Jiang, Chen Chen; Lai, Fritz; Thorne, Rick F.; Yang, Fan; Liu, Hao; Hersey, Peter; Zhang, Xu Dong. “MEK-independent survival of B-RAFV600E melanoma cells selected for resistance to apoptosis induced by the RAF inhibitor PLX4720” Clinical Cancer Research Vol. 17, Issue 4, p. 721-730 (2011)

Available from: http://dx.doi.org/10.1158/1078-0432.ccr-10-2225

Accessed from: http://hdl.handle.net/1959.13/1050490
MEK-Independent Survival of B-RAF^{V600E} Melanoma

Cells Selected for Resistance to Apoptosis Induced by the RAF Inhibitor PLX4720

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Running Title: MEK-independent survival of PLX4720-selected melanoma cells

Key Words: Melanoma, PLX4720, B-RAF, MEK, ERK

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Translational Relevance

Results from clinical studies with small molecule inhibitors of mutant B-RAF have been very encouraging in the treatment of melanoma, but frequent recurrence of tumor growth following initial remission remains a major obstacle for more successful treatment of the disease. We show in this report that B-RAF$^{V600E}$ melanoma cells selected for resistance to apoptosis induced by the RAF inhibitor PLX4720 by prolonged exposure to the inhibitor can proliferate even in the presence of the inhibitor. Survival of these cells is primarily mediated by activation of ERK that is largely independent of the conventional RAF/MEK/ERK pathway. In contrast, activation of the PI3k/Akt pathway as a consequence of extracellular stimulation appears essential for activation of ERK. These results suggest that re-growth of B-RAF$^{V600E}$ melanomas after initial responses to B-RAF inhibitors may not be responsive to MEK inhibitors, but may be susceptible to inhibitors against the PI3k/Akt pathway.
Abstract

**Purpose:** To examine mechanisms that determine long-term responses of B-RAF<sup>V600E</sup> melanoma cells to B-RAF inhibitors.

**Experimental Design:** B-RAF<sup>V600E</sup> melanoma cells were exposed to the B-RAF inhibitor PLX4720 for prolonged periods to select for cells resistant to apoptosis induced by the inhibitor. The resultant cells were analyzed for activation of ERK, MEK, and Akt and related signals. Their roles in survival of the cells were also examined.

**Results:** B-RAF<sup>V600E</sup> melanoma cells selected for resistant to PLX4720-induced apoptosis retained the V600E mutation in B-RAF, and proliferated steadily in the presence of the inhibitor, albeit with slow growth rate. These cells displayed high levels of ERK activation that is, at least in part, independent of the conventional RAF/MEK/ERK pathway, as MEK activation was low and inhibition of MEK did not significantly block activation of ERK. In contrast, extracellular signals appeared involved. This was associated with elevated activation of the PI3k/Akt pathway and could be inhibited by serum starvation and inhibition of PI3k/Akt. Inhibition of MEK did not impact on survival of these cells, whereas serum starvation, inhibition of PI3K/Akt, and inhibition of ERK1/2 reduced their viability.

**Conclusions:** These results indicate that sensitivity to induction of apoptosis may be a major determinant of long-term responses of B-RAF<sup>V600E</sup> melanomas to specific inhibitors, and suggest that rebound melanoma growth after initial treatment with the inhibitors may not be responsive to MEK inhibitors, but may be susceptible to inhibition of the PI3k/Akt pathway.
Introduction

Results from clinical studies with small molecule inhibitors of the mutant B-RAF$^{V600E}$ have been very encouraging and promise to provide a much needed breakthrough in the treatment of melanoma by targeting B-RAF$^{V600E}$ (1, 2). The latter is found in about 50% of melanomas leading to constitutive activation of the RAF/MEK/ERK pathway that is important for melanoma cell growth and survival, and is involved in resistance to many therapeutic agents (3, 4). However, a number of questions have already been raised from these studies, such as the durability of responses and why some melanomas with mutant B-RAF have not shown major responses.

It is well-established that blockade of the RAF/MEK/ERK pathway inhibits melanoma cell growth (5). Moreover, induction of apoptosis has also been shown in varying in-vitro systems, in particular, in B-RAF$^{V600E}$ melanoma cells (6-11). Apoptosis of such cells was clearly demonstrated in an ex-vivo model after administration of the B-RAF inhibitor PLX4720 that is selective for the mutant B-RAF$^{V600E}$ (6). Consistently, regression of metastatic mutant B-RAF melanomas is a frequent sign of the response to administration of PLX4032/ RG7204, an analogue to PLX4720, suggesting that induction of apoptosis may be a major biological consequence of inhibition of mutant B-RAF that causes remission of melanoma (1, 2).

