The CDK4/6 Inhibitor LY2835219 Overcomes Vemurafenib Resistance Resulting from MAPK Reactivation and Cyclin D1 Upregulation

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Abstract

B-RAF selective inhibitors including vemurafenib were recently developed as effective therapies for melanoma patients with B-RAF V600E mutation. However, most patients treated with vemurafenib eventually develop resistance largely due to reactivation of MAPK signaling. Inhibitors of MAPK signaling including MEK1/2 inhibitor trametinib failed to show significant clinical benefit in patients with acquired-resistance to vemurafenib. Here we describe that cell lines with acquired-resistance to vemurafenib show reactivation of MAPK signaling and upregulation of cyclin D1, and are sensitive to inhibition of LY2835219, a selective inhibitor of cyclin dependent kinase (CDK) 4/6. LY2835219 was demonstrated to inhibit growth of melanoma A375 tumor xenografts, and delay tumor recurrence in combination with vemurafenib. Furthermore, we developed an in vivo vemurafenib-resistant model by continuous administration of vemurafenib in A375 xenografts. Consistently, we found that the MAPK is reactivated and cyclin D1 is elevated in vemurafenib-resistant tumors, as well as in the resistant cell lines derived from these tumors. Importantly, LY2835219 exhibited tumor growth regression in vemurafenib-resistant model. Mechanistic analysis revealed that LY2835219 induced apoptotic cell death in a concentration dependent manner in vemurafenib-resistant cells while it primarily mediated cell cycle G1 arrest in the parental cells. Similarly, RNAi-mediated knockdown of cyclin D1 induced significantly higher rate of apoptosis in the resistant cells compared to parental cells, suggesting that elevated cyclin D1 activity is important for the survival of vemurafenib resistant cells. Altogether, we propose that targeting cyclin D1-CDK4/6 signaling by LY2835219 is an effective strategy to overcome MAPK-mediated resistance to B-RAF inhibitors in B-RAFV600E melanoma.
Introduction

B-RAF is the most commonly mutated driver oncogene in melanoma, with activating mutations in codon 600 occurring in almost 50% of the patients (1, 2). Treatment with B-RAF selective inhibitors such as vemurafenib or dabrafenib, has demonstrated significant benefit in melanoma patients with B-RAF V600E mutation, with extended patient progression free survival and median overall survival compared with chemotherapy (3-6). However, these responses were relatively short-lived, and drug resistance generally developed within 5-7 months (4, 6). Thus the emergence of resistance remains a considerable therapeutic challenge to achieve durable responses and prolonged survival in these patients.

A variety of molecular mechanisms are identified to be involved in resistance to B-RAF inhibition. The most common resistant mechanism is MAPK pathway reactivation, which is caused by genetic mutation of MEK or different Ras isoforms (7-9), upstream activation of receptor tyrosine kinases (RTK) such as FGFR3 and c-Met (10, 11), expression of B-RAF V600E splice variants that dimerize in presence of the B-RAF inhibitor (12), amplification of B-RAF (13, 14) and upregulation of MAP3Ks such as COT or C-RAF (15, 16). Alternatively, activation of MAPK-redundant pathways such as PI3K/Akt as a consequence of PTEN loss (17), or overexpression of RTKs such as PDGFRβ and IGF1Rβ have also been reported to induce resistance in B-RAF V600E melanoma (7, 18, 19). Additionally, secretion of growth factors such as HGF or FGF has also been implicated in resistance to B-RAF inhibition (10, 11, 20, 21). Although the resistant mechanisms are frequently associated with MAPK reactivation, treatment with the MEK inhibitor trametinib, or with trametinib plus dabrafenib, has not been very effective in patients that have previously failed B-RAF inhibitor (22), suggesting that subsequent targeting of MAPK signaling alone is not sufficient. Therefore, despite recent advances in the clinic, drug resistance upon selective B-RAF inhibition remains a considerable therapeutic challenge in clinic.

Constitutive activation of CDKs and deregulation of cell cycle are common features across several cancer types, including melanoma. P16<sup>INK4a</sup>, a tumor suppressor gene and negative regulator of CDK4, is deleted in 38% of melanoma (2, 23). In addition, germline mutations and amplification of
CDK4 gene have been identified in melanoma, which leads to unrestricted CDK4 activity and increased cell proliferation (2, 24). In general, regulation of cell cycle entry in proliferating adult mammalian cells is controlled by D-cyclins which bind and activate CDK4 and CDK6, to promote phosphorylation of retinoblastoma (Rb) protein and G1-S transition (25). The RAS-MAPK pathway is known to control cell cycle entry via upregulation of cyclin D1 in several cell types (26, 27). Inhibition of MAPK signaling by B-RAF inhibitors decrease cyclin D1 expression and upregulate CDK inhibitor p27KIP1 levels, thus blocking cell cycle entry in B-RAF V600E melanoma (26, 27). Overexpression of cyclin D1 is linked to resistance to B-RAF inhibition (28). Cyclin D1 is amplified in 11% of melanoma, including 17% of B-RAF V600E melanoma, thus suggesting a potential role of cyclin D1 in intrinsic resistance to B-RAF inhibitors (2, 28). However, the role of cyclin D1 in acquired-resistance to vemurafenib has not been described, and the therapeutic value of targeting cyclin D1/Rb axis to overcome vemurafenib-resistance has not been explored.

