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Palbociclib synergizes with BRAF and MEK inhibitors in treatment naïve melanoma but not after the development of BRAF inhibitor resistance

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Abbreviations:

CDK4 - Cyclin-dependent kinase 4

MEK - Mitogen-activated protein kinase kinase

MAPK - Mitogen-activated protein kinase

ERK - Extracellular signal-related kinase

MITF - Microphthalmia-associated transcription factor

FOXM1 - Forkhead box protein M1

RB - Retinoblastoma

CDKN2A - Cyclin-dependent kinase inhibitor 2A

ATCC- American Type Culture Collection

NCI - National Cancer Institute

STR - Short tandem repeat

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PCR- polymerase chain reaction

GI50 - Concentration required to achieve 50% growth inhibition

PBS - Phosphate buffered saline

CI- Combination index

CMFDA - 5-chloromethylfluorescein diacetate

NSG - NOD-*scid* IL-2R□ null

SDS-PAGE - Sodium dodecyl sulfate polyacrylamide gel electrophoresis

IHC - Immunohistochemistry

PI - Propidium iodide

ED50 - effective dose required to achieve 50% growth inhibition

FACS - fluorescence-activated cell sorting

shRNA – short hairpin RNA

RNA - Ribonucleic acid

Novelty and Impact Statement

This study demonstrates that in BRAF mutant melanoma adding palbociclib upfront with a BRAF and MEK inhibitor combination prevents the development of therapy resistance leading to robust durable responses. In contrast, starting palbociclib treatment after melanoma cells have adapted to BRAF inhibitors or have developed robust resistance, the durable synergistic effect of the combination is lost. These findings raise questions regarding the CDK4/6, BRAF and MEK inhibitor dosing schedule and provide a strong rationale that upfront first line treatment with all three drugs is essential for robust and durable responses in BRAF mutant melanoma patients.

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ABSTRACT

Increased CDK4 activity occurs in the majority of melanomas and CDK4/6 inhibitors in combination with BRAF and MEK inhibitors are currently in clinical trials for the treatment of melanoma. We hypothesize that the timing of the addition of CDK4/6 inhibitors to the current BRAF and MEK inhibitor regime will impact on the efficacy of this triplet drug combination. The efficacy of BRAF, MEK and CDK4/6 inhibitors as single agents and in combination were assessed in human BRAF mutant cell lines that were treatment naïve, BRAF inhibitor tolerant or had acquired resistance to BRAF inhibitors. Xenograft studies were then performed to test the *in vivo* efficacy of the BRAF and CDK4/6 inhibitor combination. Melanoma cells that had developed early reversible tolerance or acquired resistance to BRAF inhibition remained sensitive to palbociclib. In drug tolerant cells the efficacy of the combination of palbociclib with BRAF and/or MEK inhibitors was equivalent to single agent palbociclib. Similarly, acquired BRAF inhibitor resistance cells lost efficacy to the palbociclib and BRAF combination. In contrast upfront treatment of melanoma cells with palbociclib in combination with BRAF and/or MEK inhibitors induced either cell death or senescence, and was superior to a BRAF plus MEK inhibitor combination. *In vivo* palbociclib plus BRAF inhibitor induced rapid and sustained tumor regression without the development of therapy resistance. In summary, upfront dual targeting of CDK4/6 and mutant BRAF signalling enables tumor cells to evade resistance to monotherapy and is required for robust and sustained tumor regression. Melanoma patients whose tumors have acquired resistance to BRAF inhibition are less likely to have favourable responses to subsequent treatment with the triplet combination of BRAF, MEK and CDK4/6 inhibitors.

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INTRODUCTION

The high incidence of activating BRAF^{V600} mutations in cutaneous melanoma has led to the development of potent inhibitors such as vemurafenib, dabrafenib and encorafenib that specifically target activated BRAF. Despite the high response rates in patients treated with these therapies, resistance develops in 50% of patients treated with BRAF monotherapy within 6-7 months [1, 2] and by 3-5yrs this is ~90% [3]. In most patients (70-79%) relapse is associated with reactivation of the MAPK/ERK pathway [4, 5]. This led to clinical trials in which BRAF inhibitors were combined with MEK inhibitors in the expectation that this combination would prevent or delay the emergence of resistance. While dual BRAF and MEK inhibition is superior to single agent treatment, increasing progression free and overall survival, in most patients it only delays the emergence of resistance [3, 6-8]. Furthermore, in patients who have developed resistance to BRAF inhibitors when given as monotherapy, subsequent treatment with either a MEK inhibitor or a combination of BRAF and MEK inhibitors is also ineffective in most cases [5, 9]. During the early phase of BRAF or MEK inhibitor treatment rewiring of melanoma cell signalling networks occurs leading to an initial drug tolerant state that is reversible and not due to acquired mutations [10-15]. With continued drug pressure this reversible drug tolerant state eventually develops into non-reversible acquired drug resistance, potentially via the outgrowth of mutated melanoma cells that have acquired novel mutations or cells that were inherently resistant [16]. Both increased expression of MITF and c-JUN have been implicated in mediating the early reversible drug tolerant state. In these drug tolerant cells co-targeting BRAF and c-JUN leads to enhanced cell death [13, 14], while depleting MITF renders these cells sensitive to BRAF inhibition [15]. Thus combining BRAF and/or MEK inhibitors with drugs that overcome the early drug tolerant state will likely be an effective strategy to improve these targeted therapies and thus the treatment of melanoma patients.

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Another highly deregulated pathway in melanoma is the p16/CDK4-cyclinD/pRb pathway (CDK4 pathway). Cyclin-dependent kinase 4 (CDK4) is a serine/threonine kinase that is a central regulator of the G1-S transition of the cell cycle. CDK4, when complexed with the D-type cyclins, promotes cell cycle progression mainly through the phosphorylation of pRb [17]. p16^{INK4A} specifically inhibits the assembly and activation of the CDK4-CyclinD1 complex, thus loss of p16^{INK4A} leads to increased CDK4 signaling. The CDK4 pathway is deregulated in approximately 70% of melanomas mainly due to loss of functional p16^{INK4A} as a result of gene deletion, promoter methylation, or genetic mutation [18]. The majority of melanoma cell lines are sensitive to CDK4 inhibition, with p16^{INK4A} loss a biomarker of sensitivity [19]. The CDK4 pathway converges with the MAPK/ERK pathway at the CDK4-Cyclin D1 complex. CDK4 activation by ERK-induced CyclinD1 expression is critical for the ability of BRAF to promote cell cycle progression [20, 21] and increased CDK4 signaling can increase resistance to BRAF inhibitors [4, 22, 23], which may account for the association of CDKN2A genetic alterations with poorer overall and progression free survival in patients on dabrafenib plus trametinib treatment [24]. Thus, combining CDK4 inhibitors with RAF, MEK, and/or ERK inhibitors may be an approach to overcome resistance to BRAF inhibitors and improve patient responses. Indeed, preclinical studies using human melanoma cell lines have demonstrated that the majority of acquired and inherently BRAF inhibitor resistant cells respond to CDK4 inhibition. Furthermore, in human melanoma xenografts once resistance to BRAF inhibition has developed the addition of a CDK4/6 inhibitor initially induces tumor regression [25, 26].

In the present study using melanoma cell lines and xenografts, we tested the hypothesis that the timing of the addition of palbociclib to BRAF and MEK inhibitor

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treatment will impact on the efficacy of these drug combinations. We have compared the response of single agent palbociclib, BRAF and MEK inhibition and their combination in matching melanoma cells that are sensitive to these drugs, those that have developed early non-mutational BRAF inhibitor tolerance and those that have acquired resistance. We demonstrate that once a BRAF inhibitor drug tolerant state develops or melanoma cells acquire resistance, these cells are sensitive to palbociclib but the synergistic response with BRAF or MEK inhibition observed in treatment naïve cells is lost. *In vivo* upfront treatment with palbociclib and BRAF inhibition in BRAF mutant melanoma leads to a synergistic response overcoming the early BRAF inhibitor drug tolerant state and leading to sustained tumor regression.

