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Combinatorial treatments that overcome PDGFR β -driven resistance of melanoma cells to ^{V600E}B-RAF inhibition

Hubing Shi¹, Xiangju Kong¹, Antoni Ribas^{2,3,4}, and Roger s. Lo^{1,4,5}

¹Division of Dermatology/Department of Medicine, David Geffen School of Medicine, University of California, LA, California 90095-1662 USA

²Division of Surgical Oncology/Department of Surgery, David Geffen School of Medicine, University of California, LA, California 90095-1662 USA

³Division of Hematology & Oncology/Department of Medicine, David Geffen School of Medicine, University of California, LA, California 90095-1662 USA

⁴Jonsson Comprehensive Cancer Center, David Geffen School of Medicine, University of California, LA, California 90095-1662 USA

Abstract

^{V600E}B-RAF mutation is found in 50–60% of melanomas, and the novel agents PLX4032/vemurafenib and GSK2118436 that inhibit the ^{V600E}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 *in vivo* in tumor-matched short-term cultures or *in vitro* in melanoma cell lines is not caused by acquisition of secondary mutations in ^{V600E}B-RAF but rather by upregulating PDGFR β or N-RAS which results in resistance or sensitivity to MEK 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 PDGFR β upregulated (PPRM cell lines), achieving synergistic growth inhibition and cytotoxicity. PPRM cell lines show dual p-ERK and p-AKT upregulation, and their growth inhibitory responses to specific small molecule inhibitors correlated with p-ERK, p-AKT, p-p70S6K levels. Coordinate inhibition of ^{V600E}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, 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 pre-identified subsets of patients who relapse during treatment with ^{V600E}B-RAF inhibitors.

Keywords

melanoma; targeted therapy; acquired resistance; B-RAF; AKT; vemurafenib

⁵Corresponding author 52-121 CHS Dept. of Medicine/Dermatology, 10833 Le Conte Ave, Los Angeles, CA 90095-1750. Phone: (310) 825-5420, Fax: Fax (310) 206-9878, rlo@mednet.ucla.edu.

Disclosure of Potential Conflicts of Interest

The authors declare no conflicts of interest.

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 ^{V600E/K}B-RAF (> 99% of B-RAF mutations) in patients with advanced melanoma with PLX4032/vemurafenib or GSK2118436 (4, 5) demonstrated exquisite ^{V600E/K}B-RAF “oncogene addiction.” 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 ^{V600E/K}B-RAF inhibition point to either MAPK reactivation (*via* secondary N-RAS mutations (6) or COT/MAP3K8 kinase over-expression (7)), or activation of a MAPK-redundant survival pathway (*via* receptor tyrosine kinases (RTKs) such as PDGFR β (6) or IGF1R (8)).

The phosphatidylinositol 3-kinase to AKT to mammalian target of rapamycin (PI3K-AKT-mTOR) pathway appears to provide this MAPK-redundant survival pathway (8, 9). Crosstalk 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 crosstalk point to treatment-induced compensatory signaling as a potential barrier to effective targeted cancer therapy. For instance, inhibition of the MAPK pathway by targeting 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 PDGFR β 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 h) rebound survival 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 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 p-ERK recovery and augmented apoptotic induction in conjunction with dual PI3K and mTORC1/2 inhibition. Because all these novel inhibitors are undergoing clinical evaluation (ClinicalTrials.gov), their combination represents a promising and translatable approach to overcome a subset of melanomas escaping BRAF inhibitors.

Methods

Cell culture, lentiviral constructs and infections

All cell lines were maintained in DMEM with 10 or 20% heat-inactivated FBS (Omega Scientific), 2 mmol/L glutamine in a humidified, 5% CO₂ incubator and 1 μ M PLX4032 (if drug resistant). Lentiviral constructs for wild type PDGFR β over-expression and knockdown (shPDGFR β) have been described (6). Three-dimensional spheroid growth assay was performed as described (19). Briefly, PPRM cells were seeded into ultra-low attachment plates (Costar), and spheroids (72 h) 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 performed in a 96 well format (five replicates) and drug treatments initiated at 24 h post-seeding for 72 h. Stocks and dilutions of PLX4032

(Plexxikon, Berkeley, CA), AZD6244, BEZ235, MK2206 (Selleck Chemicals), AKTi (Merck), AZD8055 (ChemieTek), sunitinib, imatinib (LC Laboratories), rapamycin (Sigma-Aldrich), LY294002 and wortmannin (Promega) were made in DMSO. Cells were quantified using CellTiter-GLO Luminescence (Promega) following the manufacturer's recommendations. Spheroids were treated with either DMSO or indicated drugs at day 1 post implantation 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, PDGFR β , p-AKT (S473), p-AKT (T308), total AKT1/2, p-p70S6K (T389), total p70S6K, p-PDGFR β (Y751) and (Y771), and p-H2AX (S140), total H2AX (all from Cell Signaling Technologies) and tubulin (Sigma). Apoptotic cells were stained with Annexin V-V450 (BD Pharmingen) and PI for 15 min at room temperature. Flow cytometry data were analyzed by the FACS Express V2 software.