In this study, we have generated multiple in vitro cell lines and an in vivo model of resistance to vemurafenib, and discovered that MAPK reactivation and cyclin D1 elevation are common in these resistant models. We describe that cyclin D1 is an important mediator of vemurafenib-resistance and provides a potential therapeutic target to overcome resistance to B-RAF inhibition in B-RAF V600E melanoma. Using these in-vitro and in-vivo models, we show that cyclin D1 is generally elevated, and functions as a critical node for the survival of vemurafenib-resistant cells. We further demonstrate that LY2835219, a selective dual CDK4/6 inhibitor currently in phase 2 clinic studies (29), can overcome vemurafenib resistance in these resistant models. Altogether, this study sheds new light on mechanisms of resistance to B-RAF inhibition, identifies cyclin D1 elevation concurrent with MAPK reactivation as a common resistant mechanism, and proposes targeting cyclin D1 through CDK4/6 inhibition by LY2835219 as an effective therapeutic strategy to overcome B-RAF resistance.
Materials and Methods

Cell culture, reagents and transfections- A375, SH4 and A2058 cells were obtained from ATCC on 5/7/12, 7/2/12, 6/27/06 respectively. M14 cells were purchased from NCI on 3/10/05. Cells were stored within a central cell bank that performs cell line characterizations. All these cells were passaged for fewer than 2 months after which time new cultures were initiated from vials of frozen cells. Characterization of the cell lines was done by a third party vendor (RADIL, Columbia, Missouri, USA, which included profiling (by PCR) for contamination by various microorganisms of bacterial and viral origin. As a result, no contamination was detected. The samples were also verified to be of human origin without mammalian inter-species contamination. The alleles for 9 different genetic markers were used to determine that the banked cells matched the genetic profile that has been previously reported.

All cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM, Thermo Scientific) supplemented with 10% fetal bovine serum (FBS, Invitrogen). B-RAF selective inhibitor vemurafenib and CDK4/6 dual inhibitor LY2835219 were synthesized by Eli Lilly and Company. The mesylate salt of LY2835219 (LY2835219.CH₄O₃S) was used in all the in vitro studies. All siRNAs were obtained from Dharamcon (OnTargetPlus SiRNA). SiRNA transfections were performed using Lipofectamine™ RNAiMAX transfection reagent (Invitrogen) as per manufacturer’s instructions. Cyclin D1 and non-specific control shRNA lentiviral particles were obtained from Sigma-Aldrich (Mission® shRNA). Cells were transduced with the virus for 72 hours as per manufacturer’s instructions. Antibodies against phospho-ERK1/2 (4370), phospho-Akt1 (S473) (4060), phospho-MEK (9154), cyclin D1 (2978), cyclin D2 (3741), phospho-Rb (S807/811) (8516), p27 (2552), cleaved PARP (9541) and cleaved caspase-3 (9664) were purchased from Cell Signaling Technology. Antibodies against B-RAF (sc-166, Santa Cruz), GAPDH (Santa Cruz), tubulin (ab7291, AbCam), phospho-histone H3 (S10) (Millipore), phospho-Rb (S780) (BD Biosciences) were obtained from the indicated companies.
Generation of vemurafenib resistant cell lines – A375R1, A375R3, M14R and SH4R models with acquired-resistance to vemurafenib were generated by treating respective parental cells with gradually increasing concentrations of vemurafenib, up to 2 µM, as previously described (10). A375R2 cells were generated by treating A375 cells with a high concentration of vemurafenib (2 µM) for 1 week, followed by culturing with vemurafenib (1 µM) for up to 20 passages. Upon establishment of resistance, the inhibitor was withdrawn and all of the cell lines were maintained in regular media for subsequent passages. For A375R4 cells, the NRas Q61K was stably transfected into A375 cells, and single cell clone was selected and characterized for this study. All of the models retained resistance to vemurafenib for up to 20 passages (data not shown). In this study, all resistant cell lines with less than 10 passages were utilized for experiments.

Cell proliferation assay- Cells (1-3X10³) were normally plated in 96 well plates (BD Biosciences). Cells were treated the next day for 96 hours, and then assessed for viability using CellTiter Glo (Promega), as per manufacturer’s instructions and a luminescence plate reader (Victor, Perkin Elmer). GraphPad Prism 4 software was used to generate sigmoidal dose-response curves and calculate the proliferation IC₅₀.