MATERIALS AND METHODS

Cell Lines

Human melanoma cell lines A375 and HT144 were obtained from ATCC and SK-Mel28 from NCI, Frederick. Individuality of melanoma cell lines were routinely confirmed by a PCR based short tandem repeat (STR) analysis using 6 STR loci. Drug tolerant cells were generated by incubating the cells with 500nM of PLX4720 for 3 weeks and acquired resistant cells by incubating cells with 1μM PLX4720 for at least 3 months. Stable A375 cell lines expressing GIPZ human lentiviral vectors (Dhamacon) containing shRNA hairpins for MITF (V2LHS_259964) or non-silencing control (RHS4346) were generated by lentiviral transduction and selected by flow cytometry.

Therapeutics

Palbociclib (6-acetyl-8-cyclopentyl-5-methyl-2-((5-(piperazin-1-yl)pyridin-2-yl) amino) pyrido [2,3-d] pyrimidin-7 (8H) -one) a specific CDK4 and CDK6 inhibitor [27] was obtained from Pfizer Oncology. PLX4720 was purchased from Euroasian

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Chemicals (India). Dabrafenib, cobimetinib and trametinib were purchased from Selleckchem.

Proliferation and dose response assays

For proliferation assays cells were plated at low density in the presence of drug and drug refreshed every 6-7 days. Cell number was assessed daily via a live cell imaging system (Incucyte, Essen Instruments). To assess the GI50 of each drug 6 day dose response assays were performed as previously described [19]. PLX4720 drug tolerance in shMITF and shControl cell lines was assessed by 96h dose response assays and proliferation assays in the presence of 1μM PLX4720.

Clonogenic Assay

Cells were seeded in 6-well plates at a density of 100 (A375) or 3000 (HT144) cells per well allowed to plate down for 16 hours and then treated for three weeks with single agents or in combination. Medium ± drug was changed weekly. Where drug was removed, cells were washed with PBS twice and then medium added. Colonies were fixed with methanol, stained with 0.1% crystal violet solution and then counted. Colonies were visualized under a dissecting microscope and more than 50 cells was considered to be a colony. To assess drug synergy cells were seeded in 6-well plates and treated with a fixed combination ratio of the two drugs based on the GI50 for each individual drug. The highest and lowest combination ratio was at most 1.5 times and 1/4th the GI50, respectively. A mutually nonexclusive combination index (CI) was determined using CalcuSyn (Biosoft) where: CI<0.9 synergy; CI>1.1 antagonism; CI=0.9-1.1 additive [28].

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ß-Galactosidase and whole cell staining

Cells were grown on coverslips placed in 6-well plates for 24 hours then treated with palbociclib (1μM) and PLX4720 (1μM) as single agent or in combination for 6 days. For whole cell staining cells were incubated for 30 minutes with 5μM CellTracker Green CMFDA (5-chloromethylfluorescein diacetate) dye (ThermoFisher Scientific, C7025). Cells were then fixed with 4% paraformaldehyde for 10 minutes and stained with DAPI (1μg/mL DAPI, 50mM Tris pH7.5, 0.2% Triton X) for 20 minutes. Cells were imaged at 20x magnification using an $EVOS_{fl}$ microscope and cell size analysis performed using the Multi Wavelength Cell Scoring application in Metamorph (version 7.10.1 Molecular Devices). Cells were stained for ß-Galactosidase as described previously [29] and Definiens Tissue Studio®, version 3.6 (Definiens AG, München, Germany) was used to quantitate the images.

Immunoblotting

Western blotting was performed as described previously [29]. Primary antibodies include: P-ERK T202/Y204 (CS9101), total ERK (CS9102), P-pRb Ser780 (CS9307), P-pRb Ser807/811 (CS9308), total pRb (BD Pharmingen 554136), FOXM1 (CS5436), Cyclin D1 (C-20: SC-718), P-c-JUN (CS9261), c-JUN (CS9165) and MITF (MAB3747). Anti-mouse and anti-rabbit horseradish peroxidase-conjugated secondary antibodies were used (BioRad #170-6516, #170-6515). Protein was quantitated using Image Lab v 5.2.1 (Bio-Rad) to determine band intensity which was nomalised to vehicle band intensity.

In vivo studies

All animal studies were performed with approval from the Peter MacCallum Animal Experimentation Ethics Committee. Female Balb/c:Foxn1^{nu} mice (Nude: Animal Resources Centre, Western Australia) or NOD-*scid* IL-2Rγ null mice (NSG; bred in-

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house) were injected subcutaneously in the right flank with 3 x 10^6 A375 or HT144 cells in 50% Matrigel. When tumors reached ~100 mm^3, mice were randomized into treatment groups and dosed daily with vehicle, PLX4720 (40 mg/kg), palbociclib isethionate (120 mg/kg) or PLX4720 and palbociclib as appropriate. For western analysis protein extracts prepared from tumors harvested 4 hour post dosing were subjected to SDS-PAGE as described above. For the assessment of proliferating tumor cells bromodeoxyuridine (BrdU:100mg/kg) was injected intraperitoneally 1 hour prior to harvest.

Immunohistochemistry and β-Galactosidase staining of tumor samples

Immunohistochemistry (IHC) for BrdU staining and quantitation was performed as described previously [30]. Phosphorylated-pRb (P-pRb) was assessed by using rabbit anti-human P-pRb (Ser 807/811) primary antibody. Three representative low magnification images from each tumor were analyzed using Metamorph Image Analysis software and the number of positively stained cells was quantitated. ß-galactosidase staining was performed as previously described [30] and imaged by the *ScanScope® XT* system (*Aperio Technologies*, Inc., Vista, *California*) for quantitation by Definiens Tissue Studio®, version 3.6 (Definiens AG, München, Germany). Images were split into RGB, inverted, and the red and blue channels recombined. The red channel was used to identify nuclei by intensity and morphology, and the cytoplasm was determined by growing the margins of each nucleus. Each cell was considered ß-galactosidase positive, if either the nuclear or the cytoplasmic ß-galactosidase staining (blue channel) exceeded a defined threshold consistent for all samples. The ß-galactosidase positivity for each sample is expressed as the number of ß-galactosidase positive nuclei divided by the total number of nuclei.

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Cell Cycle analysis

A375 cells were treated for 24h or 7 days with either single agent or the combination of 1µM palbociclib and 1µM PLX4270, and for HT144 cells 250nM palbociclib and 500nM PLX4270 was used. BrdU/PI cell cycle analysis was performed as previously described [19]

Cell Death Analysis

A375 and HT144 cells were treated with palbociclib and PLX4720 for 7 days as above. Supernatant and trypsinized cells were centrifuged and resuspended in 200µL of 10µg/mL propidium iodide (PI). Analysis was performed on a BD FACSCantoII flow cytometer, with 10,000 single DNA events collected, and the proportion of dead cells was determined by PI positivity in the FL3 channel.

Gene Expression Analysis

Total RNA was isolated using the RNeasy Mini kit (Qiagen), and cDNA was synthesized using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative real-time PCR was performed using Fast SYBR Green Master Mix (Applied Biosystems) on a StepOnePlus Real-Time PCR System (Applied Biosystems) according to the manufacturer's instructions. mRNA amounts were normalized to amplification of the reference NONO RNA and are plotted as relative expression values. The primer sequences used were:

cJUN-FW, GCATGAGGAAACGCATCGCTGCCTCCAAGT

cJUN-RV, GCGACCAAGTCCTTCCCACTCGTGCACACT

MITF-FW, TGCCCAGGCATGAACACAC

MITF-RV: TGGGAAAAATACACGCTGTGAG

NONO-FW: CATCAAGGAGGCTCGTGAGAAG

NONO-RV: TGGTTGTGCAGCTCTTCCATCC

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Statistical Analysis

Student's t-test or one-way analysis of variance followed by Tukey's Multiple Comparison Test or Fisher's LSD test was performed using GraphPad PRISM. Kaplan–Meier survival plots were compared using a log-rank (Mantel–Cox) test. Differences of $P<0.05$ were considered significant. All data are expressed as mean $\pm$ SEM and at least 3 biological replicates were performed.

