Combinatorial Treatments That Overcome PDGFRβ-Driven Resistance of Melanoma Cells to \( V_{600E} \) B-RAF Inhibition

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Abstract

\( V_{600E} \) B-RAF mutation is found in 50% to 60% of melanomas, and the novel agents PLX4032/vemurafenib and GSK2118436 that inhibit the \( V_{600E} \) B-RAF kinase achieve a remarkable clinical response rate. However, as might be expected, acquired clinical resistance to these agents arises in most melanoma patients. PLX4032/vemurafenib resistance that arises \textit{in vivo} in tumor matched short-term cultures or \textit{in vitro} in melanoma cell lines is not caused by acquisition of secondary mutations in \( V_{600E} \) B-RAF but rather is caused by upregulating platelet-derived growth factor receptor β (PDGFRβ) or N-RAS which results in resistance or sensitivity to mitogen-activated protein (MAP)/extracellular signal-regulated (ERK; MEK) kinase inhibitors, respectively. In this study, we define a targeted combinatorial strategy to overcome PLX4032/vemurafenib resistance in melanoma cell lines or short-term culture where the resistance is driven by PDGFRβ upregulation, achieving synergistic growth inhibition and cytotoxicity. PDGFRβ-upregulated, PLX4032-resistant (PPRM) cell lines show dual phospho (p)-ERK and p-AKT upregulation, and their growth inhibitory responses to specific small molecule inhibitors correlated with p-ERK, p-AKT, and p-p70S6K levels. Coordinate inhibition of \( V_{600E} \) B-RAF inhibition and the RTK–PI3K–AKT–mTORC axis led to functionally significant rebound signaling, illustrating a robust and dynamic network connectivity. Combined B-RAF, phosphoinositide 3-kinase (PI3K), and mTORC1/2 inhibition suppressed both immediate early and delayed compensatory signaling, resulting in a highly synergistic growth inhibitory response but less efficient cytotoxic response. In contrast, the combination of MEK1/2, PI3K, and mTORC1/2 inhibitors consistently triggered apoptosis in a highly efficient manner. Together, our findings offer a rational strategy to guide clinical testing in preidentified subsets of patients who relapse during treatment with \( V_{600E} \) B-RAF inhibitors. Cancer Res; 71(15); 5067–74. ©2011 AACR.

Introduction

The majority of human melanomas constitutively activate the mitogen-activated protein kinase (MAPK) pathway through oncogenic mutations in either N-RAS or B-RAF (1–3). Targeted inhibition of \( V_{600E}/B-\)RAF (>99% of B-RAF mutations) in patients with advanced melanoma with PLX4032/vemurafenib or GSK2118436 (4, 5) showed exquisite B-RAF- \textquotedblleft oncogene addiction.\textquotedblright However, acquired resistance in the majority of patients presents a formidable obstacle to long-term tumor responses. Recent works aimed at understanding how melanomas acquire resistance to B-RAF inhibition point to either MAPK reactivation (via secondary N-RAS mutations (6) or COT/MAP3K8 kinase overexpression (7), or activation of a MAPK-redundant survival pathway (via receptor tyrosine kinases (RTK) such as platelet-derived growth factor receptor β (PDGFRβ; ref 6) or insulin-like growth factor 1 receptor (8).

The PI3K–AKT–mTOR pathway seems to provide this MAPK-redundant survival pathway (8, 9). Cross-talk between MAPK and PI3K–AKT–mTOR has been reported in various cancer types (10–14). The extensive network relationships between these signaling pathways (15) at nodes of cross-talk point to treatment-induced compensatory signaling as a potential barrier to effective targeted cancer therapy. For instance, inhibition of the MAPK pathway by targeting [MAP/extracellular signal-regulated kinase (ERK; MEK)] MEK1/2 in melanoma cell lines can result in treatment-induced AKT activation, mediating resistance (16).