Caspase 3/7 activity assay – Cells (5X10³) were plated in 96 well plates (BD Biosciences). Cells were treated the next day for 24-48 hours and then assessed for caspase-3 activity by Caspase-Glo-3/7 Assay (Promega), as per manufacturer’s instructions and a luminescence plate reader (Victor, Perkin Elmer).

Preparation of cell lysates and immunoblotting – Cells and tumor tissues was lysed using either RIPA lysis buffer (Bio-Rad) (cell lines) or 1% SDS solution (tumor lysates), containing 1X phosphatase and proteinase inhibitor cocktail (Pierce). Tumor lysates were prepared with freshly frozen tumor samples. Approximately 200 mg of tumor tissue was homogenized, and lysed using 0.5 mL of lysis buffer. The protein concentration of individual samples was determined with DC Protein Assay Kit (Bio-Rad). SDS-PAGE was performed on cell lysates containing 20 µg of total protein using 4-20% Novex® tri-glycine gradient gels (Invitrogen). Protein was transferred onto 0.2 µm nitrocellulose membranes using Trans-
Blot Turbo Transfer system (Biorad) as per manufacturer’s instructions. Proteins were detected using the Odyssey Infrared Imaging System (Li-COR Biosciences).

**Flow Cytometry**- Cell pellets were fixed in 70% ethanol for 30 minutes at -20°C, then washed with PBS. Fixed cells were stained with propidium iodide/Triton X-100 staining solution and incubated for 30 minutes at room temperature. Fixed cells were then subjected to flow cytometric analysis on the Beckman Coulter FC 500 Cytomics flow cytometer. Data was analyzed with ModFit LT 3.0 (Verity House Software).

**In vivo experiments and drug administration**- All animal studies were performed in accordance with American Association for Laboratory Animal Care institutional guidelines. The Eli Lilly and Company Animal Care and Use Committee approved all the experimental protocols. Athymic nude female mice were inoculated with 0.2 ml of 1 x 10^7 A375 cells, prepared in a 1:1 matrigel to media mixture, in the hind flank region. A total of 60 mice, 8-10 in each group were used for compound treatments and vemurafenib-resistant model development in each study. Vemurafenib was formulated by dissolving in DMSO in a volume equivalent to 5% of the final formulation volume, and then the remaining volume was added to the solution of 1% methylcellulose in distilled water. CDK4/6 inhibitor LY2835219 was formulated in 1% HEC in 20 mM phosphate buffer, pH2.0. Treatment was administered orally (gavage) with the dose schedules described in each study. Tumor growth and body weight were monitored over time to evaluate efficacy.

**Generation of cell lines from xenograft tumors**- Tumors were aseptically removed from the animals, washed with cold PBS, then minced and trypsinized in 10 cm Petridish containing 5 mL of TrypLE reagent (Invitrogen) for 15 minutes at 37°C. Dislodged cells were collected, resuspended and plated in DMEM (Thermo Scientific) supplemented with 50% FBS (Invitrogen) and 10% Penicillin-Streptomycin (Invitrogen). After one week, cells were switched to regular media (DMEM + 10% FBS).
Results

Development of vemurafenib resistant cell lines- To study the resistant mechanisms of B-RAF inhibition, we generated several vemurafenib resistant cells outlined in Table 1. A375R1, A375R3, M14R and SH4R cells were generated by treating their respective parental cells with gradually increasing concentrations of vemurafenib, up to 2 µM as described previously (10). The resistant mechanism of A375R1, A375R3 and M14R cells was associated with RTK/RAS activation and MAPK reactivation (10). MAPK reactivation was also observed in SH4 cells, but the underlying mechanism has not been fully characterized. A375R2 cells were generated by treating A375 cells with a constant 2 µM vemurafenib as described (12). Consistent with the earlier reports, the resistance of A375R2 cells to vemurafenib is conferred by expression of B-RAF splice variant (Supplementary S1A). The A375R4 cells were generated through stable transfection of NRas Q61K mutant and single clone selection, which resulted in vemurafenib resistance (Supplementary figure S1B).

MAPK reactivation and cyclin D1 elevation in vemurafenib resistant cells and their sensitivity to CDK4/6 inhibitor LY2835219- As shown in table 1, all in vitro generated resistant cell lines, A375R1-R4, M14R and SH4R demonstrated resistance to vemurafenib. Importantly, these resistant cells showed enhanced MAPK activation and cyclin D1 elevation (Figure 1). MAPK pathway activation and loss of cell cycle control are generally the hallmarks of melanoma (2). Therefore, we used a selective CDK4/6 dual inhibitor LY2835219, and evaluated its growth inhibitory effects in a panel of melanoma cell lines that are either sensitive or resistant to vemurafenib mediated by diverse mechanisms (Table 1). Interestingly, B-RAF V600E melanoma cells that are either sensitive to vemurafenib, such as A375, M14 and SH4, or resistant to vemurafenib, such as A375R1-4, M14R and SH4R, demonstrated comparable sensitivity to LY2835219 with IC50s ranging from 0.3-0.6 µM, (Table 1). On the contrary, BRAF V600E mutant A2058 cells with de novo resistance to vemurafenib via MAPK-independent mechanism (i.e PTEN deletion) was relatively insensitive to LY2835219 (Table 1).
Anti-tumor effects of vemurafenib, LY2835219 and their combination in A375 xenograft model-