RESULTS

In vitro palbociclib plus a BRAF or MEK inhibitor is more effective at inhibiting melanoma cell proliferation compared to the combination of BRAF plus MEK inhibitor

To assess how palbociclib in combination with PLX4720 compared with current standard of care for BRAF mutant melanoma, which is dual BRAF and MEK inhibition we determined melanoma cell proliferation with these combinations. $BRAF^{V600E}$ mutant A375 cell line was plated at low density and treated with vehicle, PLX4720 (1µM), palbociclib (1µM), cobimetinib (10nM) or their combination. From dose response assays these drug concentrations inhibited cell proliferation by 75-90% after 6 days of treatment (Supplementary Table 1). Cell confluency was monitored daily and every 6-7 days medium with or without drugs was replenished. In A375 cells continuous dual dosing with palbociclib in combination with either PLX4720 or cobimetinib induced sustained inhibition of cell proliferation (Figure 1A). This was also seen in two other $BRAF^{V600E}$ mutant cell lines HT144 and SK-Mel28 (Supplementary Figure 1A). In contrast, cells treated with the combination of PLX4720 and cobimetinib continued to proliferate albeit at a slower rate than untreated A375 cells. Similar data were obtained with dabrafenib and trametinib and

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furthermore, the triplet combination of dabrafenib, trametinib and palbociclib was as effective as the dual combinations of dabrafenib or trametinib plus palbociclib (Figure 1B).

To determine the proportion of melanoma cells that survive treatment and retain their ability to proliferate we performed clonogenic assays using both A375 and HT144 cells. After three weeks, untreated control plates were completely confluent so that individual colonies could not be counted. In contrast, continuous single agent PLX4720 and palbociclib both significantly decreased the number of colonies and there were no colonies with the combination therapy. Following the removal of combination therapy a few colonies appeared indicating the majority of cells had lost their proliferative potential (Figure 1C and Supplementary Figure 1B). Studies were then extended to compare the combination of dabrafenib and trametinib with palbociclib and dabrafenib or palbociclib and trametinib or the triplet combination (Figure 1D). After 2 weeks of treatment there were colonies with the single agent treatments but no colonies with any of the combinational treatments. Incubation of the plates for a further two weeks in drug free medium resulted in the appearance of colonies with all treatments; however, the number of colonies in the dabrafenib plus trametinib treated cells was far greater than those cells that had received the palbociclib combinations. In agreement with the proliferation assay the triplet combination was just as effective as the dual combinations with palbociclib (Figure 1D). The clonogenic and proliferation assays clearly demonstrate that the palbociclib plus BRAF or MEK inhibitor combination is as effective as the triplet combination and these combinations are superior to the BRAF and MEK inhibitor combination at inhibiting cell proliferation and survival.

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To assess if the combination was synergistic a mutually nonexclusive combination index (CI) was determined using CalcuSyn where a CI of less than 0.9 demonstrates synergy [28]. In HT144 clonogenic assays the CI value at the effective dose required to achieve 50% growth inhibition (ED50) was 0.65, the CI value at the ED75 was 0.38 and the CI value at the ED90 was 0.24 (Supplementary Figure 2A). In two independent A375 clonogenic assays the CI value at the ED50 was 0.86 and 0.93, at the ED75 was 0.79 and 0.79 and at the ED90 was 0.74 and 0.71 (Supplementary Figure 2B).

Taken together, these data show clear synergy between BRAF and CDK4 inhibition and furthermore, demonstrate that although the combination of a BRAF and MEK inhibitor is initially effective at inhibiting cell proliferation, cells quickly adapt and drug resistance develops. In contrast, drug resistance is avoided when these drugs are combined with palbociclib.

Combination of PLX4720 and palbociclib induces cell death and senescence

The combination of PLX4720 and palbociclib totally inhibited proliferation after 7-14 days (Figure 1). Cell cycle analysis demonstrated that the combination of PLX4720 and palbociclib induced a G1 arrest in both A375 and HT144 cells within 24h of treatment that was sustained at 7d (Figure 2A). Assessment of cell death by propidium iodide (PI) uptake and fluorescence-activated cell sorting (FACS) analysis after 7 days of treatment shows significant cell death ($p<0.05$) with the combination therapy in both A375 ($32 \pm 4\%$) and in HT144 ($28 \pm 5\%$) cells but not with single agent treatment (Figure 2B), and not within the first 24h of treatment (Supplementary Figure 3A). Cells that survived the combination became enlarged and showed increased SA-ß-galactosidase staining (Figure 2C) both of which are characteristic of senescence [31]. Although palbociclib alone increased SA-ß-galactosidase staining it did

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not induce the large flattened phenotype. Taken together these data indicate that in response to a combination of palbociclib and PLX4720, a significant proportion of cells die but the predominant effect is inhibition of proliferation with the majority of these cells demonstrating features consistent with senescence.

The retinoblastoma transcriptional repressor (pRb) and the transcription factor FOXM1 are direct substrates of CDK4 involved in cell cycle regulation and senescence [32-35]. The MAPK/ERK pathway regulates the G1/S cell cycle progression through several mechanisms including ERK induced transcription of CyclinD1 [36], which in turn leads to activation of CDK4; ERK can also phosphorylate and regulate FOXM1 activity [37]. To investigate the impact that combined targeting of CDK4 and mutant BRAF had on cell signaling we examined FOXM1 and CyclinD1 expression and phosphorylation of both pRb and ERK by Western blot (Figure 2D). At 6 hrs the response to the combination was similar to if not better than that induced with the single agents. For example, palbociclib alone or in combination with PLX4720 induced a similar decrease in phosphorylation of pRb and FOXM1 expression in A375 cells but the decrease was more pronounced in HT144 cells with the combination. In both cell lines at 7 days the combination of palbociclib and PLX4720 was more potent at repressing FOXM1 expression and phosphorylation of pRb compared to the single agent treatment. Loss of phosphorylation of pRb was indicated by the loss of the upper hyper-phosphorylated total pRb band and a decrease at the CDK4 phosphorylation sites Ser780 and Ser807/811. The decrease in FOXM1 and phosphorylated pRb are consistent with the combination inducing cell cycle arrest and senescence. In contrast, PLX4720 and palbociclib had opposing effects on CyclinD1 expression reflecting the inhibition of CyclinD1 transcription by PLX4720 [36] and G1 cell cycle arrest induced by palbociclib [38]. Consistent with the cell proliferation and cell survival data shown in Figure 1 the triple combination of dabrafenib, trametinib

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and palbociclib and double combination of dabrafenib and palbociclib similarly decreased phosphorylation of pRb at Ser807/811 (Supplementary Figure 3B). The combination of trametinib plus palbociclib was less effective at decreasing phosphorylation of pRb, which is likely due to the suboptimal concentration of trametinib that was used as indicated by only partial inhibition of P-ERK.

Palbociclib in combination with PLX4720 overcomes the reversible drug tolerance seen with single agent BRAF inhibition

Resistance to BRAF inhibitors is associated with an initial reversible non-mutational drug tolerant state that leads to the emergence of mutated permanently resistant clones [13-16]. We have previously demonstrated that treatment of melanoma cell lines for 3 weeks with PLX4720 leads to this drug tolerant state [13]. To assess if the combination of palbociclib plus PLX4720 overcame the development of PLX4720 induced drug tolerance we treated cells for 3 weeks with PLX4720 plus palbociclib and then subsequently treated cells for two weeks with PLX4720 (1µM), palbociclib (1µM), their combination or no drug. Consistent with the clonogenic assays (Figure 1C and 1D) after drug removal a small population of cells began to proliferate (Figure 3A). In contrast, single agent PLX4720 or palbociclib treatment maintained the inhibition of proliferation demonstrating that the remaining cells that survived combination therapy were still sensitive to single agent treatment and had not developed drug tolerance.