Here, taking advantage of PLX4032/vemurafenib–acquired resistant cell lines and a short-term culture with defined PDGFRβ upregulation, we show that single target inhibition of the RTK–PI3K–AKT–mTORC pathway in the presence of B-RAF inhibition resulted in powerful and immediate early (1 hour) rebound signaling in either the MAPK pathway (downstream of B-RAF) or PI3K–AKT pathway itself. AZD8055, an inhibitor of both mTORC1 and mTORC2...
complexes (17), synergized with PLX4032/vemurafenib in the growth inhibition of PPRM cell lines. Furthermore, the combined inhibition of phosphoinositide 3-kinase (PI3K) on top of dual mTORC1/mTORC2 inhibition, achieved by using the novel inhibitor BEZ235 (18), overcame delayed, compensatory signaling at AKT, further augmenting synergy with PLX4032/vemurafenib. Delayed compensatory signaling at MEK1/2 can limit the extent of a cytotoxic response, as substituting MEK1/2 for B-RAF inhibition reduced phospho (p)-ERK recovery and augmented apoptotic induction in conjunction with dual PI3K and mTORC1/2 inhibition. Because all these novel inhibitors and similar targeted agents are undergoing clinical evaluation (ClinicalTrials.gov), their combination represents a promising and translatable approach to overcome a subset of melanomas escaping BRAF inhibitors.

Materials and Methods

Cell culture, lentiviral constructs, and infections

All cell lines were maintained in Dulbecco’s modified Eagle’s medium with 10% or 20% heat-inactivated FBS (Omega Scientific), 2 mmol/L glucose in a humidified 5% CO₂ incubator, and 1 μmol/L PLX4032 (if drug resistant). Lentiviral constructs for wild type PDGFRα overexpression and knockdown (shPDGFRα) have been described (6). Three-dimensional spheroid growth assay was conducted as described (19). Briefly, PPRM cells were seeded into ultralow attachment plates (Costar), and spheroids (72 hours) were implanted into a bovine collagen I matrix (Invitrogen). One representative spheroid was selected from each well and tracked each day by photography.

Cellular proliferation and drug treatments

Cell proliferation experiments were carried out in a 96-well format (5 replicates), and drug treatments initiated at 24 hours postseeding for 72 hours. Stocks and dilutions of PLX4032 (Plexxikon), AZD6244, BEZ235, MK2206 (Selleck Chemicals), AKTi (Merck), AZD8055 (Chemieinstitute), sunitinib, imatinib (LC Laboratories), rapamycin (Sigma–Aldrich), LY294002, and wortmannin (Promega) were made in dimethyl sulfoxide (DMSO). Cells were quantified by using CellTiter-GLO Luminescence (Promega) following the manufacturer’s recommendations. Spheroids were treated with either DMSO or indicated drugs at day 1 postimplantation into collagen. At day 6, spheroids were washed twice in PBS before calcein-AM and ethidium bromide (Molecular Probes) were added for 1 hour at 37°C prior to fluorescence microscopy.