*In vitro* analysis revealed that parental B-RAF V600E melanoma cells, such as A375 cells were sensitive to both vemurafenib and LY2835219. To compare their activities *in vivo*, we tested their antitumor effects as single agents or in combination in an A375 xenograft model. As demonstrated in figure 2A, vemurafenib treatment at 15 mg/kg twice daily induced significant tumor growth regression in the first 2 weeks of treatment. Tumor growth was also inhibited by LY2835219 in a dose dependent fashion (Figure 2B). Statistically significant tumor growth inhibition by LY2835219 was observed at 45 or 90 mg/kg once daily dose schedule. Furthermore, analysis of tumor lysates showed that LY2835219 treatment significantly reduced pS780-Rb and pS10-Histone H3 levels, indicating inhibition of cell cycle and a decrease in proliferating tumor cells as a result of CDK4/6 inhibition (Figure 2C). When xenograft tumors were treated with a combination of vemurafenib and LY2835219, an additive anti-tumor growth effect was observed (Figure 2D).

Development of *in vivo* vemurafenib resistant model and efficacy of CDK4/6 inhibitor LY2835219 in this resistant model-

To evaluate the efficacy of LY2835219 in the vemurafenib-resistant tumors, we developed an *in vivo* vemurafenib resistant model as shown in figure 3A. Following the establishment of tumors, vemurafenib at 15 mg/kg was administered orally twice a day. Consistent with the previous observations in this model, vemurafenib treated mice demonstrated significant regression in tumor volume initially. We continued dosing with vemurafenib until resistance was evident. Approximately 40-45 days after dosing, many animals relapsed and resistant tumors started to emerge. When vemurafenib resistant tumors reached sizes approximately 600-1000 mg, the animals were randomized into two groups, each 7-8 animals. One group continued to be dosed with vemurafenib, and the other group was switched to CDK4/6 inhibitor LY2835219 treatment alone at 90 mg/kg once daily schedule. As
demonstrated in Figure 3A, LY2835219 treated mice demonstrated significant tumor growth regression, whereas the tumors in vemurafenib-treated mice continued to grow. Furthermore, LY2835219 mediated tumor growth inhibition was maintained upon cessation of the treatment. To rule out the possibility that the tumors had developed a dependence on vemurafenib for continued growth as previously described (14), we repeated the same experiment with a vehicle control arm in addition to the continued vemurafenib arm and the LY2835219 treatment group. In this study both the vehicle control group and the vemurafenib group showed indistinguishable and continued growth post vemurafenib withdrawal (Supplementary figure S2). These results suggest that CDK4/6 inhibitor LY283519 as a single agent is effective in overcoming vemurafenib resistance in this in vivo model. We further investigated the molecular mechanism behind increased sensitivity of resistant tumors to LY2835219. We found hyper-elevated levels of phospho-ERK, phospho-MEK, cyclin D1 and phospho-Rb (S780, S807, S811) in these vemurafenib-resistant tumors, indicating upregulation of MAPK and CDK activity (Figure 3B). This observation is consistent with previous findings that upregulation of cyclin D1 was associated with enhanced sensitivity of cancer cells to CDK4/6 inhibitors (30, 31). Interestingly, analysis of whole exome sequencing data derived from parental A375 and vemurafenib-resistant A375R1 cells revealed a copy number gain of 11q13 region in the A375R1 cells, and amplification of genes in the 11q13 locus including \textit{CCND1}, \textit{FGF3}, \textit{FGF4} and \textit{FGF19} (Supplementary table 1). Copy number variations of \textit{CCND1} are currently being evaluated in other resistant cell lines. Taken together, our results indicate that MAPK reactivation and upregulation of cyclin D1 are associated with vemurafenib resistance and sensitivity to CDK4/6 inhibition.

**Cyclin D1 upregulation and vemurafenib resistance in cells generated from resistant xenograft tumors**- To further understand the mechanism of vemurafenib-resistance and increased sensitivity to LY2835219, we generated two cell lines from vemurafenib-resistant tumors, hereafter referred them to as A375RV1 and A375RV2 cells. Genotype analysis (STR) confirmed that A375RV1 and A375RV2 have the same genotypes of parental A375 cells. Compared to A375 cell line (vemurafenib IC\textsubscript{50}: 102 nM),
A375RV1 and A375RV2 cell lines maintained resistance to vemurafenib with IC50 of 1520 nM and 1095 nM, respectively (Figure 4A and Table 1). We further characterized these cells and found that phospho-MEK and phospho-ERK levels remained high in the resistant cells in the presence of vemurafenib concentrations as high as 3 µM compared with parental A375 cells where phospho-MEK and p-ERK were diminished at concentrations as low as 500nM (Figure 4B and supplementary figure S3). In addition, we also detected expression of p61-B-RAF splice variant in the A375RV1 cells (Figure 4B), suggesting that one mechanism of resistance to vemurafenib in this cell line is likely due to alterations in B-RAF splicing. Consistent with the observations from the analysis of the tumor lysates, the cell lines derived from the resistant tumors also showed enhanced expression of cyclin D1 (Figures 4B and S4C).