The drug tolerant state is driven by increased expression of MITF and c-JUN and is characterized by a mesenchymal phenotype [13-15]. Our data demonstrate that the combination of PLX4720 and palbociclib induces either cell death or senescence in the majority of cells, clearly indicating that the combination overcomes the PLX4720 induced mesenchymal phenotype. To determine if this is via palbociclib regulation of either MITF or c-JUN we assessed both gene expression and protein levels following

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single agent and combination therapy early on treatment (48hrs) and after 3 weeks the time at which drug tolerance has developed. As previously demonstrated PLX4720 induced the expression of both c-JUN and MITF at the mRNA and protein level (Figure 3B and 3C). Palbociclib did not alter PLX4720 induced MITF or c-JUN gene expression (Figure 3B), and at the protein level palbociclib did not significantly alter the PLX4720 induced increase in either total c-JUN or P-c-JUN. In contrast, after 3 weeks of treatment palbociclib significantly decreased both basal and PLX4720 induced MITF protein (Figure 3C). MITF is regulated transcriptionally, post-transcriptionally and post-translationally [39] thus the disconnect between palbociclib regulation of MITF gene expression and protein demonstrates that palbociclib regulates MITF at the translational and/or post-translational level.

To assess the impact of depletion of MITF on PLX4720 induced drug tolerance, MITF was knocked down using a short hairpin RNA (shRNA: Supplementary Figure 4). Knockdown of MITF did not alter sensitivity to PLX4720 (Supplementary Figure 4C). After 3 weeks of PLX4720 treatment, cells containing a non-silencing control shRNA construct developed drug tolerance indicated by an increase in MITF expression (Supplementary Figure 4B), and both an increase in GI50 and a decrease in the efficacy to inhibit cell proliferation (Figure 3D). In contrast, cells that had MITF knockdown did not develop drug tolerance showing a similar GI50 and proliferation rate compared to MITF knockdown cells that had not been pretreated with PLX4720 for 3 weeks (Figure 3D). Taken together these data demonstrate that drug tolerance does not develop when PLX4720 is combined with palbociclib, because the combination induces senescence, overcoming the mesenchymal drug tolerant phenotype and the increase in MITF.

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BRAF inhibitor tolerant and acquired resistant cells are sensitive to palbociclib but have lost efficacy to the combination

Preclinical studies on human melanoma cell lines have demonstrated that the majority of acquired BRAF inhibitor resistant cells and those that are inherently resistant respond to CDK4 inhibition [25, 26], however whether melanoma cells that have developed the early reversible drug tolerant state are sensitive to palbociclib is unknown. To assess if the combination of palbociclib alone or in combination with BRAF or MEK inhibition is effective during the early drug tolerant state and in acquired resistant cells we generated BRAF inhibitor tolerant and acquired resistant A375 and HT144 cells and compared their response to single agent and combinational therapy. Proliferation assays demonstrated that both PLX4720 tolerant and acquired resistant cells respond to palbociclib (Figure 4). In PLX4720 tolerant A375 and HT144 cells the palbociclib response was equivalent to the combination of palbociclib with either PLX4720 or cobimetinib indicating that synergy with both the BRAF and MEK inhibitor was lost (Figure 4B, 4E, 4H and 4K). When A375 drug tolerant cells were treated with a combination of PLX4720, cobimetinib and palbociclib the response was greater than the single agent palbociclib but it did not induce a complete inhibition of proliferation (Figure 4B) as seen in the sensitive A375 cells (Figure 4A). In the A375 and HT144 acquired resistant cells the combination of PLX4720 plus palbociclib also failed to induce total inhibition of cell proliferation and thus senescence (Figure 4C and 4I). In contrast cobimetinib plus palbociclib and the triplet of PLX4720, cobimetinib and palbociclib were equally effective at totally inhibiting cell proliferation in A375 PLX4720 resistant cells (Figure 4C and 4F) but not in the HT144 PLX4720 resistant cells (Figure 4L); this difference is likely due to different mechanisms of acquired resistance to PLX4720 in the two different cell lines. Our data demonstrate that once melanoma cells become BRAF inhibitor tolerant they remain sensitive to palbociclib but the synergistic response when combined with

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either PLX4720 or cobimetinib is lost as is the induction of senescence. Similarly in BRAF inhibitor acquired resistant cells palbociclib does not synergize with BRAF inhibitor but may with a MEK inhibitor depending on the mechanism of resistance.

In vivo continuous palbociclib and PLX4720 induces tumor regression

The *in vitro* analysis revealed that the most effective treatment of BRAF mutant melanoma cells was upfront dual treatment with PLX4720 and palbociclib; therefore we investigated the antitumor efficacy of this combination in both the A375 and HT144 tumor xenograft models (Figure 5A and 5B, Supplementary Figure 5). Tumor bearing mice were subjected to a continuous dosing schedule. All treatments were well tolerated and all led to a significant increase in median survival ($p < 0.0001$). The drug combination was significantly more efficacious than the single agents ($p<0.01$) resulting in rapid and sustained tumor regression in both xenograft models. In the HT144 model no tumors gained resistance whereas only one tumor developed resistance after seven weeks of treatment in the A375 model (Figure 5 and Supplementary Figure 5A).

To assess whether PLX4720 and palbociclib effectively inhibited their targets *in vivo*, tumoral phosphorylated-ERK (P-ERK) and -pRb (P-pRb) were assessed in tumor samples after three and seven days of treatment (Figure 5C). After three days of treatment P-ERK was decreased by an equivalent amount by both single agent PLX4720 and the combination, but there was no change with palbociclib. By seven days of PLX4720 treatment, P-ERK was higher compared to three days of PLX4720 treatment, consistent with the emergence of resistance to PLX4720. In contrast, inhibition of P-ERK was sustained in the combination group at seven days, demonstrating that the drug combination either overcomes or delays the early drug tolerance to PLX4720.

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To assess the proportion of cells that were still proliferating, melanoma cells positive for P-pRb (Ser807/Ser811) were assessed by immunohistochemistry. Three days of continuous treatment resulted in a significant decrease in P-pRb (Ser807/Ser811) in all treatment groups. Palbociclib alone and in combination with PLX4720 equivalently and robustly inhibited P-pRb by 95% and 99%, respectively, while PLX4720 was significantly less effective, decreasing P-pRb by 45% (Figure 5D). Following seven days of treatment, palbociclib and the combination maintained significant inhibition of P-pRb compared to vehicle control. At seven days, however, the palbociclib group exhibited a significant increase in P-pRb compared to the combination group, consistent with the development of palbociclib resistance. P-pRb levels in the PLX4720 group also increased between three and seven days, reaching control levels, which is consistent with the development of PLX4720 resistance. BrdU incorporation was performed to further assess tumor cell proliferation after seven days (Figure 5E). In agreement with the P-pRb results, palbociclib and the combination, but not PLX4720, effectively inhibited cell proliferation. Together, these results demonstrate that resistance to both PLX4720 and palbociclib develops quickly in this model of melanoma and that the combination therapy overcomes resistance to the single agent therapies.

To investigate if a senescence-like response also occurred *in vivo* in response to PLX4720 and palbociclib we examined senescence associated SA-ß-galactosidase staining in tumors after seven days of treatment. Consistent with the *in vitro* data, palbociclib and the combination of palbociclib and PLX4720 significantly increased SA-ß-galactosidase staining whereas PLX4720 did not (Figure 5F and Supplementary Figure 6). The *in vitro* data indicated that the combination therapy induces senescence in the majority of melanoma cells, however a subset of cells still had the

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potential to proliferate upon drug removal. To determine if this occurs *in vivo,* treatment of the mice responding to combination therapy was ceased after 145 days and tumor growth monitored. Six out of seven mice showed tumor growth that was detectable 10- 20 days after drug removal (Figure 5G). Combination therapy was reinitiated after four weeks off drug and all tumors responded, with one having a complete response. These data indicated that similar to the *in vitro* data, a subset of tumor cells survive the combination of palbociclib and PLX4720 treatment and are capable of resuming proliferation upon drug withdrawal. Importantly, when these tumors are rechallenged with the combination of palbociclib and PLX4720 they continue to have a robust response decreasing tumor volume, clearly demonstrating that under these conditions they do not become resistant to therapy.