Protein and apoptosis detection

Cell lysates for Western blotting were made in RIPA (Sigma) with protease (Roche) and phosphatase (Santa Cruz Biotechnology) inhibitor cocktails. Western blots were probed with antibodies against p-ERK1/2 (T202/Y204), total ERK1/2, PDGFRβ, p-AKT (S473), p-AKT (T308), total AKT1/2, p-p70S6K (T389), p-H2AX (Y159), p-ADAP (Y218), p-PDGFRβ (Y751) and (Y771), and p-AKT in PPRM cell lines (Fig. 1A). Although Pt48 R, a cell line derived from a PLX4032-acquired resistant melanoma tumor, lacks an isogenic, drug-resistant counterpart, it displayed similarly elevated levels of p-AKT and p-ERK (Fig. 1A and C). PDGFB-B–treated NIH3T3 cells upregulated the p-AKT level dramatically and the p-ERK level to a lesser extent (Fig. 1A). PDGFRβ protein upregulation in PPRM cell lines and culture are associated with its hyperphosphorylation (p-Y751 and p-Y771 for PI3K and RAS GTPase activating protein docking, respectively; Supplementary Fig. S1A), which is in turn sensitive to dose-dependent inhibition by imatinib and sunitinib (multikinase inhibitors with activity against PDGFRβ; Supplementary Fig. S1B). Additionally, overexpression of wild-type PDGFRβ with PDGF-BB stimulation in M229 and M238 parental cells caused a dramatic increase in the levels of p-AKT while having little appreciable effect on the levels of p-ERK (Supplementary Fig. S2). Conversely, stable knockdown of PDGFRβ in M238 R1 downregulated p-AKT but not p-ERK levels but paradoxically increased p-AKT in M229 R5, suggesting compensation (Supplementary Fig. S2). Thus, PDGFRβ upregulation likely drives PI3K-AKT survival signaling, whereas p-ERK accumulation in PPRM cell lines may be a consequence of prolonged culture with PLX4032 and loss of negative feedback, altered RAF usage, signal cross-talk, or as yet uncharacterized mechanisms (8, 13, 20).

We then systematically tested the potential suppressive effect of individual inhibitors of the RTK–PI3K–AKT–mTORC axis on the growth of PPRM cell lines. In cell survival assays (PPRM cell lines maintained with 1 μmol/L PLX4032, unless otherwise indicated), imatinib and sunitinib (Fig. 1B and C, top) as well as the AKT1/2 inhibitor, AKTi (Fig. 1B, top), were individually inactive against PPRM cell lines. Although AKT1/2 inhibition dramatically reduced p-AKT levels (Fig. 1B), making AKT3-mediated survival rescue unlikely, pan-AKT inhibition by using MK2206 (21) was similarly inactive against PPRM cell lines (Supplementary Fig. S3). As treatment (1 hour) with imatinib or AKTi caused a rebound increase in p-ERK at room temperature. Flow cytometry data were analyzed by the fluorescence-activated cell sorting Express V2 software.

Data processing

Statistical analyses were conducted by using InStat 3 Version 3.0b (GraphPad Software); graphical representations by using DeltaGraph or Prism (Red Rock Software); and combination index (CI) calculation by using CalcuSyn V2.1 (Biosoft).

Results and Discussion

Individual target (PDGFRβ, AKT, mTORC1) inhibition and compensatory signaling

PPRM cell lines (M229 R5 and M238 R1) harbor a V600EB-RAF transcriptional signature upon PLX4032 withdrawal, indicating persistent MAPK signaling, and their p-ERK levels remain highly sensitive to PLX4032 treatment, suggesting MAPK not being the sole survival pathway (6). We examined the relative levels of p-AKT and p-ERK in M229 R5 and M238 R1 and their isogenic parental cell lines (M229 and M238, respectively) and found highly elevated levels of both p-ERK and p-AKT in PPRM cell lines (Fig. 1A). Although Pt48 R, a PDGFRα counterpart (Supplementary Fig. S1A), which is in turn sensitive to dose-dependent inhibition by imatinib and sunitinib (multikinase inhibitors with activity against PDGFRβ; Supplementary Fig. S1B). Additionally, overexpression of wild-type PDGFRβ with PDGF-BB stimulation in M229 and M238 parental cells caused a dramatic increase in the levels of p-AKT while having little appreciable effect on the levels of p-ERK (Supplementary Fig. S2). Conversely, stable knockdown of PDGFRβ in M238 R1 downregulated p-AKT but not p-ERK levels but paradoxically increased p-AKT in M229 R5, suggesting compensation (Supplementary Fig. S2). Thus, PDGFRβ upregulation likely drives PI3K-AKT survival signaling, whereas p-ERK accumulation in PPRM cell lines may be a consequence of prolonged culture with PLX4032 and loss of negative feedback, altered RAF usage, signal cross-talk, or as yet uncharacterized mechanisms (8, 13, 20).