**LY2835219 induces G1 arrest in the parental cells but apoptosis in vemurafenib-resistant cells derived from tumors** - We tested whether the vemurafenib-resistant cells derived from tumors were sensitive to LY2835219 in cell culture, and found that LY2835219 inhibited the proliferation of the parental A375 and resistant A375RV1 and A375RV2 cells with similar potencies with IC50 values of 395 nM, 260 nM and 463 nM, respectively (Figure 5A). This is consistent with the responses of other in vitro vemurafenib-resistant cells. It is well established that inhibition of cyclin D1-CDK4/6 axis, and subsequent inactivation of Rb, arrests proliferating cells in G1 stage of cell cycle. We performed PI staining and FACS analysis of cell-cycle distribution to study the mechanism of anti-proliferative effects of LY2835219 in parental and vemurafenib-resistant cells. As expected, 92% of parental A375 cells arrested in the G1 phase upon LY2835219 treatment (Figure 5B). However, LY2835219 treatment induced cell death in the resistant cells, up to 90% in the A375RV1 and 69% in A375RV2 cells, in 48 hours as indicated by the presence of a sub-diploid peak in the cytometry histograms. Similarly, LY2835219 arrested 75% of parental M14 cells in the G1 stage but induced cell death in 70% of vemurafenib-resistant M14R cells (Supplementary figure S4A). Thus, anti-tumor effects of LY2835219 are differentially mediated in parental and resistant cells, although the IC50 values are similar in CellTiter Glo proliferation assay. We further investigated if LY2835219 induced cell death in the resistant cells is
mediated by apoptosis. As demonstrated in figures 5C and 6A, LY2835219 treatment significantly elevated caspase3/7 activity in A375RV1 and A375RV2 cells and cleaved PARP activity in A375RV1 cells in a concentration dependent fashion. Similarly, LY2835219 induced higher caspase-3/7 activity and PARP cleavage in other vemurafenib resistant A375R1 and M14R cells in a concentration dependent manner (Supplementary figure S4B), suggesting that LY2835219 induces apoptosis preferably in the vemurafenib-resistant cells.

**Upregulation of Cyclin D1 is associated with LY2835219-induced apoptosis, and important for the survival of vemurafenib-resistant tumor cells**- Treatment with LY2835219 caused a concentration dependent inhibition of phospho-Rb (S780, S807/S811) with similar potencies in parental and resistant cells, indicating inhibition of CDK4/6 activity and inactivation of Rb function. Furthermore, LY2835219 treatment induced a concentration dependent enhancement of cyclin D1/D2 proteins in A375 cells, indicative of growth arrest in these cells (Figure 6A). Consistent with the *in vivo* data, cyclin D1 levels remained high in vemurafenib-resistant tumor cells (Figure 6A, 6B and Supplementary figure S4C). We explored if vemurafenib-resistant cells are dependent on cyclin D1 for their survival. We performed shRNA mediated selective knockdown of cyclin D1 in the parental and vemurafenib-resistant cells, and found that the loss of cyclin D1 decreased phospho-Rb levels in both A375 and A375RV1 cells, but induced significantly higher levels of cleaved PARP and cleaved caspase-3 fragments in A375RV1 cells (Figure 6B). Similarly, specific knockdown of cyclin D1 via siRNA also induced PARP cleavage in A375RV1 and other vemurafenib-resistant cells including A375RV2 and A375R1 cells (Supplementary figure S4C), indicating that loss of cyclin D1 induces apoptosis in vemurafenib-resistant cells and not in the parental cells. Cyclin D1 knockdown did not affect phospho-ERK levels (Figure 6B), or levels of other D-type cyclins, such as cyclin D2 (Supplementary figure S4C). Thus, we show that vemurafenib-resistant cells are dependent on cyclin D1 for their survival.
Discussion

In this study, we generated multiple *in vitro* cell lines and an *in vivo* model resistant to vemurafenib, and discovered that MAPK reactivation and cyclin D1 elevation are associated with acquired resistance in these models. We further describe that inhibition of cyclin D1-CDK4/6 signaling by CDK4/6 inhibitor LY2835219 is an effective therapy to overcome resistance. Expression of B-RAF V600E splice variants, RTK/Ras activation, NRas mutation and B-RAF amplification are the predominant clinical mechanisms of resistance to vemurafenib that have been observed to date, and all of these resistant mechanisms together with BRAF mutation induce hyperactivation of MAPK pathway (7, 12, 13). We found that the cells resistant to vemurafenib with hyperactivation of MAPK pathway have elevated cyclin D1 expression, and that they were sensitive to CDK4/6 inhibitor LY2835219. We subsequently developed a vemurafenib resistant model in vivo and evaluated the anti-tumor effect of LY2835219 in this model. Consistent with the *in vitro* findings, phospho-ERK and cyclin D1 were elevated in these resistant tumors (Figure 3B). More importantly, LY2835219 induced regression of these vemurafenib-resistant tumors (Figure 3A). These results suggest that CDK4/6 inhibition represents an effective therapeutic strategy to overcome vemurafenib resistance due to MAPK reactivation and associated with cyclin D1 elevation.