DISCUSSION

Acquired resistance to targeted therapy is a major problem and identifying combinations and/or scheduling strategies that will delay or overcome the emergence of resistance is of great importance to the success of these agents in the clinic. Targeting BRAF and MEK has shown clear clinical efficacy in BRAF-mutant metastatic melanoma patients [7, 8, 40, 41], however the majority of patients eventually progress on either single agent or the combination treatment and in most cases, this is due to reactivation of the MAPK/ERK pathway [42]. These studies demonstrate that single or dual targeting different points along the MAPK/ERK pathway will at best delay the emergence of resistance. More importantly, the mutational heterogeneity that develops upon acquired resistance to these inhibitors further complicates subsequent effective treatment strategies. Preclinical and clinical studies indicate that progression of melanoma to a fully drug-resistant state is preceded by a reversible drug-tolerant phase that may contribute to the establishment of acquired resistance [16]. The drug tolerant phase is mediated by increased MITF and c-JUN signalling [11-16, 43],

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which is in contrast to the drug resistant cells that tend to express low levels of MITF and are marked by high AXL/WNT5A expression [44-47]. Thus BRAF inhibitor combination therapies that prevent this early drug tolerant phase may provide a unified upfront treatment strategy that will improve efficacy and duration of the therapeutic response to MAPK/ERK pathway inhibitors.

Our data show that simultaneously targeting mutant BRAF and CDK4/6 in BRAF mutant melanoma leads to rapid and sustained inhibition of cell proliferation, the induction of senescence and tumor regression. Importantly, this combination prevents the development of BRAF inhibitor resistance likely via reversing the BRAF inhibitor induction of MITF a major driver of the drug tolerant state [15]. Overcoming BRAF inhibitor tolerance allows the drug combination to divert the cells down a senescence pathway via regulation of factors including MITF, FOXM1 and pRb and in doing so stops the development of acquired resistance. The potent efficacy of this combination however, is lost on cells that have previously developed BRAF inhibitor early drug tolerance or acquired drug resistance. The addition of a MEK inhibitor to this combination fails to be effective during the drug tolerant phase but upon BRAF inhibitor acquired resistance this may be an effective treatment option for some patients. Thus a key implication of our study is that the addition of CDK4/6 inhibitors with BRAF inhibitors prevents the development of resistance. A second key implication is that melanoma patients who have developed drug tolerance or acquired resistance to BRAF inhibition are less likely to have robust or durable responses to subsequent treatment with CDK4/6 inhibitors in combination with BRAF and/or MEK inhibitors.

The robust response to the combination of PLX4720 and palbociclib was complex; in most cells it induced either cell death or senescence. However, the observation that

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tumors could regrow after withdrawal of the combination also suggests that a few cells became dormant, as opposed to senescent, maintaining their proliferative potential. This is similar to that observed in NRAS mutant melanoma when treated with the combination of palbociclib and a MEK inhibitor [48]. This concept was supported by both *in vitro* and *in vivo* data. *In vitro* when the combination treatment was removed after three weeks of treatment, a few surviving cells began to proliferate and *in vivo* although tumors were barely detectable once treatment was stopped some but not all tumors progressed. Importantly, and similar to the initial tumor response, when therapy was reinitiated there was a rapid and robust inhibition of tumor growth with at least one tumor having a complete response. This data indicated that after 5 months of continuous treatment resistance to the combination of PLX4720 and palbociclib had not occurred. More importantly it demonstrates that for those tumors that have the potential to progress, introducing a short break in treatment and thus allowing the dormant cells to reenter the cell cycle can induce a complete response upon reinitiation of the combination therapy. Our data suggest that one mechanism by which the BRAF plus CDK4 inhibitor combination overcomes acquired resistance is likely through inhibiting the MITF induced drug tolerant phase associated with BRAF inhibitor monotherapy. These observations raise two interesting possibilities for the clinical use of combined BRAF and CDK4 inhibition. First, that if patients suffered treatment-related toxicities they would be able to have a break in therapy without the risk of developing resistance and secondly, some patients may even benefit from a relatively short interval in treatment.

In clinical trials in melanoma patients assessing CDK4/6 inhibitors in combination with MAPK/ERK pathway inhibitors (NCT01777776, NCT02159066, NCT01820364, NCT02065063, NCT01543698, NCT01781572), these agents are being given concurrently and in some trials a seven day break from the CDK4/6

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inhibitor is being introduced to overcome the neutropenia associated with long term treatment [49]. To date the preliminary data from these trials is promising, of nine BRAF mutant melanoma patients treated with LEE011 (CDK4/6 inhibitor) and LGX818 (BRAF inhibitor) two patients had partial responses and six had stable disease [50]. In NRAS mutant melanoma patients treated with LEE011 in combination with binimetinib (MEK inhibitor), antitumor activity was observed in 12 out of 14 (86%) cases, six having partial responses and six with stable disease [51]. Given our data that the dual inhibition of BRAF and CDK4 will provide more durability of response but only in treatment naïve patients, it will be of major interest to determine the duration of the response in these trials in both treat naïve patients and those who have failed current standard of care.

A critical remaining question is whether combining CDK4 and BRAF inhibitors would be superior to the current standard of care, which is a combination of BRAF and MEK inhibition. Moreover, whether adding a CDK4 inhibitor to this dual combination would be more effective than the BRAF plus CDK4 inhibitor combination is also of importance. The rationale for combining a MEK inhibitor with a BRAF inhibitor is to prolong the response by delaying the onset of resistance associated with reactivation of the MAPK/ERK pathway. In addition, this combination counteracts the proliferative skin lesions that occur due to paradoxical activation of the BRAF/ERK pathway in non-BRAF mutant cells [52, 53]. Our proliferation data clearly show that CDK4 and BRAF inhibitor combination was superior to the BRAF and MEK inhibitor overcoming the development of resistance. The triplet combination of BRAF, MEK and CDK4 inhibition gave a similar response to the dual BRAF and CDK4 inhibitor response suggesting that in terms of inhibition of tumor growth the dual combination and triplet combination would be equally effective. How CDK4 inhibition impacts on BRAF inhibitor induced paradoxical

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activation of the MAPK/ERK pathway is unknown but it may negate the proliferative skin lesions associated with pathway activation given that increased CyclinD1 expression and thus CDK4 activation is a major mechanism by which this pathway increases cell proliferation.

In summary, our study demonstrates a potent and sustained therapeutic benefit of upfront dual targeting CDK4/6 and BRAF in BRAF mutant melanoma. We demonstrate that the combination is far superior to single agent BRAF and dual BRAF and MEK inhibitor therapy, because it prevents resistance associated with these treatments. Moreover, efficacy of this combination is compromised in both BRAF inhibitor tolerant and resistant cells. These findings provide a strong rational for upfront treatment of metastatic melanoma patients with a combination of CDK4/6 and BRAF inhibitors and indicate that in patients whose tumors do initially respond, the response will be sustained without the development of resistance. However, melanoma patients whose tumors have become resistant to BRAF inhibitors are less likely to have favourable responses to subsequent treatment with this combination.

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Figure Legends

Figure 1: A combination of PLX4720 and palbociclib induces sustained inhibition of A375 melanoma cell proliferation.

