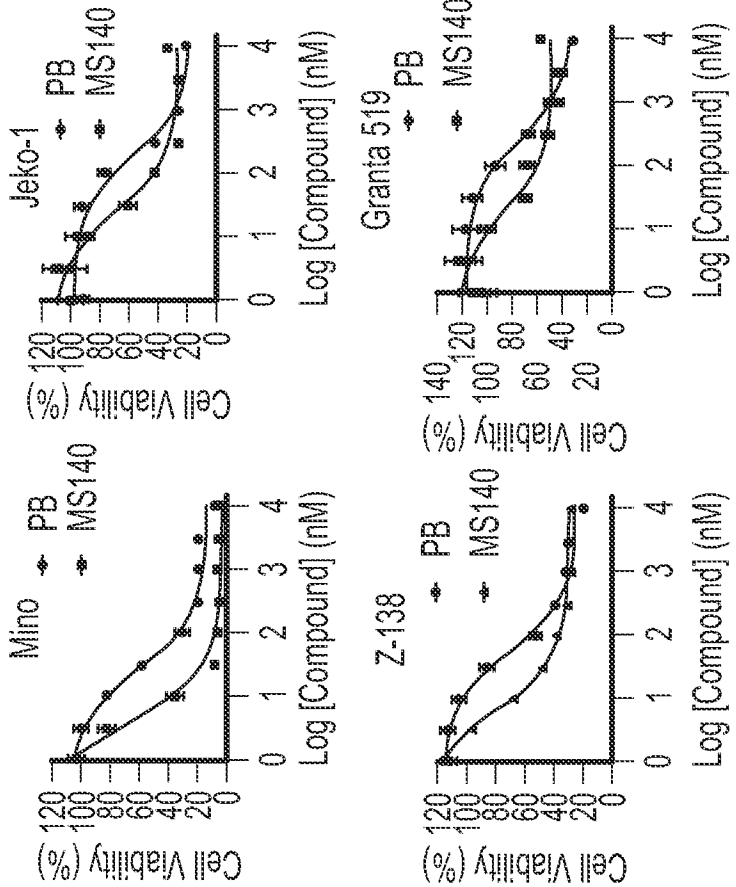


FIG. 11G

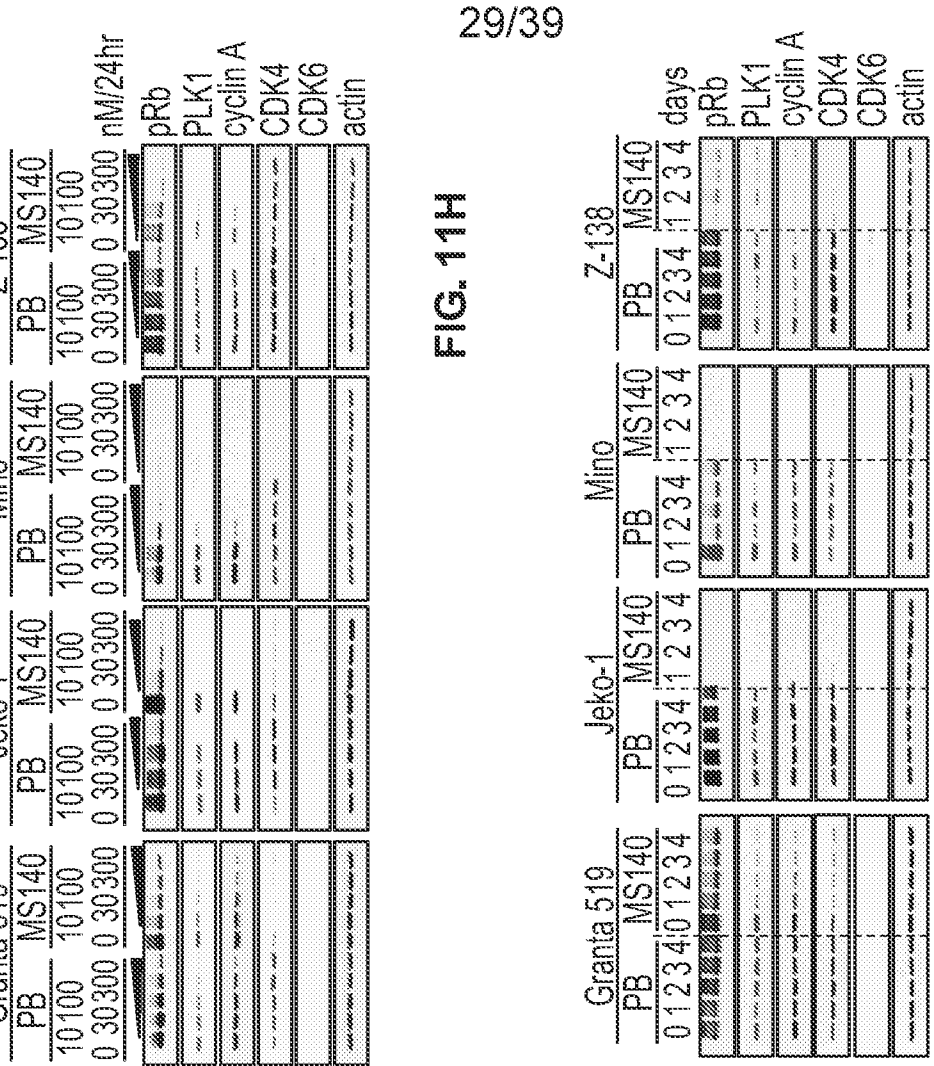


FIG. 11H

FIG. 11I

Tumor type	Cell line	PB G150 (nM)	MS140 G150 (nM)
MCL	Mino	26	4
MCL	Z138	53	6
MCL	Jeko-1	202	25
MCL	Granta519	256	20
MCL	REC-1	2421	341
MM	KMS-12-PE	80	15
AML	MOLM-14	70	45
AML	MV4-11	227	25
DLBCL	Pfeiffer	1056	79