Although molecular mechanisms that regulate sensitivity of B-RAF$^{V600E}$ melanoma cells to apoptosis induced by specific inhibitors are currently not well understood, it has been reported that acquired resistance of melanoma cells to the inhibitors was associated with rebound activation of the RAF/MEK/ERK pathway (12, 13). Signaling to activate MEK/ERK has been shown to switch to C-RAF when mutant B-RAF was inhibited due to elevated C-RAF expression (12). Moreover, most melanoma cells harboring the B-RAF$^{V600E}$
mutation retain the wild-type B-RAF allele (14). These cells can be rescued by signals mediated by extracellular growth factors when B-RAF<sup>V600E</sup> was knocked down (14). In addition, mutant B-RAF inhibitors can cause activation of ERK by transactivation of C-RAF in cells carrying wild-type B-RAF (15-17). On the other hand, constitutive activation of other survival signaling pathways such as the PI3k/Akt pathway has been reported to mediate resistance of mutant B-RAF melanoma cells to inhibition of B-RAF (18, 19).

To understand the mechanism(s) that determines long-term responses of mutant B-RAF melanoma cells to specific inhibitors, we generated B-RAF<sup>V600E</sup> melanoma cells resistant to PLX4720-induced apoptosis by prolonged exposure to the inhibitor. We show in this report that these cells display high levels of activation of ERK that is important for survival of the cells. Unexpectedly, our results indicate that activation of ERK in the cells is largely independent of C-RAF and MEK. Instead, extracellular signals appear of importance in ERK activation. This is, at least in part, mediated by increased activation of the PI3k/Akt pathway.
Materials and Methods

Cell Culture and Reagents

Human melanoma cell lines Mel-RMu and Mel-CV have been described previously (20). They were cultured in DMEM containing 5% FCS (Commonwealth Serum Laboratories, Melbourne, Vic, Australia). The mouse MAbs against phospho-ERK (p-ERK) (Thr202/Tyr204), phospho-Akt (p-Akt) (Ser473), phospho-MEK (p-MEK), ERK, ERK1, ERK2, Akt, Akt1, Akt2, Akt3, MEK1, C-RAF, and B-RAF, were purchased from Cell Signalling Technology (Beverly, MA). The rabbit polyclonal Ab against Caspase-3 was from Stressgen (Victoria, BC, Canada). Isotype control Abs used were the ID4.5 (mouse IgG2a) MAb against Salmonella typhi supplied by Dr. L. Ashman (Institute for Medical and Veterinary Science, Adelaide, Australia), the 107.3 mouse IgG1 MAb purchased from PharMingen (San Diego, CA), and rabbit IgG from Sigma Chemical Co. (Castle Hill, Australia). PLX4720 was provided from Plexxikon Inc. (Berkeley, CA). It was dissolved in DMSO and made up in stock solutions of 4mM. The MEK inhibitor U0126 and the PI3k inhibitor LY294002 were purchased from Promega Corporation (Madison, WI, USA).

Generation of melanoma cells resistant to PLX4720-induced apoptosis from B-RAFV600E melanoma cell lines

Mel-RMu and Mel-CV cells were seeded in wells of 24-well plates or 6-well plates till they reached 70-80% confluence. Cells were then switched into medium containing PLX4720 (10μM). The surviving cells were fed every 3 days with medium containing PLX4720 for 6-8 weeks until they reached 70-80% confluence. The resulting cells were then passaged and cultured in the presence of PLX4720 for at least 20 weeks. The resulting cell
lines were designated PLX-selected melanoma cells (Mel-RMu.S and Mel-CV.S, respectively).

**Apoptosis**

Quantitation of apoptotic cells was carried out by measurement of sub-G1 DNA content using propidium iodide (PI) on a flow cytometer as described elsewhere (20, 21).

**Cell Viability Assays (MTS Assays)**

Cell viability assays were performed as reported previously (20). In brief, cells were seeded at 5000 cells/well onto flat-bottomed 96-well culture plates and allowed to grow for 24 hours followed by the desired treatment. Cells were then labeled with the VisionBlue™ reagent and detected by Synergy™ 2 multi-detection microplate reader (BioTek, VT) according to the manufacturer’s instruction.