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levels (Fig. 1B, bottom; Pt48 R data not shown), we asked whether 0.5 μmol/L of the MEK inhibitor AZD6244, which by itself is inactive against PPRM cell lines up to 10 μmol/L (6), could enhance the growth-inhibitory activity of imatinib, sunitinib, or AKTi (Fig. 1B and C, top). AZD6244 was effective in sensitizing only M229 R5 to imatinib (IG50 from not reached, NR; to 2 μmol/L), Pt48 R to sunitinib (IG50 from 1.5 to 0.4 μmol/L), and M229 R5 to AKTi (IG50 from 3 to 0.03 μmol/L). Only AZD6244-induced sensitization of Pt48 R to sunitinib correlated with p-p70S6K suppression (Fig. 1B and C, bottom). On the other hand, despite strong suppression of both p-ERK and p-AKT by combined AKTi + AZD6244 or sunitinib + AZD6244 treatments in M238 R1, the p-p70S6K level remained refractory to suppression (Fig. 1B and C, bottom), suggesting a cell context-dependent differences in mTOR-p70S6K activation, PDGFRβ phosphorylation, RAS-GTP levels, and/or C-RAF usage (8, 22). The inconsistent growth-suppressive effects of combining RTK (imatinib or sunitinib) and MAPK (PLX4032 or AZD6244) inhibition are contrasted to the cell-cycle arresting effects of stable PDGFRβ knockdown as reported earlier (6), as the functional effects of small molecule inhibitors and short hairpin (shRNA)-mediated genetic knockdown should not be viewed as equal given that the latter (multiple independent shRNAs) produces a more sustained and specific inhibition of the intended target than the former (‘dirty’ kinase inhibitors).
Rapamycin, an inhibitor of the mTORC1 complex, induced significant rebound increases in both p-ERK and p-AKT levels, which were ineffectively counter inhibited by AZD6244 and AKTi, respectively (Fig. 1D, top). In this context, rapamycin-induced mTORC1 inhibition has been shown to activate the MAPK pathway through a PI3K-dependent feedback loop in cancer cells (10). Thus, despite near complete downregulation of p-p70S6K with rapamycin treatment (Fig. 1D, bottom; Pt48 R data not shown), rapamycin titration in the presence of AZD6244 or both AZD6244 and AKTi was still ineffectual in growth-inhibiting PPRM cell lines (Fig. 1D, top). In fact, rapamycin rendered M229 R5 insensitive to combination treatment with PLX4032, AZD6244, and AKTi (Fig. 1B, top). The lower degree of rapamycin-induced p-ERK rebound in M238 R1 may account for the greater degree of p-ERK suppression on cotreatment with AZD6244 (Fig. 1D, top). Together, these data argue for extensive RTK–MAPK–AKT pathway connectivity in PPRM cell lines that affords compensatory survival signaling even with simultaneous inhibition of multiple targets.