FIG. 11J

FIG. 11K

FIG. 11M

FIG. 11L

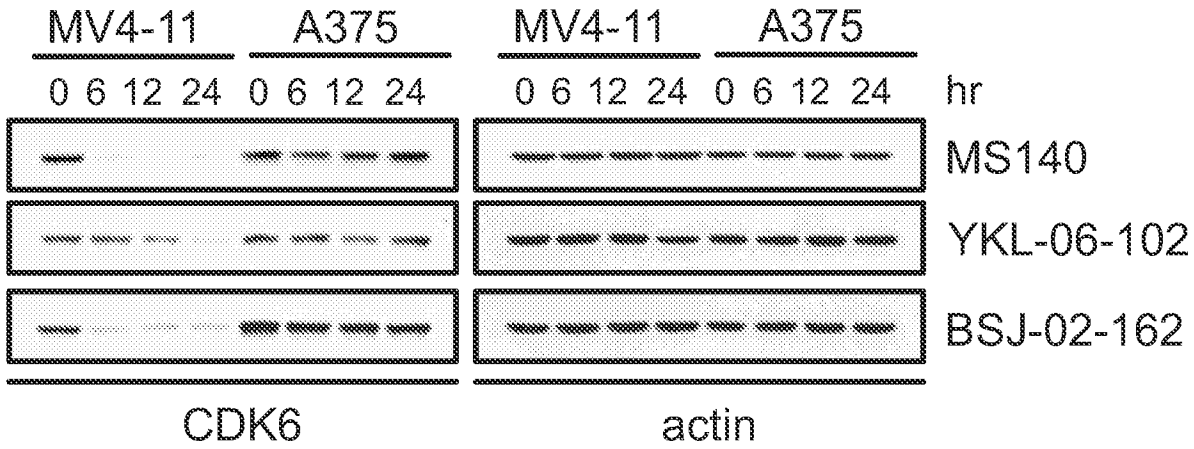


FIG. 12A

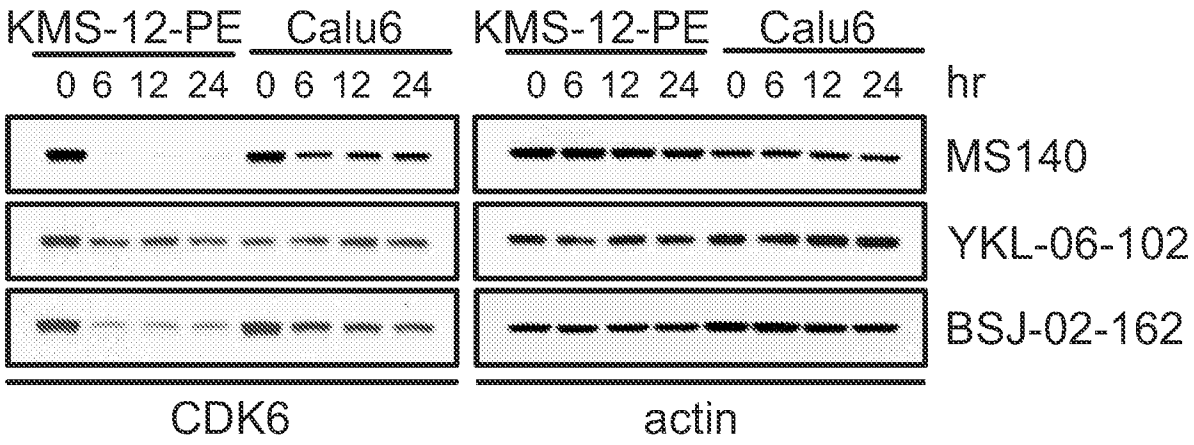