**Flow Cytometry**

Immunostaining on intact and permeabilized cells was carried out as described previously (20, 21).

**Western Blot analysis**

Western blot analysis was carried out as described previously (20, 21). Labeled bands were detected by Immun-Star™ HRP Chemiluminescent Kit, and images were captured and the intensity of the bands was quantitated with the Bio-Rad VersaDoc™ image system (Bio-Rad, Regents Park, NSW, Australia).

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Small RNA interference (siRNA)

The siRNA constructs used were obtained as the siGENOME SMARTpool reagents (Dharmacon, Lafayette, CO), MEK1 siGENOME SMARTpool (M-003571-01-0005), C-RAF siGENOME SMARTpool (M-003601-02-0010), ERK1 siGENOME SMARTpool (M-003592-03-0010), ERK2 siGENOME SMARTpool (M-003555-04-0010), Akt1 siGENOME SMARTpool (M-003000-03-0010), Akt2 siGENOME SMARTpool (M-003001-02-0010), Akt3 siGENOME SMARTpool (M-003002-02-0010) and siGENOME Non-targeting siRNA pool (D-001206-13-20). Transfection of siRNA pools was carried out as described previously (20, 21).
Results

**Prolonged Exposure to PLX4720 Selects B-RAF^V600E Melanoma Cells Resistant to PLX4720-Induced Apoptosis**

We exposed two melanoma cell lines, Mel-CV that carried B-RAF^V600E and Mel-RMu that harbored B-RAF^V600E and an activating mutation in EGFR (L747_S752/P753S deletion), to the B-RAF^V600E inhibitor PLX4720 at 10μM for prolonged periods. This concentration was chosen because it induces high levels of apoptosis (>50% apoptotic cells) in most B-RAF^V600E melanoma cell lines and fresh melanoma isolates (20). In addition, it is conceivably relevant to applications in vivo, as plasma concentrations of a closely related inhibitor PLX4032/RG7204 of around 60μM were not associated with significant adverse effects in phase I clinical trials (1, 22). As expected, initial exposure to PLX4720 at 10μM for 3 days induced apoptosis in more than 60% of Mel-RMu and Mel-CV cells. However, the levels of apoptosis in the remaining cells exposed to fresh PLX4720 decreased progressively (Figure 1A). At the end of three weeks, the levels of apoptosis of the cells in the presence of PLX4720 were equivalent to those of the parental counterparts without any treatment (≤5%), indicative of successful selection of B-RAF^V600E melanoma cells resistant to apoptosis induced by PLX4720 (Figure 1A). For standardization, only the cells that had been exposed to PLX4720 for at least twenty weeks were designated as PLX-selected cells and used for subsequent experiments. Notably, both PLX-selected Mel-RMu (Mel-RMu.S) and Mel-CV (Mel-CV.S) cells retained the V600E mutation in B-RAF, whereas Mel-CV.S cells also retained the activating mutation in EGFR (data not shown).

Despite resistance to apoptosis, the PLX-selected cells proliferated significantly slower than their parental counterparts (Figure 1B). Consistently, the cells displayed increased levels of p21 and p27, and decreased levels of cyclin D1 and CDK4 (Figure 1C). Nevertheless, these
cells were able to form stable populations and be passaged in the presence of PLX4720 (Figure 1D).

**PLX-Selected B-RAF<sup>V600E</sup> Melanoma Cells Exhibit High Levels of ERK Activation that is largely Independent of MEK**

As reported before, initial exposure to PLX4720 at 10μM resulted in marked inhibition of ERK activation, with the inhibitory effect sustained up to 3 days in Mel-RMu and Mel-CV cells (20) (Figure 2A). However, activation of ERK in the remaining cells was steadily recovered in the presence of fresh PLX4720. By 9 days, the levels of ERK activation were comparable to those in cells without exposure to PLX4720 (Figure 2A). Notably, the levels appeared even higher in the PLX-selected cells (the cells that had been exposed to PLX4720 for at least 20 weeks) than their parental counterparts.