A: A375 cell proliferation (confluency) was measured every 12-24hrs using an IncuCyte, both media and drugs were replaced every 6-7 days. **A.** Treatment was continuous with single agents PLX4720 (1μM), palbociclib (Palbo:1μM) and cobimetinib (Cobi:10nM) or their combination. **B.** Treatment was continuous with single agents dabrafenib (Dab:100nM), palbociclib (Palbo:1μM) and trametinib (Tram:10nM) or their combination. **C.** Cells treated continuously for three weeks with vehicle (Con), palbociclib (1μM), PLX4720 (1μM) or their combination were assessed for colony formation. One set of plates was stained at the time of drug removal (three weeks treatment: left panel) and another (right panel) following two weeks in drug free medium. The graph represents data after three weeks of treatment. All groups were significantly different from control and each other. n=3 $\pm$ SEM, One-way ANOVA with post-hoc Tukey, * $p<0.05$. **D:** For colony formation assays A375 cells were treated continuously for two weeks with vehicle (control), palbociclib (palbo:1μM), dabrafenib (Dab:100nM) trametinib (Tram:10nM) or their combination. One set of plates was stained at the time of drug removal (two weeks treatment: left panel) and another (right panel) following two weeks in drug free medium.

Figure 2: A combination of PLX4720 and palbociclib induces both cell death and senescence.

A375 cells were treated with palbociclib (Palbo:1μM), PLX4270 (PLX:1μM) or their combination (Combo) . HT144 cells were treated with 250nM palbociclib and 500nM PLX4720 or their combination. **A:** BrdU/PI cell cycle analysis of viable A375 and

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HT144 cells. Data are the mean ± SEM, n=3: White stars indicate a significant increase in G0/G1 phase together with a significant decrease in S phase compared to vehicle, * p< 0.05 . **B:** Cell death assessed by propidium iodide exclusion after 7days of treatment. Data are mean ± SEM n=3: * p< 0.05 compared to vehicle control. **C:** Cell morphology and senescence was assessed by staining cells with either celltracker green (whole cell stain) or ß-galactosidase (green) with overlaid Dapi (nuclear: grey) staining. Data are mean ± SEM n=3: * p< 0.05. Size bars are 200μm. **D:** Representative immunoblots of pRb, ERK1/2, Cyclin D1 and FOXM1 in cells treated with PLX4720 (1μM), palbociclib (1μM) or their combination.

Figure 3: Combination of PLX4720 and palbociclib prevents the development of resistance

A: A375 cell confluency was measured every 12-24hrs using an IncuCyte. Media and drugs were replaced every 6-7 days. Cells were treated continuously with PLX4720 (1μM), palbociclib (1μM) or their combination. At 3 weeks combination therapy was removed and replaced with media only (drug free), single agent therapy or the combination and proliferation monitored for a further 2 weeks. **B:** C-JUN and MITF gene expression in A375 cells after 48hrs and 3 weeks of treatment with PLX4720 (1μM), palbociclib (1μM) or their combination. Data are the mean ± SEM, n=3: One-way ANOVA with post-hoc Tukey, * p< 0.05. **C:** Western blot of A375 cells after 48hrs and 3 weeks of treatment with PLX4720 (1μM), palbociclib (1μM) or their combination. Two biological replicates are shown for each time point. Quantitation of MITF band intensity following 3 weeks of treatment is shown relative to control. Data are mean ± SEM, n=3: One-way ANOVA with post-hoc Tukey, * p< 0.05. **D:** shCtrl and shMITF knockdown cells were treated continuously with 500nM PLX4720 (blue) or DMSO vehicle (black) for 3 weeks, then analyzed for sensitivity to PLX4720 by 96h dose response assays or cell confluency over time in 1μM PLX4720.

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Figure 4: Combination of palbociclib and MAPK pathway inhibitors is synergistic in treatment naïve cells, but not cells with BRAF inhibitor tolerance or acquired resistance

A375 and HT144 cell confluency was measured every 12-24hrs using an IncuCyte. Media and drugs were replaced every 6-7 days. Cells were treated continuously with PLX4720 (1μM), palbociclib (Palbo:1μM), cobimetinib (Cobi:10nM) or the indicated combinations. **A, D, G** and **J**: Proliferation assays in response to drug treatment in control cells that are naïve to drug treatment. **B, E, H** and **K**: Proliferation assays in response to drug treatment in cells that have been pretreated with PLX4720 for 2-3 weeks to induce PLX4720 drug tolerance. **C, F, I** and **L**: Proliferation assays in response to drug treatment in cells that have permanent acquired resistance to PLX4720. Mean $\pm$ SEM, n=4-5 technical replicates.

Figure 5: Combination of PLX4720 and palbociclib induces sustained inhibition of A375 and HT144 xenograft tumor growth.

A: A375 or **B:** HT144 tumor bearing mice were treated daily with vehicle (black), palbociclib (red), PLX4720 (blue) or their combination (green) and tumor volumes were measured every 3-4 days. Mean tumor volumes (±SEM) are shown and the grey shaded area indicates tumor regression. A Mantel-Cox log-rank test was used to compare Kaplan–Meier survival plots. For both A375 and HT144 tumor bearing mice, all treatments led to a significant improvement in overall survival compared to control treated animals. n= 8-10, p<0.0001. For biomarker studies (**C, D, E, F**) A375 tumors were harvested after 3 or 7 days of continuous daily treatment. **C:** Immunoblot of tumors. **D:** The percent of P-pRb positive cells as determined by immunohistochemistry in the tumor. **E:** The percent of BrdU positive cells in tumors. **F:** The percent of ß-galactosidase positive cells present in the tumor. Data in D, E and

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F were analyzed using ANOVA followed by a Fisher's LSD test. For all data * indicates groups are significantly different from control and lines show significant difference between treatment groups. ($p<0.05$). All data are expressed as mean ± SEM of 3 -8 biological replicates. **G:** Mice from the combination group shown in (**A**) were monitored for A375 tumor growth after cessation of drug treatment on day 145. Treatment was recommenced and stopped again as indicated by the vertical lines. Palbociclib (Palbo) and PLX4720 (PLX). Tumor volumes were measured every 3-4 days and each colored line represents an individual mouse.

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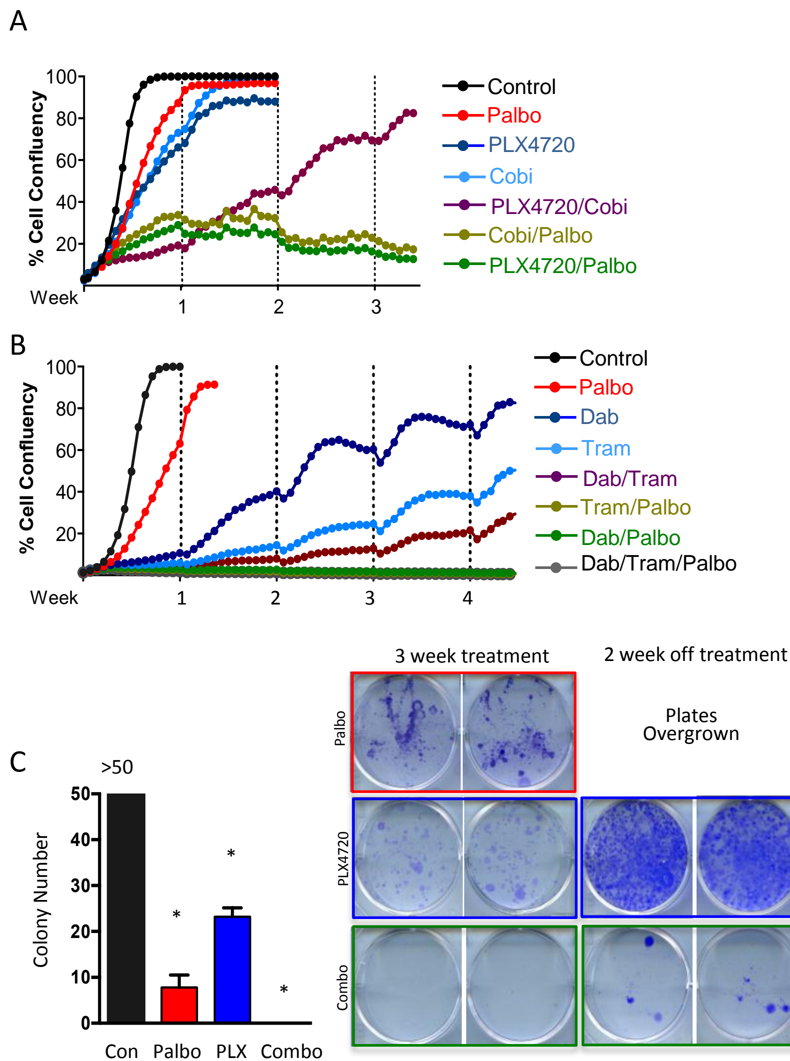