FIG. 12B

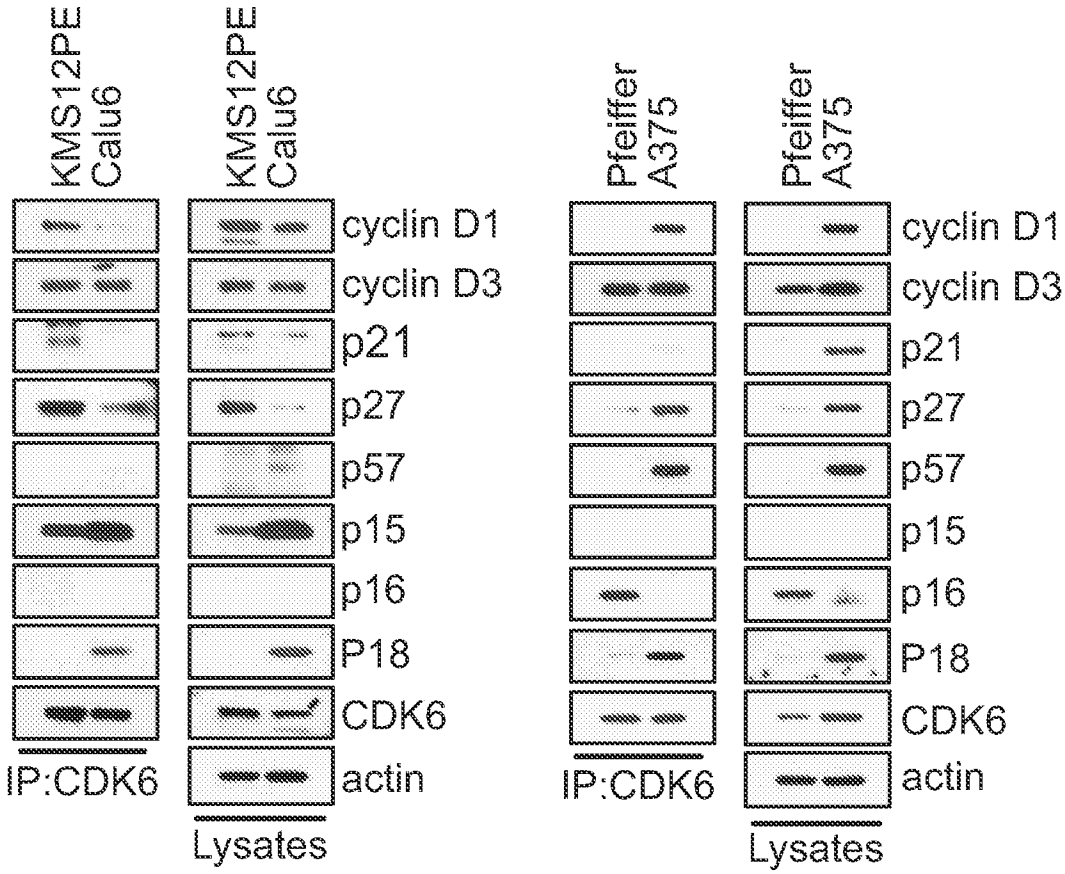
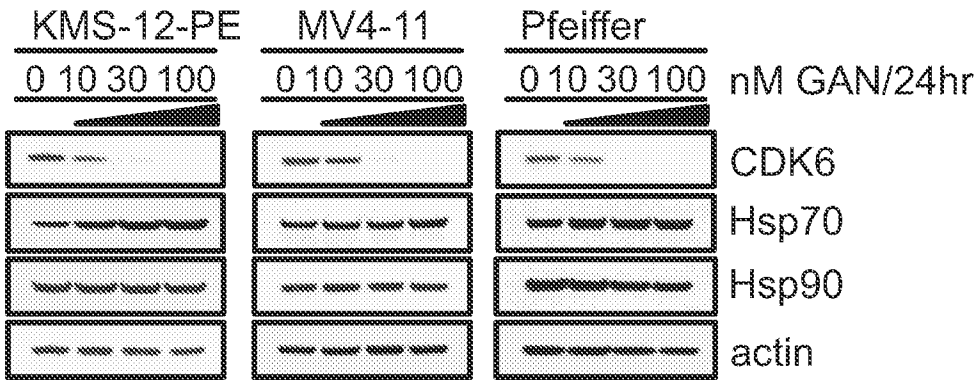