To investigate the mechanism of LY2835219 sensitivity and vemurafenib resistance, we generated resistant cells lines from vemurafenib-resistant xenograft tumors. Molecular analysis of the tumor-derived resistant cell lines revealed hyperactivation of MAPK pathway and an increase in cyclin D1 expression just as had been observed in the *in vitro* resistant cells. Similar to the xenograft studies in mice, resistant cells derived from tumors retained resistance to vemurafenib, as well as sensitivity to LY2835219 (Figures 4A and 5A). We further examined the mechanism of anti-proliferative effects of LY2835219 in parental and resistant cells. As expected, LY2835219 arrested parental cells in the G1 stage (Figure 5B and supplementary figure S4A), consistent with the role of CDK4/6 function in progression of cells from G1 to S stage of cell cycle (25). However, treatment of resistant cells with LY2835219 induced apoptosis within 24-48 hours in a concentration-dependent fashion (Figures 5B, 5C,
6A, and supplementary figures S4A and S4B). The surprising pro-apoptotic effects of LY2835219 were observed across a variety of vemurafenib-resistant cell lines, including cells derived from resistant xenograft tumors. Thus, vemurafenib-resistant B-RAF V600E melanomas with hyperactivated MAPK signaling and enhanced cyclin D1 expression are prone to apoptosis upon CDK4/6 inhibition by LY2835219, suggesting that these resistant cells are more dependent on cyclin D1/CDK4/6 signaling for survival.

Both vemurafenib and LY2835219 were demonstrated to inhibit tumor growth of BRAF V600E melanoma in single agent, and combination of these two resulted in an additive tumor growth inhibition (Figure 2). This suggests that upfront combination of CDK4/6 and B-RAF inhibitors may be more efficacious than single agent therapy. However, a more robust tumor growth regression was observed when CDK4/6 inhibitor LY2835219 was utilized for treatment of xenograft tumors acquired resistance to vemurafenib (Figure 3A). These results suggest that vemurafenib followed by LY2835219 treatment schedule might be a more effective approach than upfront combination of these two agents in delaying and overcoming resistance. Additional studies in defining these dose schedules are ongoing in preclinical models.

Previous studies revealed that cyclin D1 overexpressing cells demonstrate constitutive CDK activity, and increased sensitivity to CDK4/6 inhibitors (30, 31). Cyclin D1 was also demonstrated to be implicated in resistance to inhibitors of ERBB2, EGFR and ER signaling (32, 33). Recent studies suggest that cyclin D1 overexpression may be sufficient to render B-RAF V600E melanoma cells resistant to B-RAF inhibition (28). CCND1 is amplified in 11% of melanoma (2), including 17% of B-RAF V600E melanoma (28), indicating that it could be a mechanism of de novo resistance to B-RAF inhibitors. Molecular analysis of our vemurafenib-resistant tumors revealed significant upregulation of cyclin D1 levels relative to parental tumors (Figure 3B). Consistently, cyclin D1 was also upregulated across most vemurafenib-resistant cell lines, including cell lines derived from resistant xenograft tumors. The cyclin D1 upregulation could simply results from MAPK reactivation since it is a downstream effector of MAPK signaling. To date, we have not fully characterized the cyclin D1 amplification in every resistant
cell line. However, in at least A375R1 case we found that the vemurafenib resistance was associated with 11q13 copy number gain (Table S1). This chromosomal region includes the genes for FGF ligands as well as CCND1, potentially explaining both the cyclin D1 upregulation and CDK4/6 dependence described here, as well as the previously reported FGF pathway activation (10). Analysis of cyclin D1 amplification in other resistant cells is ongoing. We further investigated if cyclin D1 is required for proliferation and survival of vemurafenib-resistant cells. Consistent with the pro-apoptotic effects of LY2835219, knockdown of cyclin D1 by shRNA also induced higher levels of apoptosis in vemurafenib-resistant vs parental cells (Figure 6B and supplementary figure S4C). However, these data do not entirely exclude the possibility that either off-target effects of LY2835219 or inhibition of Rb-independent pathways by cyclin D1 knockdown might also contribute to the induction of apoptosis observed selectively in vemurafenib-resistant cells. Altogether, we demonstrate that cyclin D1 is important for survival of vemurafenib-resistant cells with hyperactivation of MAPK pathway and cyclin D1 upregulation, and propose LY2835219, a CDK4/6 dual inhibitor as a potential therapy to overcome such resistance.