To study the mechanism by which ERK is highly activated in PLX-selected cells, we examined the activation status of MEK that is directly up-stream of ERK in the RAF/MEK/ERK pathway. Figure 2B shows that while phosphorylated (activated) MEK was readily detected in the parental Mel-RMu and Mel-CV cells, it was expressed at markedly lower levels in Mel-RMu.S and Mel-CV.S cells. This suggests that activation of MEK may not play a major role in activation of ERK in the cells. Consistently, treatment with the MEK inhibitor U0126 caused only moderate inhibition of phosphorylation (activation) of ERK in these cells, whereas the levels of phosphorylated (activated) ERK were reduced markedly in the parental Mel-RMu and Mel-CV cells (Figure 2B). The similar effect was observed with another MEK inhibitor AZD6244 (Data not shown). Furthermore, siRNA knockdown of MEK1 significantly reduced the levels ERK phosphorylation in the parental cells, but had no detectable effect on activation of ERK in Mel-RMu.S and Mel-CV.S cells (Figure 2C).
We studied whether signaling to activate ERK was switched to C-RAF in the PLX-selected cells. As shown in Figure 2D, the levels of C-RAF in Mel-RMu.S and Mel-CV.S cells were increased in comparison with their parental counterparts. However, inhibition of C-RAF by siRNA did not reduce the levels of ERK activation in the PLX-selected cells, nor did it inhibit activation of ERK in the parental counterparts. These results suggest that the increase in C-RAF in B-RAF\(^{V600E}\) melanoma cells after prolonged inhibition of B-RAF\(^{V600E}\) may not be responsible for rebound activation of ERK.

**Activation of ERK Contributes to Survival of PLX-Selected Melanoma Cells**

We examined the role of activation of ERK in survival of the PLX-selected melanoma cells by transfecting siRNA pools for ERK1 and ERK2 into the Mel-RMu.S and Mel-CV.S cells, respectively (Figure 3A). As shown in Figure 3B, inhibition of ERK1 or ERK2 by siRNA induced moderate levels of apoptosis (25%-35%) in both lines. This was associated with activation of caspase-3 (Figure 3C). In contrast, siRNA knockdown of ERK1 or ERK2 did not induce apoptosis in the parental counterparts (data not shown). These results indicate that activation of ERK is responsible for survival of B-RAF\(^{V600E}\) melanoma cells after long-term exposure to PLX4720.

**Extracellular Signaling is Critical for Activation of ERK in the PLX4720-Selected B-RAF\(^{V600E}\) Melanoma Cells**

Extracellular signals are essential for activation of ERK in the absence of activating mutations of its up-stream kinases (23, 24). We therefore examined if ERK activation in the PLX-selected cells is due to extracellular stimulation afforded by FCS in culture medium by reducing its concentration from 5% to 0.5%. Twenty-four hours after culturing in medium with 0.5% FCS, the levels of activation of ERK were decreased in both the PLX-selected cells.
and the parental counterparts (Figure 4A). Of note, while the levels of ERK activation remained relatively stable in the parental counterparts, the levels in the PLX4720-selected cells continued to decrease. By 72 hours, activation of ERK was hardly detectable in both Mel-RMu.S and Mel-CV.S cells (Figure 4A).

We also examined whether the PLX4720-selected cells rely on extracellular signals for their survival. After switching cells to medium with 0.5% FCS, the growth rate was reduced by approximately 30% in parental Mel-RMu and Mel-CV cells at 48 hours, which remained stable thereafter (Figure 4B). These cells otherwise appeared healthy with cell volumes and morphology comparable to those grown in medium with 5% FCS (Figure 4C). In contrast, the growth rate of Mel-RMu.S and Mel-CV.S cell was reduced by approximately 60% and 75%, respectively, at 48 hours. This was associated with induction of apoptosis (Figure 4D). Nevertheless, by the end of 2 weeks, sparse colonies were found in the PLX-selected cell cultures (Figure 4C).

**Activation of ERK Is Associated with Increased Activation of Akt in PLX-Selected B-RAF<sup>V600E</sup> Melanoma Cells**

Another important survival signaling pathway in melanoma is the PI3k/Akt pathway that can be activated in response to extracellular signaling and is able to activate ERK independent of MEK (18, 25). As shown in Figure 5A, the levels of Akt activation were elevated in both Mel-RMu.S and Mel-CV.S cells, but activation was decreased after the cells were switched into medium containing only 0.5% FCS. While Akt activation was inhibited by the PI3k inhibitor LY294002, the levels of activation of ERK were also decreased in the PLX-selected cells treated with the inhibitor (Figure 5B). In contrast, LY294002 did not have any notable effect on ERK activation in the parental counterparts.
To further confirm the role of the PI3k/Akt pathway in activation of ERK in PLX4720-selected cells, we knocked down Akt1, Akt2, and Akt3 in Mel-CV and Mel-CV.S cells, respectively, by siRNA. Consistent with previous reports that Akt3 is the major isoform of Akt in melanoma (19, 26), knockdown of Akt3, but not knockdown of Akt1 or Akt 2, resulted in marked reduction in the levels of phosphorylated Akt in both the PLX-selected and the parental cells (Figure 5C). Similar to inhibition of PI3k by LY294002, knockdown of Akt3 by siRNA partially inhibited ERK activation in Mel-CV.S cells. In contrast, knockdown of Akt1 or Akt2 did not reduce the levels of ERK activation in the cells (Figure 5C). Neither knockdown of Akt3 nor knockdown of Akt1 or Akt2 had notable effect on activation of ERK in the parental counterparts (Figure 5C).