FIG. 13A



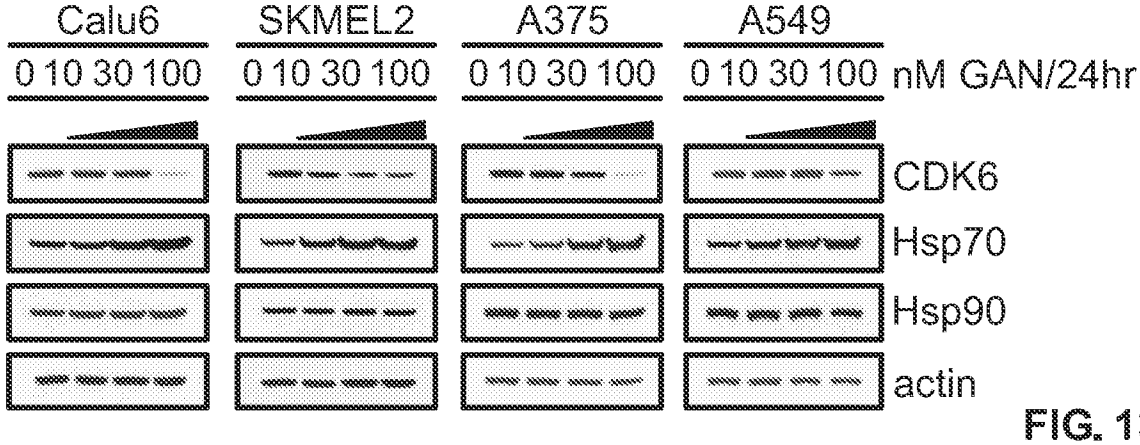


FIG. 13B

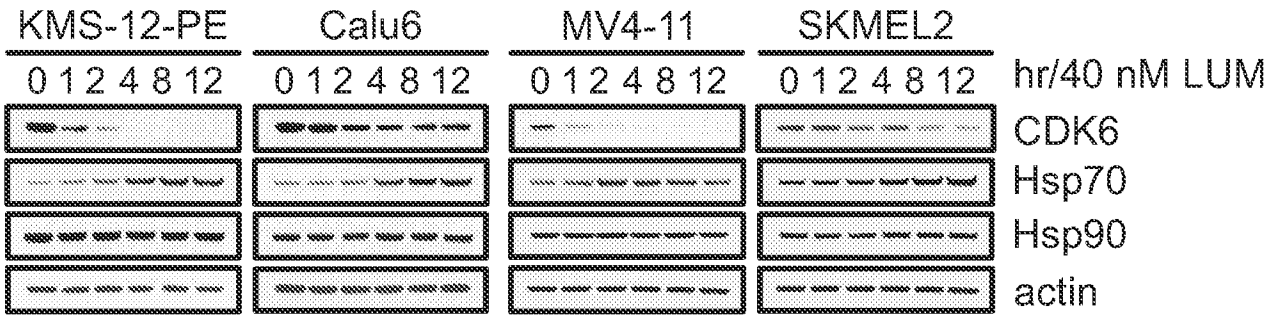


FIG. 13C

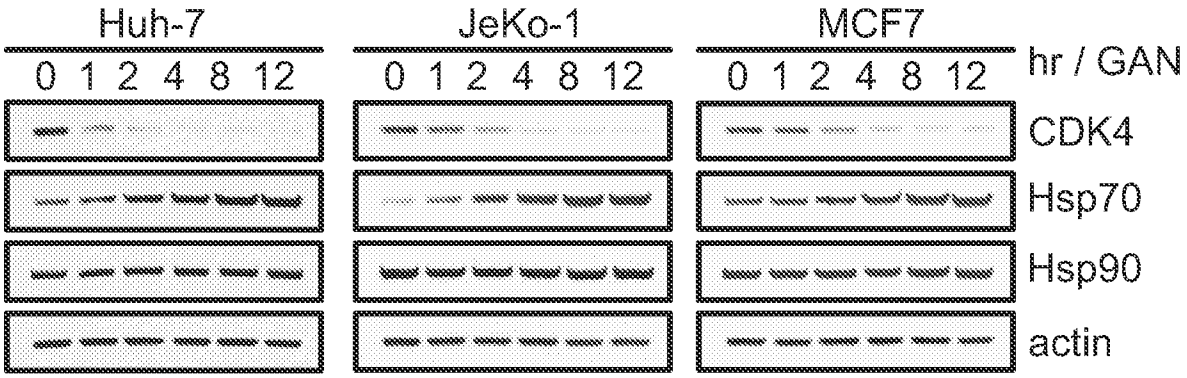


FIG. 13D

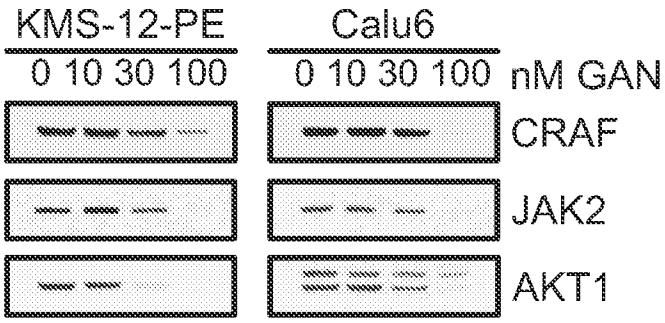


FIG. 13E

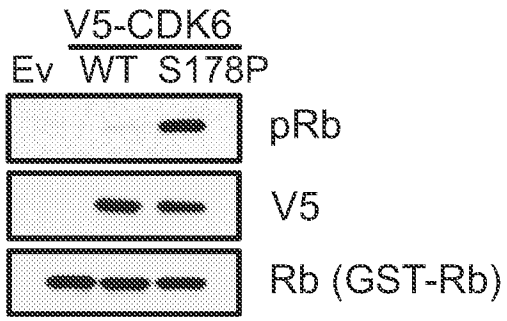


FIG. 13F

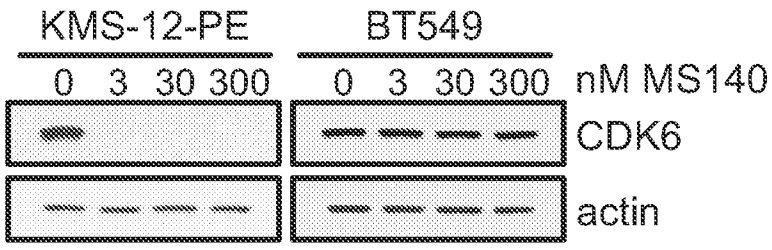


FIG. 13G

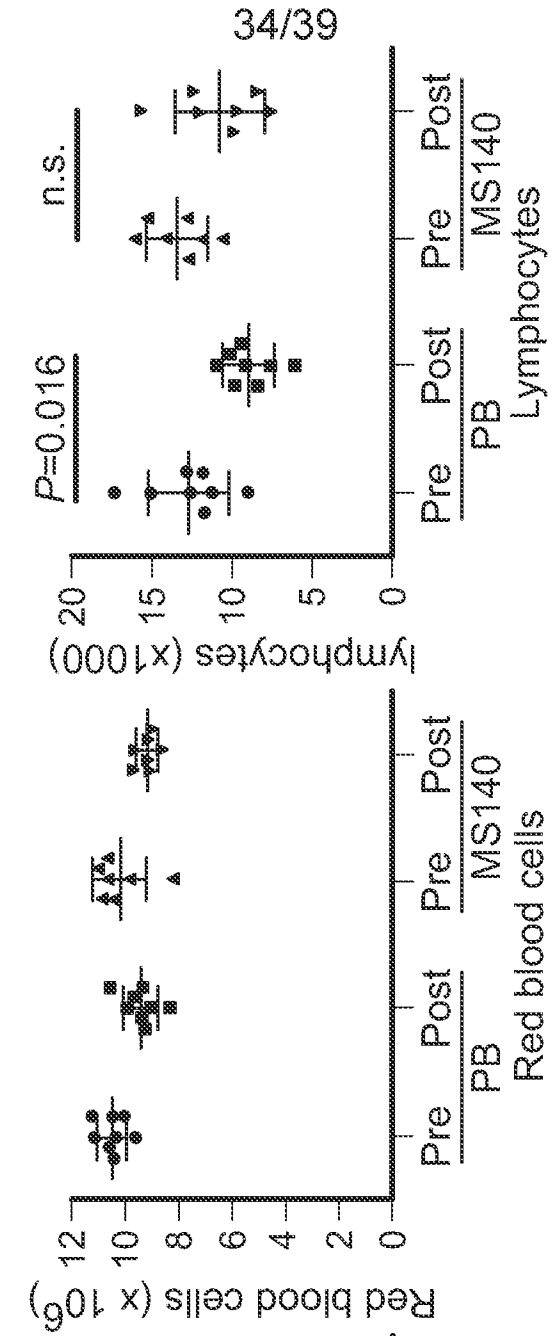
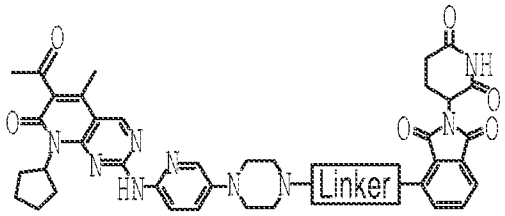

White blood cells (x1000)
20
15
10
5
0
P=0.006
n.s.
Pre Post Pre Post
PB MS140
White blood cells
Red blood cells (x 10^6)
12
10
8
6
4
2
0
Pre Post Pre Post
PB MS140
Red blood cells
lymphocytes (x1000)
20
15
10
5
0
P=0.016
n.s.
Pre Post Pre Post
PB MS140