Data processing

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

Results and Discussion

Individual target (PDGFR β , AKT, mTORC1) inhibition and compensatory signaling

PPRM cell lines (M229 R5 and M238 R1) harbor a V600E-B-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 culture derived from a PLX4032-acquired resistant melanoma tumor, lacks an isogenic, drug-sensitive counterpart, it displayed similarly elevated levels of p-AKT and p-ERK (Fig. 1A). PDGF-BB-treated NIH3T3 cells upregulated the p-AKT level dramatically and the p-ERK level to a lesser extent (Fig. 1A). PDGFR β protein upregulation in PPRM cell lines and culture are associated with its hyper-phosphorylation (p-Y751 and p-Y771 for PI3K and RAS GTPase activating protein docking, respectively) (fig. S1A), which is in turn sensitive to dose-dependent inhibition by imatinib and sunitinib (multi-kinase inhibitors with activity against PDGFR β (fig. S1B). Additionally, over-expression of wild type PDGFR β 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 (fig. S2). Conversely, stable knockdown of PDGFR β in M238 R1 downregulated p-AKT but not p-ERK levels but paradoxically increased p-AKT in M229 R5, suggesting compensation (fig. S2). Thus, PDGFR β upregulation likely drives PI3K-AKT survival signaling, while 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 crosstalk, 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 μ M PLX4032, unless otherwise indicated), imatinib and

sunitinib (Fig. 1, B 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 using MK2206 (21) was similarly inactive against PPRM cell lines (fig. S3). As treatment (1 h) with imatinib or AKTi caused a rebound increase in p-ERK levels (Fig. 1B, bottom; Pt48 R data not shown), we asked whether 0.5 μ M of the MEK inhibitor AZD6244, which by itself is inactive against PPRM cell lines up to 10 μ M (6), could enhance the growth-inhibitory activity of imatinib, sunitinib, or AKTi (Fig. 1, B and C, top). AZD6244 was effective in sensitizing only M229 R5 to imatinib (IG_{50} from not reached, NR, to 2 μ M), Pt48 R to sunitinib (IG_{50} from 1.5 μ M to 0.4 μ M), and M229 R5 to AKTi (IG_{50} from 3 μ M to 0.03 μ M). Only AZD6244-induced sensitization of Pt48 R to sunitinib correlated with p-p70S6K suppression (Fig. 1, B 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, PDGFR β 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 PDGFR β knockdown as reported earlier (6), as the functional effects of small molecule inhibitors and 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 down-regulation 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 co-treatment 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 PPRM cell line growth and 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 combination index (CI) values was consistent with a synergistic effect (Fig. 2B; table S2). While the PI3K inhibitors, LY294002 or wortmannin, in the presence of PLX4032 were individually inactive against PPRM cell lines (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 to their parental cells (M229, IG_{50} = 0.2 μ M *vs.* M229 R5, IG_{50} = 0.007 μ M; M238, IG_{50} = 0.5 μ M *vs.* M238 R1, IG_{50} = 0.0001 μ M). Pt48 R was similarly highly sensitive to BEZ235 (IG_{50} = 0.01 μ M). Notably, we found that PLX4032 dramatically

enhanced the efficacy of BEZ235 (Fig. 2C), resulting in a highly synergistic CI (Fig. 2B; 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 *vs.* the PLX4032 and AZD8055 combination could not be explained by the level of p-ERK, p-AKT, and p-p70S6K suppression at one hour of drug treatments alone or in combination (Fig. 2, A and C, bottom). Thus, we posited that PI3K may serve as a node of delayed retrograde, rebound signaling and its co-inhibition may facilitate sustained p-AKT suppression. Indeed, at 24 h (*vs.* 1 h, Fig. 2, A 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-PDK1-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 h. This differential p-AKT recovery did not appear to impact p-p70S6K levels, occurred regardless of PLX4032 treatment (Fig. 3A), was appreciable as early as 6 h post treatment, and became even more dramatic by 48 h (Fig. 3B). Although the *in vitro* potencies of AZD8055 *vs.* 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 (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 h post PLX4032 treatment (Fig. 3A) which was not apparent at 24 h post AZD6244 treatment (Fig. 3C). We thus tracked delayed compensatory p-ERK recovery post PLX4032 *vs.* AZD6244 treatments, with or without AZD8055 or BEZ235 treatments (Fig. 3D). Indeed, PLX4032 treatment was followed by rapid (6h) 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 to PLX4032 treatment, strongly attenuated p-ERK recovery extending to 48 h (Fig. 3D), even in the presence of mTORC1/2 co-targeting. This dramatic difference in p-ERK recovery in response to PLX4032 *vs.* AZD6244 treatment is not likely to be due to differential inhibitor retention/metabolism, as the difference was noted as early as 6 h post treatments, the inhibitor concentrations used were identical (1 μ M), and the IG_{50} values of PLX4032 and AZD6244 against the parental line M229 are quite similar, suggesting similar pharmacokinetic and MAPK pharmacodynamic properties (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 two-dimensional cultures in three-dimensional spheroid assays of live *vs.* dead cells in a collagen I matrix (Fig. 4A and fig. S7). To quantify apoptotic cells directly, we measured propidium iodide/annexin-V staining and asked whether AZD6244, which is associated with attenuated p-ERK recovery compared with PLX4032, would synergize with BEZ235 to induced 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 using an alternative marker (p-H2AX levels, 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 in order to generalize potential translatable approaches to overcome drug resistance.