**The PLX4720-selected B-RAF^{V600E} Melanoma Cells Are not Sensitive to MEK inhibitors, but Are Susceptible to Inhibition of PI3k/Akt**

MEK-independent activation of ERK in PLX-selected cells suggests that these cells may not susceptible to inhibition of MEK (Figures 2B, 5B and C). To test this, we treated Mel-RMu.S and Mel-CV.S cells with U0126 for 72 hours. The addition of U0126 did not cause significant apoptosis of the selected cells (Figure 6A). In contrast, co-treatment with U0126 and PLX4720 resulted in moderate enhancement in induction of apoptosis in the parental counterparts (Figure 6A). A similar effect on survival of PLX-selected melanoma cells was observed with another MEK inhibitor AZD6244 (data not shown).

We also examined the effect of inhibition of the PI3k/Akt pathway on survival of the PLX4720-selected B-RAF^{V600E} melanoma cells. As shown in Figure 6B, the addition of the PI3k inhibitor LY294002 induced apoptosis in 36% and 45% of Mel-RMu.S and Mel-CV.S cells, respectively. Notably, LY294002 did not cause apoptotic cell death in the parental counterparts, but it enhanced PLX4720-induced apoptosis of the cells (Figure 6B). The effect...
of the PI3k/Akt pathway on survival of the PLX-selected melanoma cells was also shown by siRNA knockdown of Akt3 that similarly induced apoptosis of Mel-CV.S cells (Figures 5C and 6C).
Discussion

The above results show that B-RAF<sup>V600E</sup> melanoma cells selected for resistance to apoptosis induced by PLX4720 can proliferate, albeit with reduced growth rate, in the presence of the inhibitor. Survival of these cells is associated with rebound activation of ERK that is largely independent of the conventional RAF/MEK/ERK pathway. Instead, elevated activation of the PI3k/Akt pathway as a result of extracellular stimulation appears to be important. The results suggest that melanoma recurrence in patients treated with selective mutant B-RAF inhibitors may not respond to MEK inhibitors, but may be susceptible to inhibitors against the PI3k/Akt pathway.

Although the RAF/MEK/ERK pathway was initially thought to be mainly responsible for regulation of cell proliferation (27, 28), it is now clear that this pathway also plays an important role in regulation of cell survival (27, 28). Blockade of this pathway can cause not only inhibition of cell growth but also induction of apoptosis in melanoma cells (6-9). Mutant B-RAF-specific inhibitors display the dual effects on B-RAF<sup>V600E</sup> melanoma cells, but the relative contributions of inhibition of cell growth and induction of apoptosis to the therapeutic effect remain ambiguous (6, 13). Despite the high rate of clinical responses in patients treated with mutant B-RAF-specific inhibitors such as PLX4032/RG7204 and GSK2118436, longer follow-up has shown frequent recurrence of tumor growth (13, 30). It is conceivable that induction of apoptosis may occur at early stages after inhibition of mutant B-RAF, but the remaining cells that are presumably resistant to apoptosis may retain low proliferation potential even in the presence of the inhibitors, and regain high growth rate after the inhibition is removed. In corroboration with this, we found in this study that although initial exposure to PLX4720 induced apoptosis in most melanoma cells harboring B-RAF<sup>V600E</sup>, the remaining cells could proliferate, albeit with slow growth rate, even in the presence of a relatively high concentration of the inhibitor for prolonged periods. These results highlight
the importance of induction of apoptosis in determining long-term responses of B-RAF\textsuperscript{V600E} melanoma cells to specific inhibitors, and suggest that it is critical to administer the inhibitors at dosages that efficiently induce apoptosis to melanomas that are naïve to the inhibitors.