Lymphocytes

FIG. 13H



palbociclib-based

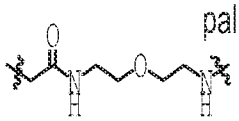
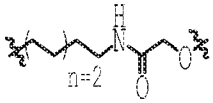
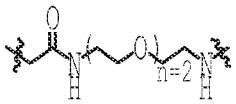
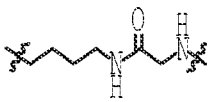
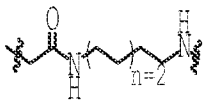
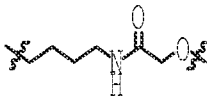
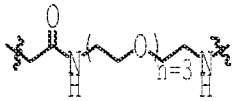
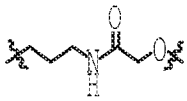
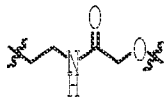
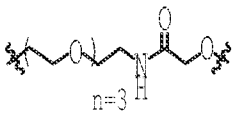
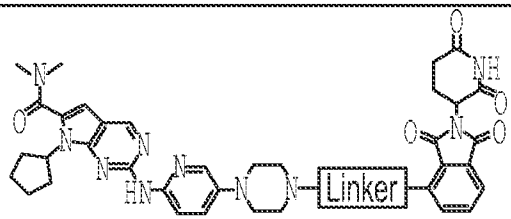
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BSJ-03-007		BSJ-02-162	
BSJ-03-008		BSJ-03-204	
YKL-06-102		BSJ-03-135	
BSJ-03-009		BSJ-03-189	
BSJ-03-123		BSJ-04-060	No linker