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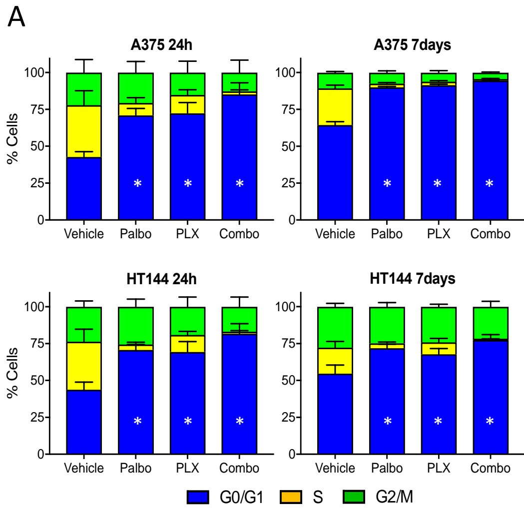

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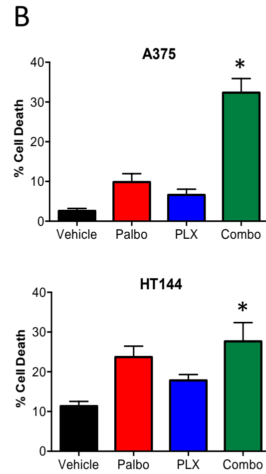

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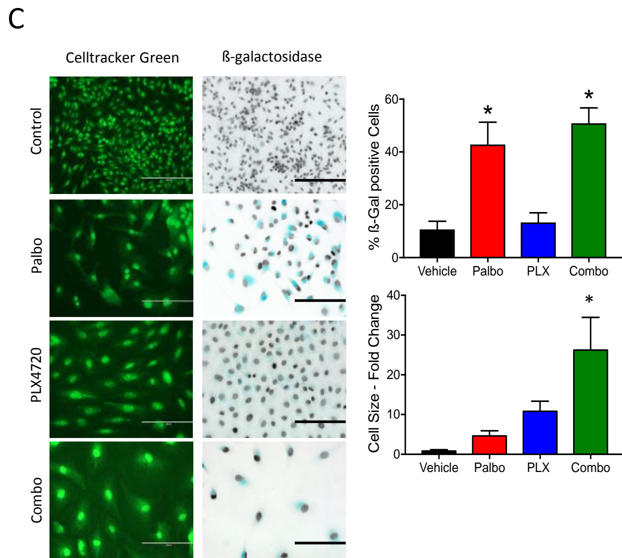

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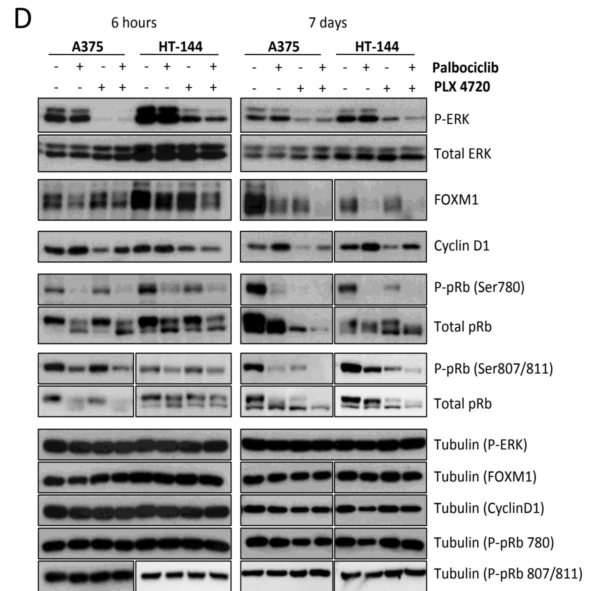

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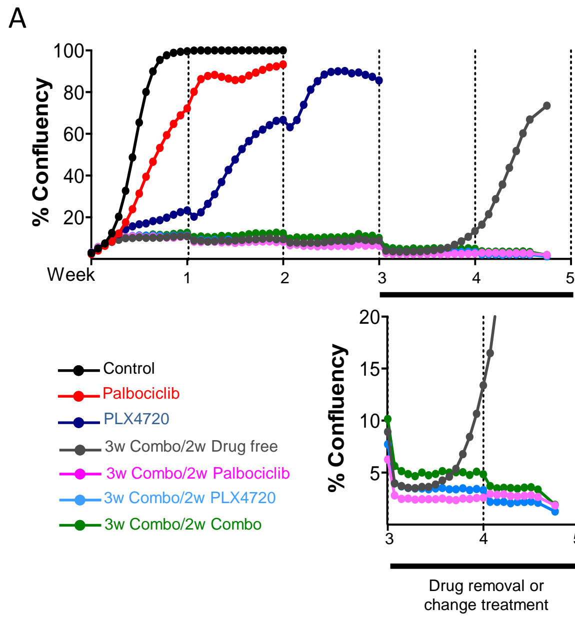