Reactivation of ERK is a common finding after inhibition of the RAF/MEK/ERK pathway (30). In melanoma cells harboring wild-type B-RAF, this is largely due to relief of negative feedback mediators such as the Sprouty proteins and the mitogen activated protein kinase (MAPK) phosphatases (DUSPs) (31-33). However, the feedback is impaired in melanoma cells carrying activating mutations in B-RAF (33, 34). This is associated with the inability of mutant B-RAF to bind to sprouty family proteins (34, 35). Nevertheless, we found in this study that ERK was highly activated in PLX-selected B-RAF\textsuperscript{V600E} melanoma cells. Although elevated C-RAF has been reported to be a mechanism of reactivation of MEK/ERK and acquired resistance to the RAF inhibitor AZ628 in mutant B-RAF melanoma cells (12), the increase in C-RAF did not appear to play a major role in reactivation of ERK in the PLX-selected B-RAF\textsuperscript{V600E} cells in this study. The exact reason for this discrepancy is unknown, but it may be related to different biochemical properties of the two inhibitors used in the studies (12).

The present results show that reactivation of ERK appears largely independent of MEK. This was demonstrated by the lack of MEK activation and the failure of MEK inhibitors and siRNA knockdown of MEK to inhibit ERK activation in the cells. The lack of activation of MEK may be due to impaired up-stream signaling, as the levels of MEK in the PLX-selected cells remained comparable to those in the parental counterparts. Another possible explanation is loss-of-function mutations in MEK, which has been reported to be responsible for the failure of melanoma cells to respond to the MEK inhibitor AZD6244 (36). Regardless of the mutational status of MEK in the PLX-selected melanoma cells, our results clearly indicated that mechanisms other than MEK can substitute its role in activation of ERK.
Available evidence suggests that ERK activation was dependent on activation of the PI3k/Akt pathway in that Akt activation was increased in the PLX-selected melanoma cells, and that inhibition of the pathway by inhibitors of PI3k or knockdown of Akt3 inhibited activation of ERK in the cells. Increased activation of the PI3k/Akt pathway in the PLX-selected cells appeared to result from extracellular signals as reduction in concentrations of FCS in culture medium caused not only marked decreases in activation of ERK, but also decreases in activation of Akt. These observations are in line with the existence of cross-talk between these two major survival pathways (18, 25, 37), and indicate that ERK can be activated by the PI3k/Akt pathway when its conventional up-stream signaling is switched off in melanoma cells.

The rebound activation of ERK in PLX-selected melanoma cells appeared necessary for survival of the cells, because siRNA knockdown of either ERK1 or ERK2 caused reduction in cell viability. This was recapitulated by inhibition of the PI3k/Akt pathway and reduction in extracellular stimulation, consistent with their roles in activation of ERK in the PLX-selected melanoma cells. Notably, inhibition of ERK did not induce apoptosis in the parental counterparts, suggesting that the PLX-selected cells were more addicted to activation of ERK for their survival. It is of note that inhibition of MEK did not impinge on survival of PLX-selected melanoma cells, although it enhanced PLX-induced apoptosis in the parental counterparts (13). Therefore, activation of ERK is responsible for survival of the PLX-selected B-RAF^{V600E} melanoma cells, which may not be susceptible to inhibition of MEK, but may be responsive to inhibition of PI3k/Akt.

In conclusion, we have shown in this study that 1) B-RAF^{V600E} melanoma cells resistant to apoptosis induced by PLX4720 selected by prolonged exposure to the inhibitor can proliferate even in the presence of the inhibitor; 2) the conventional RAF/MEK/ERK pathway is dispensable for survival of the PLX-selected melanoma cells; and 3) activation of ERK by the
PI3k/Akt pathway resulting from extracellular stimulations is responsible for survival of B-RAF\textsuperscript{V600E} melanoma cells after long-term exposure to PLX4720; These results suggest that 1) induction of apoptosis is a major determinant of long-term responses of melanoma cells to mutant B-RAF inhibitors; 2) re-growth of B-RAF\textsuperscript{V600E} melanomas after discontinuation of BRAF inhibitors may not respond to single-agent MEK inhibitor; and 3) Inhibition of the PI3k/Akt pathway may be a useful strategy in the treatment of recurrent B-RAF\textsuperscript{V600E} melanomas after discontinuation of B-RAF inhibitors. In addition, these results, along with others (12), suggest that combination of B-RAF inhibitors and those against MEK and/or PI3k/Akt should be considered at the initial treatment of melanomas carrying activating mutations in B-RAF.
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Acknowledgements