FIG. 14A



ribociclib-based

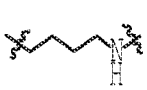
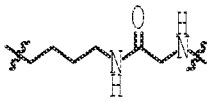
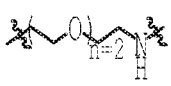
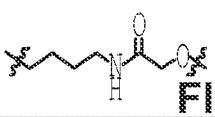
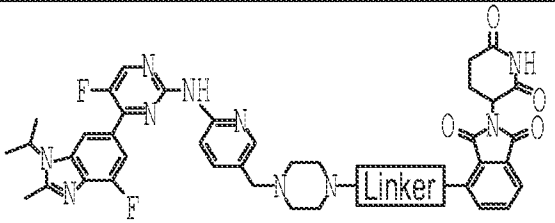
BSJ-01-151		BSJ-01-187	
BSJ-01-152		BSJ-04-132	

FIG. 14B



abemaciclib-based

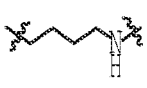
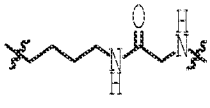
BSJ-01-158			

FIG. 14C

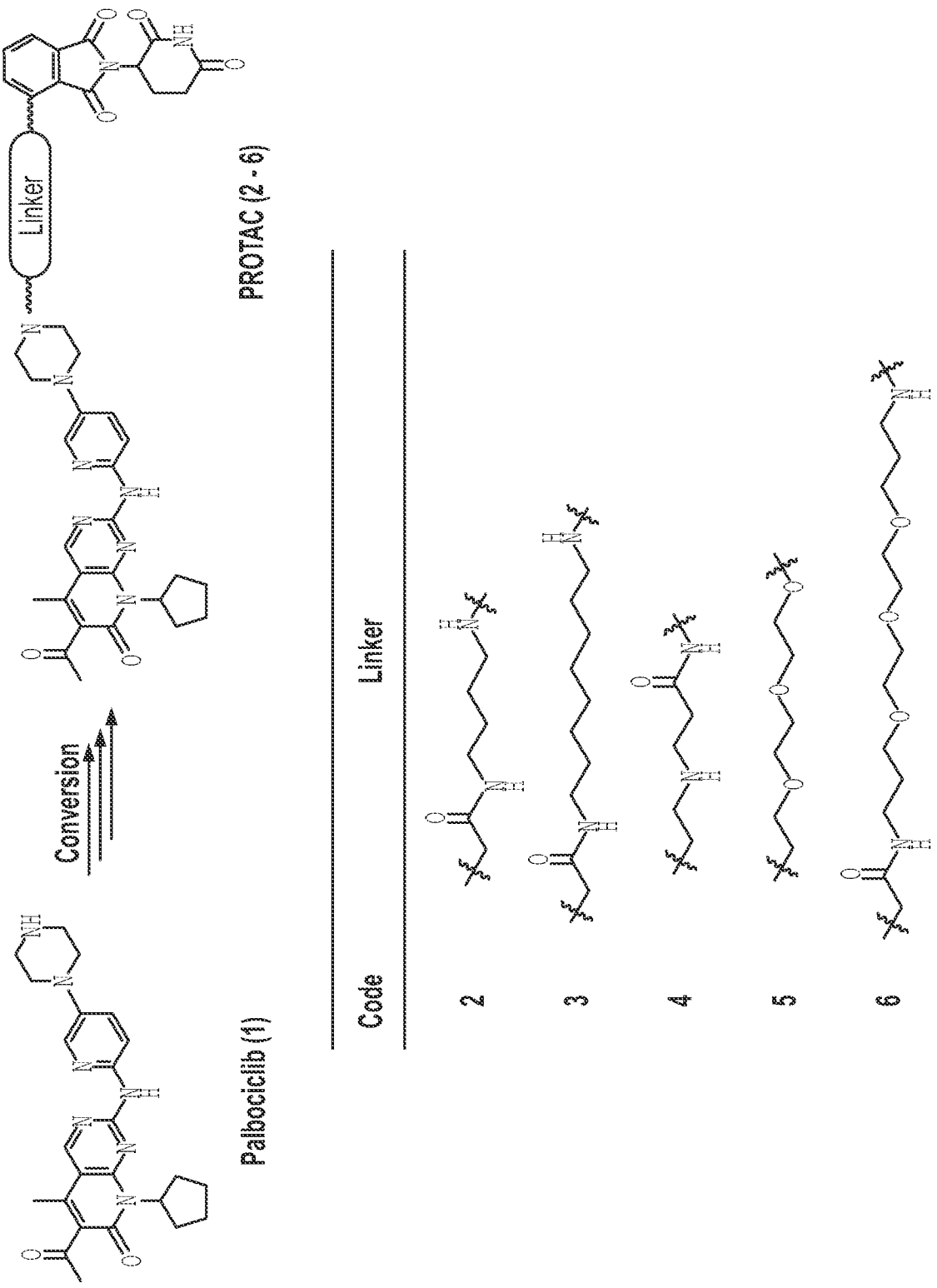
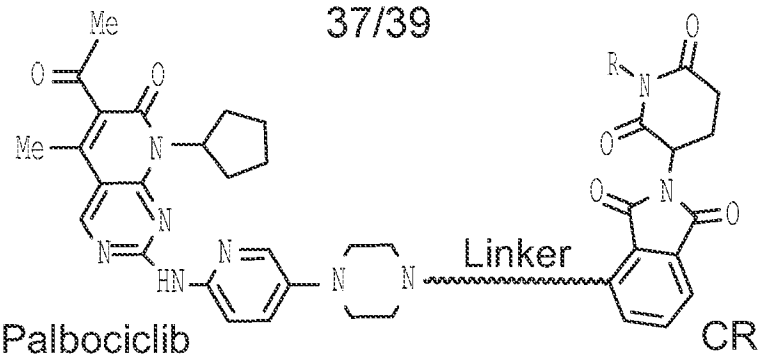