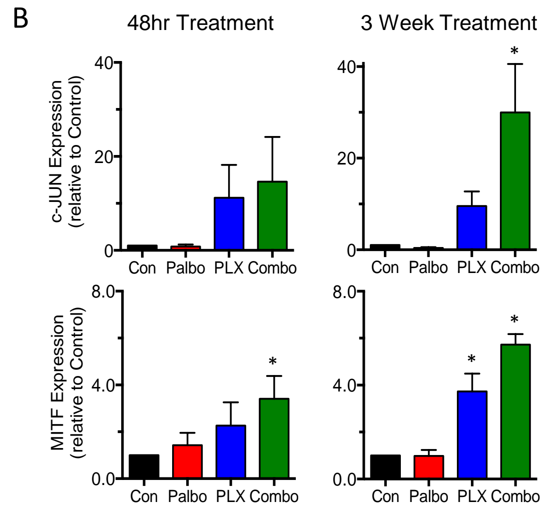

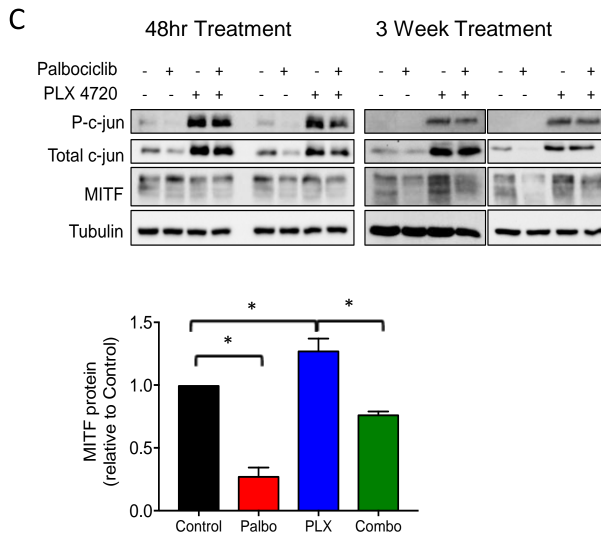

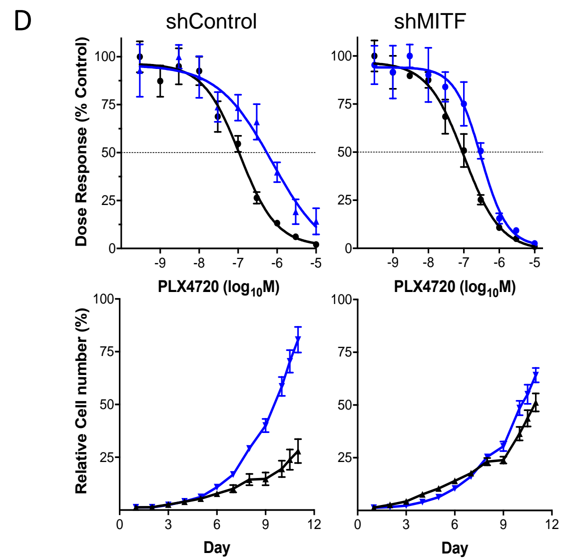

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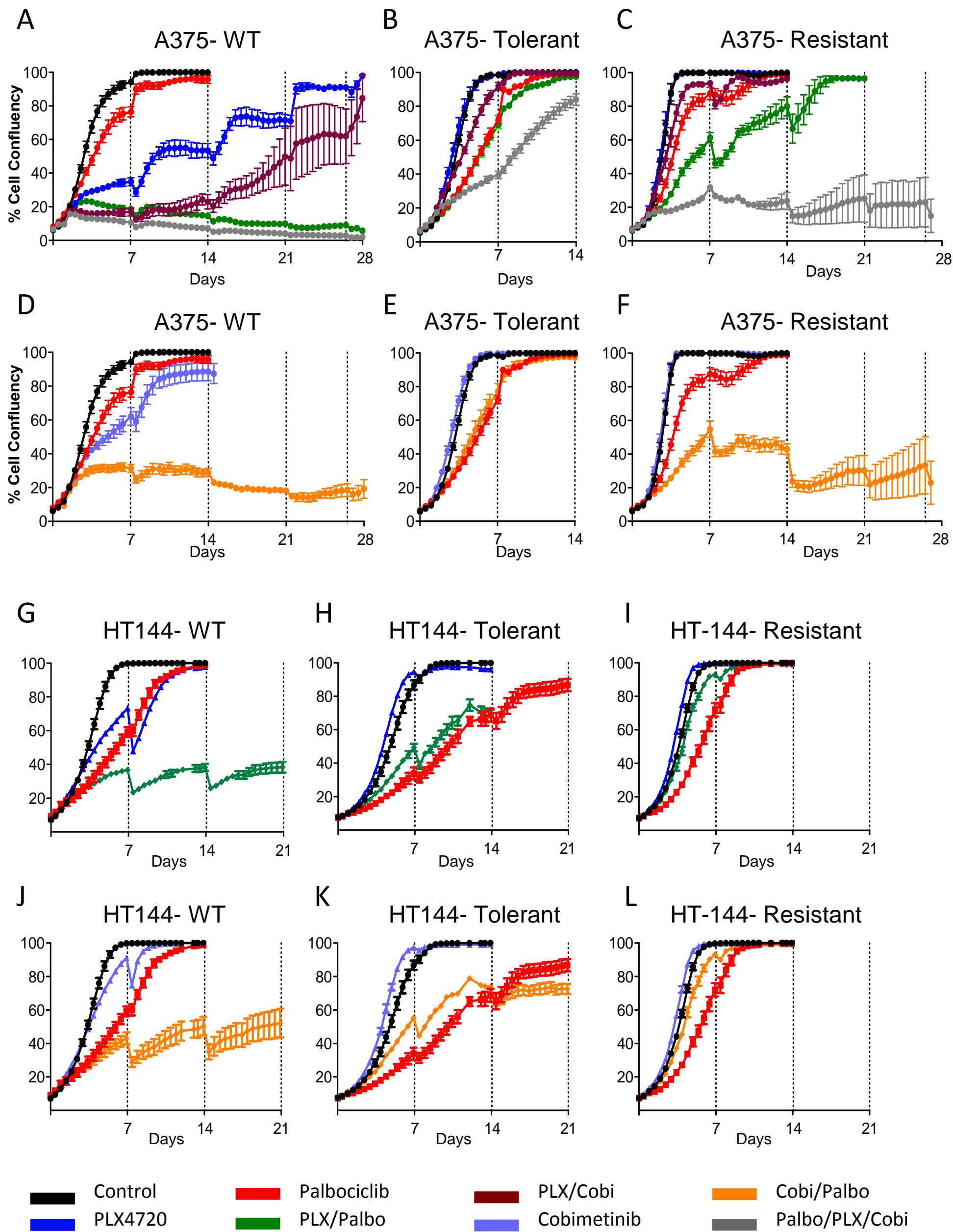

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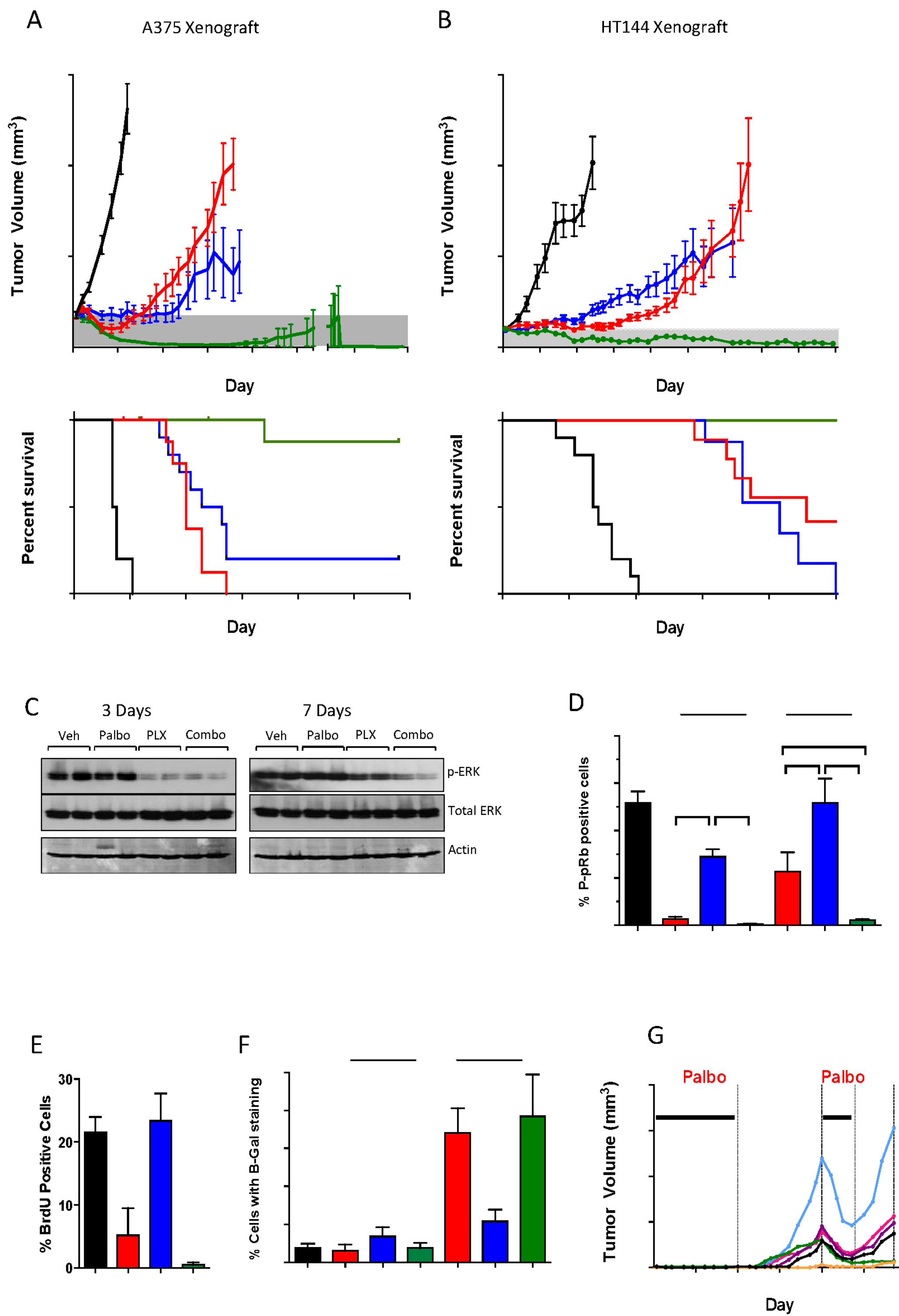

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