This work was supported by the NSW State Cancer Council, and National Health and Medical Research Council, Australia. X.D. Zhang and R.F. Thorne are Cancer Institute NSW Fellows.
Figure Legends

Figure 1 – Selection of B-RAF\textsuperscript{V600E} melanoma cells resistant to apoptosis induced by PLX4720 by prolonged exposure to the inhibitor

A. B-RAF\textsuperscript{V600E} Mel-RMu and Mel-CV cells seeded in wells of 24-well plates were exposed to PLX4720 (10\(\mu\)M). Culture medium containing PLX4720 was changed every 3 days. Apoptosis was measured in cells from representative wells at indicated time points by the PI method. Data shown are the mean ± SE of results from three individual culture wells.

B. Mel-RMu.S and Mel-CV.S cells and their parental counterparts were subjected to colony formation assays. The data shown are representative of three individual experiments.

C. Whole cell lysates from Mel-RMu.S and Mel-CV.S cells and their parental counterparts were subjected to Western blot analysis of p21, p27, CDK4, Cyclin D1, and GAPDH (as a loading control). The data shown are representative of three individual experiments.

D. Representative of microscopic photographs of Mel-RMu.S and Mel-CV.S cells and their parental counterparts grown in 75mm\(^2\) culture flasks.

Figure 2 – Activation of ERK in the PLX-selected melanoma cells is largely independent of MEK and C-RAF.

A. Left panel: Whole cell lysates from Mel-RMu and Mel-CV cells treated with PLX4720 (10\(\mu\)M) for indicated periods were subjected to Western blot analysis of pERK and ERK. Right panel: Whole cell lysates from Mel-RMu.S and Mel-CV.S cells and their parental counterparts were subjected to Western blot analysis of phosphorylated ERK and ERK. The data shown are representative of three individual experiments.
B. Left panel: Whole cell lysates from Mel-RMu.S and Mel-CV.S cells and their parental counterparts were subjected to Western blot analysis of phosphorylated MEK and MEK. Right panel: Whole cell lysates from Mel-RMu.S and Mel-CV.S cells and their parental counterparts with or without treatment with U0126 (20μM) for 3 hours were subjected to Western blot analysis of phosphorylated ERK and ERK. The data shown are representative of three individual experiments.

C. Mel-RMu.S and Mel-CV.S cells and their parental counterparts were transfected with the control and MEK1 siRNA, respectively. Twenty-four hours later, whole cell lysates were subjected to Western blot analysis of MEK1, phosphorylated ERK, and ERK. The data shown are representative of three individual experiments.

D. Left panel: Whole cell lysates from Mel-RMu.S and Mel-CV.S cells and their parental counterparts were subjected to Western blot analysis of C-RAF and GAPDH (as a loading control). Right panel: Mel-RMu.S and Mel-CV.S cells and their parental counterparts were transfected with the control and C-RAF siRNA, respectively. Twenty-four hours later, whole cell lysates were subjected to Western blot analysis of C-RAF, phosphorylated ERK, and ERK. The data shown are representative of three individual experiments.

Figure 3 – Activation ERK contributes to survival of the PLX-selected melanoma cells

A. Mel-RMu.S and Mel-CV.S cells were transfected with the control, ERK1, and ERK2 siRNA, respectively. Twenty-four hours later, whole cell lysates were subjected to Western blot analysis of ERK and GAPDH (as a loading control). The data shown are representative of three individual experiments.

B. Mel-RMu.S and Mel-CV.S cells were transfected with the control, ERK1, and ERK2 siRNA, respectively. Forty-eight hours later, cells were subjected to quantitation of apoptosis using the PI methods. The data shown are the mean ± SE of results from three individual experiments.
C. Mel-RMu.S and Mel-CV.S cells were transfected with the control, ERK1, and ERK2 siRNA, respectively. Forty-eight hours later, whole cell lysates were subjected to Western blot analysis of caspase-3 and GAPDH (as a loading control). The data shown are representative of three individual experiments.

Figure 4 – Extracellular signals are critical for activation of ERK and survival of the PLX-selected melanoma cells.

A. Mel-RMu.S and Mel-CV.S cells and their parental counterparts were cultured in medium containing 5% FCS till approximate 50% confluence. Cells were then switched into medium containing 0.5% FCS for indicated periods. Whole cell lysates were subjected to Western blot analysis of phosphorylated ERK and ERK. The data shown are representative of three individual experiments.