Palbociclib (1)
Conversion
Linker
PROTAC (2 - 6)
Code
Linker
2
3
4
5
6

FIG. 15



Palbociclib CRBN Recruting

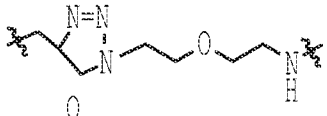
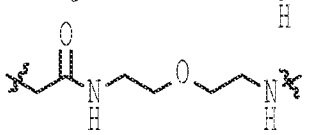
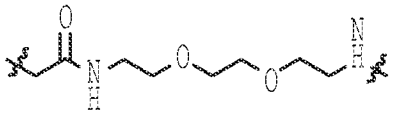
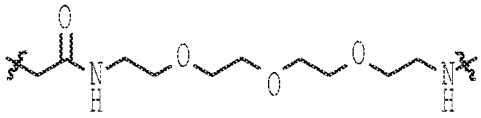
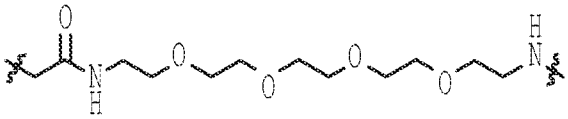
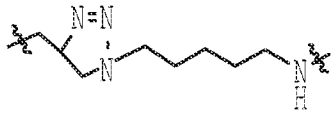
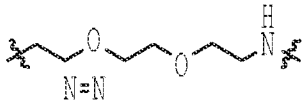
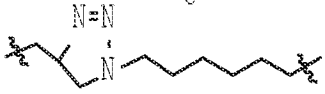
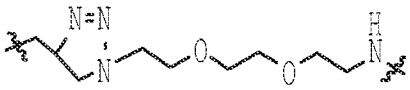
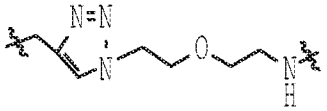
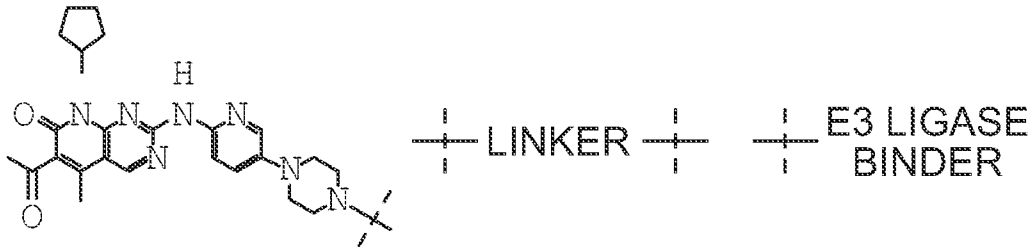
Compounds	Linker	R	DC50/nM CDK6	DC50/nM CDK4
CP-10		H	2.1	>100
CP-5		H	1.1	>100
CP-6		H	24.2	>100
CP-7		H	14.3	>100
CP-8		H	31.0	>100
CP-13		H	5.3	>100
CP-14		H	10.6	>100
CP-15		H	1.6	>50
CP-16		H	1.7	>50
CP-21		H	81.6	>100
CP-22		H	86.8	>100
CP-9		CH_2CH_3	ND	ND
CP-23		CH_2CH_3	ND	ND

FIG. 16



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]