Supplementary Material

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Figure 1.

Compensatory signaling limits the growth-inhibitory responses of PPRM cell lines to co-targeting of V^{600E} -B-RAF with RTK, AKT or mTORC1. A, Upregulation of p-AKT (Ser 473) and p-ERK in PPRM cell lines. Western blots of lysates showing relative levels of indicated proteins in PPRM (1 μ M PLX4032) and parental cell lines. NIH3T3, treated with PDGF-BB (20 ng/ml, 1 h) or PBS. Tubulin, loading control. B, Survival curves of PPRM cell lines (treated with 1 μ M PLX4032, unless otherwise indicated) in response to imatinib or AKTi titration with or without 0.5 μ M AZD6244 (72 h) (top; mean $\pm$ SD, n=5). Dashed line, 50% growth inhibition. Western blots showing indicated protein levels from cells treated (1 h) with DMSO, 0.5 μ M imatinib (or 0.5 μ M AKTi), 0.5 μ M AZD6244 or both (bottom). C, Survival curves in response to sunitinib titration with or without 0.5 μ M AZD6244 (72 h) (top) and Western blots showing indicated protein levels from cells treated (1 h) with DMSO, 0.2 μ M sunitinib, 0.5 μ M AZD6244, or both (bottom). D, Western blots showing indicated proteins levels in cells treated (1 h) with DMSO, 0.1 μ M rapamycin, rapamycin + 0.5 μ M AZD6244, or rapamycin + AZD6244 + 0.5 μ M AKTi (top) and survival curves in response to rapamycin titration alone or in the presence of 0.5 μ M AZD6244 or AZD6244 + 0.5 μ M AKTi (bottom).

Figure 2.

MAPK targeting strongly synergizes with co-suppression of PI3K and mTORC1/2 in growth inhibiting PPRM cell lines. A, Survival curves for PPRM cell lines titrated with the mTORC1/2 inhibitor AZD8055 with or without 1 μ M PLX4032 for 72 h (top) and Western blots showing indicated proteins levels treated with DMSO, PLX4032 (1 μ M), AZD8055 (0.1 μ M) or both for 1 h (bottom). B, Relative synergy of indicated drug combinations (expressed as $\log_{10}$ of CI values). C, Survival curves for PLX4032-sensitive parental lines *vs.* PLX4032-resistant sub-lines (1 μ M PLX4032) titrated with BEZ235 (top) or for all PPRM cells titrated with BEZ235 with or without of 1 μ M PLX4032 (middle). Western blots showing indicated proteins levels from PPRM cells were treated with DMSO, 0.1 μ M BEZ235, 1 μ M PLX4032, or both drugs for 1 h (bottom).

Figure 3.

Co-targeting of specific signaling nodes attenuates AKT and ERK signal recovery. A. B. RAF targeting combined with co-suppression of PI3K and mTORC1/2 prevented delayed (24 h) p-AKT recovery. Western blots of lysates from PPRM cells treated with DMSO, 1 μ M PLX4032, 0.1 μ M AZD8055, PLX4032 + AZD8055, 0.1 μ M BEZ235, or PLX4032 + BEZ235 for 24 h. B. Western blots showing indicated protein levels in PPRM cell lines treated with DMSO or a single dose of AZD8055 (0.1 μ M) or BEZ235 (0.1 μ M) for the indicated durations (h) in the presence of 1 μ M PLX4032. C. MEK1/2 targeting combined with co-suppression of PI3K and mTORC1/2 prevented delayed (24 h) p-ERK recovery. Western blots showing indicated protein levels from indicated cell lines treated with DMSO, 0.1 μ M BEZ235, 1 μ M AZD6244, or both for 24 h. D. Western blots showing indicated protein levels in PPRM cell lines treated with DMSO or a single dose of 1 μ M PLX4032 or 1 μ M AZD6244, with or without co-treatment with AZD8055 (0.1 μ M) or BEZ235 (0.1 μ M), for the indicated durations (h).

Figure 4.

MEK1/2, rather than B-Raf, targeting in combination with dual PI3K and mTORC1/2 suppression augments apoptosis induction. A, Live (green) vs. dead (orange) cells in PPRM spheroids treated with DMSO, 1 μ M PLX4032, 1 μ M AZD6244, 0.1 μ M BEZ235 or the indicated combinations (dosed every 48 h) for 6 days. B, PPRM cells treated with DMSO (D), 1 μ M PLX4032 (P), 0.1 μ M BEZ235 (B) or both (P+B) for 5 days (M229 R5, Pt48 R) or 6 days (M238 R1); levels of apoptosis plotted on the right. C, Treatment with DMSO, 1 μ M AZD6244 (A), 0.1 μ M BEZ235, or both and apoptosis plotted.