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References


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**TABLE 1.** Overview of drug sensitivities, mutational status and mechanisms of resistance of melanoma cell lines tested in this study

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Mutation</th>
<th>Sensitivity to Vemurafenib</th>
<th>Resistance Mechanism</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (nM)</th>
<th>Apoptosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>A375</td>
<td>BRAF&lt;sup&gt;V600E&lt;/sup&gt;</td>
<td>Sensitive</td>
<td></td>
<td>Average 412, SD 103</td>
<td>No</td>
</tr>
<tr>
<td>A375-R1</td>
<td>BRAF&lt;sup&gt;V600E&lt;/sup&gt;</td>
<td>Resistant</td>
<td>FGF-mediated ERK activation</td>
<td>Average 4541, SD 939</td>
<td>Yes</td>
</tr>
<tr>
<td>A375-R2</td>
<td>BRAF&lt;sup&gt;V600E&lt;/sup&gt;</td>
<td>Resistant</td>
<td>p61-BRAF splicing</td>
<td>Average 3364, SD 1449</td>
<td>Yes</td>
</tr>
<tr>
<td>A375-R3</td>
<td>BRAF&lt;sup&gt;V600E&lt;/sup&gt;</td>
<td>Resistant</td>
<td>FGF-mediated ERK activation</td>
<td>Average 3035, SD 1781</td>
<td>Yes</td>
</tr>
<tr>
<td>A375-R4</td>
<td>BRAF&lt;sup&gt;V600E&lt;/sup&gt;</td>
<td>Resistant</td>
<td>N-Ras Q61K</td>
<td>Average 8214, SD 2640</td>
<td>-</td>
</tr>
<tr>
<td>M14</td>
<td>BRAF&lt;sup&gt;V600E&lt;/sup&gt;</td>
<td>Sensitive</td>
<td></td>
<td>Average 303, SD 97</td>
<td>No</td>
</tr>
<tr>
<td>M14-R</td>
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FIGURE LEGENDS

FIGURE 1. MAPK reactivation and Cyclin D1 elevation in vemurafenib resistant melanoma cell lines.

FIGURE 2. Anti-tumor activity of vemurafenib and LY2835219 in A375 xenograft model. A. Antitumor activity of vemurafenib in A375 xenografts. Mice bearing subcutaneous A375 tumors were dosed with vehicle (n=8) or 15 mg/kg vemurafenib (n=10) twice daily. Y axis is mean tumor volume ± s.e.m. B. Anti-tumor activity of CDK4/6 inhibitor LY2835219 in A375 xenograft model. Mice bearing subcutaneous A375 tumors were dosed with vehicle (n=7), 22.5 mg/kg (n=5), 45 mg/kg (n=5) or 90 mg/kg (n=5) LY2835219 once daily for 21 days. C. Dose dependent reduction of CDK4/6 activity in A375 xenograft tumors by LY2835219. Mice bearing subcutaneous A375 tumors were dosed with either vehicle, 22.5 mg/kg, 45 mg/kg or 90 mg/kg of LY2835219 once daily. Tumors were collected 24 hours after the third dose. Tumor lysates were prepared and analyzed by immunoblotting using indicated antibodies. D. Activities of LY2835219, vemurafenib and their combination in A375 xenograft model. Mice bearing subcutaneous A375 tumors were dosed for 21 days with either vehicle (n=8), 45 mg/kg LY2835219 (n=8), 10 mg/kg vemurafenib (n=8) or combination of 45 mg/kg LY2835219 and 10 mg/kg vemurafenib (n=8). LY2835219 was dosed once daily and vemurafenib was dosed twice daily. The brown line below the X-axis indicates the dosing (Rx) period in all studies. Y axis is mean tumor volume ± s.e.m. The pairwise comparisons of each treatment versus control are all statistically significant (p < 0.001). The tests between the combination group and each single agent group are also statistically significant (p < 0.001). The p-value for the two-way interaction to determine if combinations are different from additive is not statistically significant (p = 0.657), thus indicating that the combination of these two agents is additive rather than synergistic.

FIGURE 3. Development of in vivo vemurafenib resistant A375 xenograft model and activity of CDK4/6 inhibitor LY2835219 in the resistant model. A. Efficacy of LY2835219 in an in vivo model of acquired resistance to vemurafenib. Mice bearing subcutaneous A375 tumors were dosed with vehicle or
15 mg/kg vemurafenib twice daily after tumors were established. Dosing was continued until the resistant was evident. Upon emergence of resistant tumors with size 600-1000 mg, mice were randomized and dosed with 90 mg/kg LY2835219 once a day, or continued dosing with 15 mg/kg vemurafenib twice daily. The blue or red line under the X axis indicates the dosing (Rx) period of vemurafenib or LY2835219, respectively. Y axis is mean tumor volume ± s.e.m.