FIG. 17

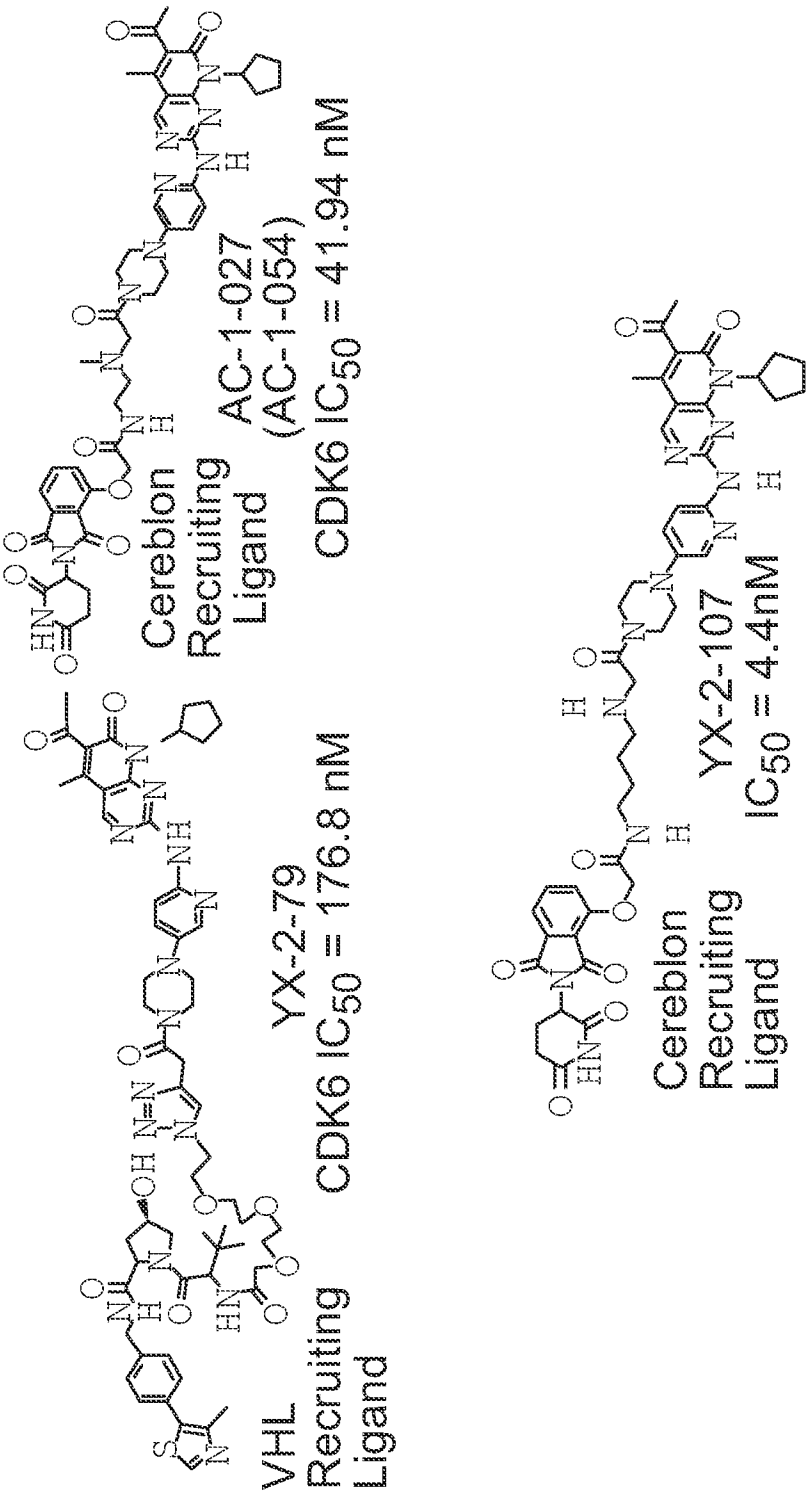

VHL Recruiting Ligand
YX-2-79
CDK6 IC50 = 176.8 nM
Cereblon Recruiting Ligand
AC-1-027 (AC-1-054)
CDK6 IC50 = 41.94 nM
Cereblon Recruiting Ligand
YX-2-107
IC50 = 4.4nM

FIG. 18

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 21/14697

A. CLASSIFICATION OF SUBJECT MATTER

IPC - A61K 35/74, A61P 3/00, C12N 1/00, C12N 15/52, C12N 15/70 (2021.01)

CPC - A61K 35/74, A61P 3/00, C12N 1/00, C12N 15/52, C12N 15/70

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
See Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
See Search History document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	US 2003/0039998 A1 (GELFAND et al.) 27 February 2003 (27.02.2003) abstract, para [0016], [0036], [0040], [0124], [0138], Claim 21, Claim 22	8-9, 17, 22-23 --- 1-5, 18, 24
X -- Y	WO 2018/106870 A1 (ICAHN SCHOOL OF MEDICINE AT MOUNT SINAI) 14 June 2018 (14.06.2018) pg 1, ln 22-29, pg 12, ln 9-12, pg 12, ln 13-26, pg 15, ln 1-5, pg 15, ln 32-33, pg 16, ln 6-21, pg 16, ln 22-34	20 ---- 1-5, 24
Y	US 2007/0202555 A1 (TANAKA et al.) 30 August 2007 (30.08.2007) Claim 1, Claim 5, para [0044], [0077]	18
Y	ANDERS et al. "Count-based differential expression analysis of RNA sequencing data using R and Bioconductor" Nature Protocols, September 2013, Vol 8, No 9, pp 1765-1786. abstract, pg 1784, para 3	24

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"D" document cited by the applicant in the international application
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
29 March 2021	APR 15 2021
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer Lee Young Telephone No. PCT Helpdesk: 571-272-4300

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 21/14697

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. ☒ forming part of the international application as filed:

      ☒ in the form of an Annex C/ST.25 text file.

      ☐ on paper or in the form of an image file.

   b. ☐ furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. ☐ furnished subsequent to the international filing date for the purposes of international search only:

      ☐ in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

      ☐ on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 21/14697

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 6-7, 10-16, 19, 21
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.