Synergistic growth inhibition by combining PI3K, mTORC1/2, and B-RAF suppression

We then tested the growth-inhibitory effect of the dual mTORC1/2 inhibitor, AZD8055, on PPRM cell lines. As seen in Figure 2A, AZD8055 treatment alone suppressed p-ERK levels. Expectedly, PLX4032 treatment alone suppressed p-AKT and p-p70S6K levels. Expectedly, PLX4032 treatment alone suppressed p-ERK levels. Combination treatment with AZD8055 and PLX4032 was more effective than either drug treatment alone in growth inhibition,
suggesting that PLX4032-mediated suppression of p-ERK levels is critical for this synergistic growth inhibition. A calculation of CI values was consistent with a synergistic effect (Fig. 2B; Supplementary Table S2). Although the PI3K inhibitors, LY294002 or wortmannin, in the presence of PLX4032 were individually inactive against PPRM cell lines (Supplementary Fig. S4), the dual PI3K and mTORC1/2 inhibitor BEZ235 was highly specific (vs. parental lines) and potent in growth-inhibiting PPRM cell lines (Fig. 2C). Both M229 R5 and M238 R1 were highly sensitive to BEZ235 compared with their parental cells (M229, IC_{50} = 0.2 μmol/L vs. M229 R5, IC_{50} = 0.007 μmol/L; M238, IC_{50} = 0.5 μmol/L vs. M238 R1, IC_{50} = 0.0001 μmol/L). Pt48 R was similarly highly sensitive to BEZ235 (IC_{50} = 0.01 μmol/L). Notably, we found that PLX4032 dramatically enhanced the efficacy of BEZ235 (Fig. 2C), resulting in a highly synergistic CI (Fig. 2B; Supplementary Table S2). Despite the potent growth-inhibitory effect of BEZ235 combined with PLX4032, incomplete growth inhibition at high drug concentrations suggests ineffective cell death induction over the duration of the assay (see below). A high degree of synergy between AZD8055 (or BEZ235) and PLX4032 in growth-inhibiting PPRM cell lines strongly indicates that MAPK remains a functional survival pathway redundant to and cooperative with the AKT pathway.

**Importance of sustained pathway suppression**

The higher degree of synergy observed with the PLX4032 and BEZ235 versus the PLX4032 and AZD8055 combination could not be explained by the level of p-ERK, p-AKT, and p-p70S6K suppression at 1 hour of drug treatments alone or in combination (Fig. 2A and C, bottom). Thus, we posited that PI3K may serve as a node of delayed retrograde, rebound signaling and its coinhibition may facilitate sustained p-AKT suppression. Indeed, at 24 hours (vs. 1 hour; Fig. 2A and C, bottom) of drug treatment, we found a significant re-accumulation of p-AKT with mTORC1/2 suppression by AZD8055, suggesting a delayed PI3K-PDK-1–dependent phosphorylation and activation of p-AKT (Fig. 3A). In contrast, PI3K and mTORC1/2 concomitant suppression by BEZ235 led to sustained p-AKT suppression at 24 hours. This differential p-AKT recovery did not seem to impact p-p70S6K levels, occurred regardless of PLX4032 treatment.

**Figure 3.** Cotargeting of specific signaling nodes attenuates AKT and ERK signal recovery. A, B-RAF targeting combined with cosuppression of PI3K and mTORC1/2 prevented delayed (24-hour) p-AKT recovery. Western blots of lysates from PPRM cells treated with DMSO, 1 μmol/L PLX4032, 0.1 μmol/L AZD8055, PLX4032 + AZD8055, 0.1 μmol/L BEZ235, or PLX4032 + BEZ235 for 24 hours. B, Western blots showing indicated protein levels in PPRM cell lines treated with DMSO or a single dose of AZD8055 (0.1 μmol/L) or BEZ235 (0.1 μmol/L) for the indicated durations (h) in the presence of 1 μmol/L PLX4032. C, MEK1/2 targeting combined with co-suppression of PI3K and mTORC1/2 prevented delayed (24-hour) p-ERK recovery. Western blots showing indicated protein levels from indicated cell lines treated with DMSO, 0.1 μmol/L BEZ235, 1 μmol/L AZD6244, or both for 24 hours. D, Western blots showing indicated protein levels in PPRM cell lines treated with DMSO or a single dose of 1 μmol/L PLX4032 or 1 μmol/L AZD6244, with or without cotreatment with AZD8055 (0.1 μmol/L) or BEZ235 (0.1 μmol/L), for the indicated durations (h).
was appreciable as early as 6 hours posttreatment, and became even more dramatic by 48 hours (Fig. 3B). Although the \textit{in vitro} potencies of AZD8055 versus BEZ235 toward mTORC1/2 are reportedly similar (17, 18), we cannot rule out differential accumulated levels of these small molecule inhibitors over time. Nevertheless, it is important to not only track immediate early but also delayed compensatory signaling.
A critical role for MEK1/2 inhibition in cooperative cytotoxicity