B. Mel-RMu.S and Mel-CV.S cells and their parental counterparts were cultured in medium containing 5% FCS till approximate 50% confluence. Half of the cultures were then switched into medium with 0.5% FCS, and the rest were switched into fresh medium containing 5% FCS for the same periods. Cell viability was quantitated at indicated time points using MTS assays. Results from cells cultured in medium with 0.5% FCS were compared with those from cells cultured in medium with 5% FCS for the same periods and were expressed as the percentages of the latter. The data shown are the mean ± SE of results from three individual culture wells.

C. Representative microscopic photographs of Mel-RMu.S and Mel-CV.S cells and their parental counterparts cultured in medium containing 0.5% FCS for indicated periods.

D. Mel-RMu.S and Mel-CV.S cells and their parental counterparts were cultured in medium containing 5% FCS till approximate 50% confluence. Cells were then switched into medium with 0.5% FCS for 48 hours. Apoptosis was quantitated by the
PI method. The data shown are the mean ± SE of results from three individual experiments.

Figure 5 – Increased activation of Akt is responsible for activation of ERK in the PLX-selected melanoma cells.

A. Mel-RMu.S and Mel-CV.S cells and their parental counterparts were cultured in medium containing 5% FCS till approximate 50% confluence. Half of the cultures were then switched into medium with 0.5% FCS, and the rest were switched into fresh medium containing 5% FCS for 24 hours. Whole cell lysates were subjected to Western blot analysis of phosphorylated Akt and Akt. The data shown are representative of three individual experiments.

B. Mel-RMu.S and Mel-CV.S cells and their parental counterparts with or without exposure to LY294002 (20μM) for 6 hours were subjected to Western blot analysis of phosphorylated Akt, Akt, phosphorylated ERK, and ERK. The data shown are representative of three individual experiments.

C. Mel-CV and Mel-CV.S cells were transfected with the control, Akt1, Akt2, and Akt3 siRNA, respectively. Twenty-four hours later, whole cell lysates were subjected to Western blot analysis of phosphorylated Akt, Akt1, Akt2, Akt3, phosphorylated ERK, and ERK. The data shown are representative of three individual experiments.

Figure 6 – Inhibition of PI3K/Akt, but not inhibition of MEK induces apoptosis of the PLX-selected melanoma cells.

A. Mel-RMu and Mel-CV cells were treated with U0126 (20μM), PLX4720 (10μM), or the combination of both for 72 hours. Mel-RMu.S and Mel-CV.S were also exposed to U0126 (20μM) (The selected cells were routinely cultured in the presence of PLX4720) for 72 hours. Apoptosis was quantitated by the PI method. The data shown are the mean ± SE of results from three individual culture wells.
B. Mel-RMu and Mel-CV cells were treated with LY294002 (20μM), PLX4720 (10μM), or the combination of both for 72 hours. Mel-RMu.S and Mel-CV.S were also exposed to LY294002 (20μM) (The selected cells were routinely cultured in the presence of PLX4720) for 72 hours. Apoptosis was quantitated by the PI method. The data shown are the mean ± SE of results from three individual culture wells.

C. Mel-CV and Mel-CV.S cells were transfected with the control and Akt3 siRNA, respectively. Forty-eight hours later, apoptosis was quantitated by the PI method. The data shown are the mean ± SE of results from three individual culture wells.
Figure 1

A

% Apoptotic cells

Days after exposure to PLX4720

B

Mel-RMu  Mel-RMu.S

Mel-CV  Mel-CV.S

C

Mel-RMu  Mel-RMu.S  Mel-CV  Mel-CV.S

-p21

-p27

-CDK4

-Cyclin D1

-GAPDH

D

Mel-RMu  Mel-RMu.S

Mel-CV  Mel-CV.S
Figure 2
Figure 3

A

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<tr>
<th></th>
<th>Mel-RMu.S</th>
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<tr>
<td>Con siRNA</td>
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<td>ERK1 siRNA</td>
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<td>ERK2 siRNA</td>
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![Western blot images of ERK-1 and ERK-2]

B

% Apoptotic cells

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![Bar graph showing apoptosis levels]

C

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![Western blot images of procaspase-3 and cleaved caspase-3]

-GAPDH
Figure 4
Figure 5
Figure 6
Figure 1
Figure 2
Figure 3

A

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B

% Apoptotic cells

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- Pro-caspase-3
- Cleaved caspase-3
- GAPDH
Figure 4
Figure 6