**FIGURE 4.** Cell lines derived from vemurafenib-resistant tumors maintain resistance to vemurafenib. A. Sensitivity of A375, and tumor-derived resistant A375RV1 and A375RV2 cells to vemurafenib. Cell viability was accessed using CellTiter-Glo. B. Cyclin D1 elevation and MAPK pathway reactivation in tumor-derived A375RV1 cells in the presence of vemurafenib. Cells were treated with indicated concentrations of vemurafenib for 24 hours. Cell lysates were analyzed by Immunoblotting using indicated antibodies.

**FIGURE 5.** CDK4/6 inhibitor LY2835219 is active against and induces apoptosis in tumor-derived and vemurafenib-resistant cells. A. Sensitivity of A375, A375RV1 and A375RV2 cells to LY2835219. Cell viability was accessed using CellTiter-Glo. B. Cell cycle analysis of A375 and A375RV1 cells treated by LY2835219. Cells were treated with indicated concentration of LY2835219 for 48 hours and subjected to PI staining and subsequent FACS analysis of cell-cycle distribution. Dead cells are indicated as debris. C. Caspase-3/7 activities in LY2835219 treated A375, A375RV1 and A375RV2 cells. Cells were treated with increasing concentration (0, 0.3 µM, 1 µM, 3.3 µM and 5 µM) of LY2835219 for 24 hours and Caspase 3/7 activity was determined using Caspase-Glo 3/7 Assay.

**FIGURE 6.** Cyclin D1 and CDK4/6 signaling is required for the survival of vemurafenib-resistant B-RAF V600E melanoma cells. A. Cell signaling analysis in A375 and A375RV1 cells treated with CDK4/6 inhibitor LY2835219. Cells were treated with indicated concentrations of LY2835219 for 48 hours. Cell lysates were analyzed by immunoblotting using antibodies indicated. B. Cyclin D1 knock
down by shRNA induced apoptosis in vemurafenib-resistant cells. Cells were transduced with lentivirus encoding either control or cyclin D1 shRNA. Cell lysates were collected 72 hours post-infection and analyzed by immunoblotting using indicated antibodies. Cell lysates were collected 72 hours post-transfection and analyzed by immunoblotting using indicated antibodies.
Figure 1

A375 A375-R1 A375-R2 A375-R3 A375-R4 M14 M14-R SH4 SH4-R

pMEK1/2 pERK1/2 pAKT Cyclin D1 Tubulin
Figure 2

A

B

C

D

Legend:
- **5% DMSO in 1% Methylcellulose, 0.2 ml, PO, BID**
- **Vemurafenib, 15 mg/kg, PO, BID**
- **LY2835219, 22.5 mg/kg, PO, QD x 21**
- **LY2835219, 45 mg/kg, PO, QD x 21**
- **LY2835219, 90 mg/kg, PO, QD x 21**
- **1% HEC in 25 mM PB pH2, 0.2 ml, PO, QD x 21**
- **Vemurafenib, 10 mg/kg, PO, BID x 21**
- **Vemurafenib, 15 mg/kg, PO, BID**
- **LY2835219, 45 mg/kg, PO, QD x 21**

**LY2835219**

- Vehicle
- 22.5 mg/kg
- 45 mg/kg
- 90 mg/kg

**pS70-Rb**

**pS10-Histone H3**

**GAPDH**

**Tumor Volume, mm³**

**Day**

**Rx**
Figure 3

A

- 5% DMSO in 1% Methylcellulose, 0.2 ml, PO, BID x 28
- Vemurafenib, 15 mg/kg, PO, BID x 119
- Vemurafenib, 15 mg/kg, PO, BID x 92 / LY2835219, 90 mg/kg, PO, QD x 28

B

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- pMEK1/2
- pERK1/2
- Cyclin D1
- pRb S807/S811
- pRb S780
- pAkt S473
- Tubulin
Figure 4

A

![Graph showing relative luminescence units against log concentration of Vemurafenib (nM). The graph compares A375, A375-RV1, and A375-RV2.]

B

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<tr>
<th>Vemurafenib (µM)</th>
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- BRAF
- p61-Splice variant
- pMEK1/2
- pERK1/2
- Cyclin D1
- pRb S780
- Tubulin
Figure 5

A

Relative Luminiscence

Log Concentration LY2835219 (nM)

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<tr>
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DMSO | 1.5 µM | 5 µM |

LY2835219

C

Relative Caspase-3 Activity (%)

Log Concentration LY2835219 (nM)
Figure 6

A

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LY2835219 (µM)

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B

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LY2835219 (µM)

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Control ShRNA

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Cyclin D1 ShRNA

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Molecular Cancer Therapeutics

The CDK4/6 Inhibitor LY2835219 Overcomes Vemurafenib Resistance Resulting from MAPK Reactivation and Cyclin D1 Upregulation

Vipin Yadav, Teresa F. Burke, Lysiane Huber, et al.

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