Effective growth inhibition of PPRM cell lines with BEZ235 and PLX4032 correlated with little if any appreciable apoptosis at day 4 (Supplementary Fig. S5), despite strong suppression of p-AKT and p-p70S6K levels (Fig. 3A and B). To address this apparent paradox, we noted significant p-ERK recovery at 24 hours post-PLX4032 treatment (Fig. 3A), which was not apparent at 24 hours post-AZD6244 treatment (Fig. 3C). We thus tracked delayed compensatory p-ERK recovery post-PLX4032 versus AZD6244 treatments, with or without AZD8055 or BEZ235 treatments (Fig. 3D). Indeed, PLX4032 treatment was followed by rapid (6 hours) and significant levels of p-ERK recovery (back to the baseline p-ERK levels in M229 R5 and M238 R1). Curiously, mTORC1/2 inhibition enhanced p-ERK recovery after PLX4032 treatment (Fig. 3D), consistent with earlier experiments (Fig. 2A and C, bottom). Importantly, AZD6244 treatment, compared with PLX4032 treatment, strongly attenuated p-ERK recovery extending to 48 hours (Fig. 3D), even in the presence of mTORC1/2 cotargeting. This dramatic difference in p-ERK recovery in response to PLX4032 versus AZD6244 treatment is not likely to be because of differential inhibitor retention/metabolism, as the difference was noted as early as 6 hours posttreatments, the inhibitor concentrations used were identical (1 μmol/L), and the IC50 values of PLX4032 and AZD6244 against the parental line M229 are quite similar, suggesting similar pharmacokinetic and MAPK pharmacodynamic properties (Supplementary Fig. S6).

We then confirmed the potent growth inhibitory combination of MAPK (via PLX4032 or AZD6244) and dual PI3K and mTORC1/2 (via BEZ235) suppression uncovered in 2-dimensional cultures in 3-dimensional spheroid assays of live versus dead cells in a collagen I matrix (Fig. 4A and Supplementary Fig. S7). To quantify apoptotic cells directly, we measured Annexin V-propidium iodide staining and asked whether AZD6244, which is associated with attenuated p-ERK recovery compared with PLX4032, would synergize with BEZ235 to induce cytotoxicity. By day 5 (M229 R5, Pt48 R) or day 6 (M238 R1) of drug treatments, low levels of apoptosis were detectable with BEZ235 treatment alone or combined treatment with PLX4032 (Fig. 4B). PLX4032 (vs. DMSO) treatment alone expectedly caused minimally elevated levels of apoptosis. In Pt48 R, the BEZ235/PLX4032 combination induced a level of apoptosis that was more than additive (Fig. 4B), correlating with the least degree of p-ERK recovery on PLX4032 treatment in this cell line (Fig. 3A and D). Notably, AZD6244 strongly synergized with BEZ235 in apoptosis induction in all PPRM cell lines (Fig. 4C), consistent with apoptosis tracking by using an alternative marker (p-H2AX levels, Supplementary Fig. S8).

Thus, studies of these cell line models of RTK driven, PLX4032/vemurafenib acquired resistance pointed to a common feature of hyperactive AKT which extensively crosstalks with the MAPK pathway, permitting compensatory survival signaling with insufficient number of target inhibition. Sustained and combined suppression of the MAPK pathway and the PI3K–AKT–mTORC axis appeared to be critical to overcome PLX4032/vemurafenib acquired resistance. This study highlights the need not only to identify mechanisms of acquired resistance to targeted kinase inhibitors but also to understand the signaling network associated with each mechanism to generalize potential translatable approaches to overcome drug resistance.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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