

## Combinations of BRAF, MEK, and PI3K/mTOR Inhibitors Overcome Acquired Resistance to the BRAF Inhibitor GSK2118436 Dabrafenib, Mediated by *NRAS* or *MEK* Mutations

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### Abstract

Recent results from clinical trials with the BRAF inhibitors GSK2118436 (dabrafenib) and PLX4032 (vemurafenib) have shown encouraging response rates; however, the duration of response has been limited. To identify determinants of acquired resistance to GSK2118436 and strategies to overcome the resistance, we isolated GSK2118436 drug-resistant clones from the A375 BRAF<sup>V600E</sup> and the YUSIT1 BRAF<sup>V600K</sup> melanoma cell lines. These clones also showed reduced sensitivity to the allosteric mitogen-activated protein/extracellular signal-regulated kinase (MEK) inhibitor GSK1120212 (trametinib). Genetic characterization of these clones identified an in-frame deletion in *MEK1* (MEK1<sup>K59del</sup>) or *NRAS* mutation (NRAS<sup>Q61K</sup> and/or NRAS<sup>A146T</sup>) with and without MEK1<sup>P387S</sup> in the BRAF<sup>V600E</sup> background and NRAS<sup>Q61K</sup> in the BRAF<sup>V600K</sup> background. Stable knockdown of *NRAS* with short hairpin RNA partially restored GSK2118436 sensitivity in mutant *NRAS* clones, whereas expression of NRAS<sup>Q61K</sup> or NRAS<sup>A146T</sup> in the A375 parental cells decreased sensitivity to GSK2118436. Similarly, expression of MEK1<sup>K59del</sup>, but not MEK1<sup>P387S</sup>, decreased sensitivity of A375 cells to GSK2118436. The combination of GSK2118436 and GSK1120212 effectively inhibited cell growth, decreased ERK phosphorylation, decreased cyclin D1 protein, and increased p27<sup>kip1</sup> protein in the resistant clones. Moreover, the combination of GSK2118436 or GSK1120212 with the phosphoinositide 3-kinase/mTOR inhibitor GSK2126458 enhanced cell growth inhibition and decreased S6 ribosomal protein phosphorylation in these clones. Our results show that *NRAS* and/or *MEK* mutations contribute to BRAF inhibitor resistance *in vitro*, and the combination of GSK2118436 and GSK1120212 overcomes this resistance. In addition, these resistant clones respond to the combination of GSK2126458 with GSK2118436 or GSK1120212. Clinical trials are ongoing or planned to test these combinations. *Mol Cancer Ther*; 11(4); 909–20. ©2012 AACR.

### Introduction

Melanoma is a highly aggressive form of skin cancer for which effective treatment has been limited. The identification of a frequent mutation (>50%) at valine position 600 of the B-isoform of RAF kinase (BRAF<sup>V600</sup>) in melanoma (1) has held great promise for therapeutic intervention. Recent clinical trials with agents that target BRAF, GSK2118436 (dabrafenib), and PLX4032 (vemurafenib)

have shown impressive clinical responses in melanoma patients with mutant BRAF<sup>V600</sup> (2–4). The U.S. Food and Drug Administration approved vemurafenib for the treatment of BRAF<sup>V600E</sup> mutation positive, inoperable, or metastatic melanoma (5). However, the duration of response has been limited as resistance to BRAF inhibitors develops within months following initiation of treatment. These clinical observations make it imperative to determine factors that promote resistance to BRAF inhibitors and to identify potential therapies that will overcome this resistance.

Development of tumor resistance to single-targeted agents is inevitable. Gatekeeper mutations, which have been identified as one mechanism of acquired resistance to other targeted kinase inhibitors (6, 7), have also been proposed as a potential mechanism of BRAF inhibitor resistance (8). However, additional *BRAF* mutations have yet to be observed *in vitro* or in the clinic (9, 10). Instead, development of mutations in other genes, such as *NRAS* (9) or *MEK* (10), seems to reestablish RAS–MEK–ERK signaling in tumors exposed to BRAF

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inhibitors. Activation of platelet-derived growth factor PDGF receptors (PDGFR; ref. 9), IGF1R (11), AKT3 (12), and COT1 (13) have also been identified, either in clinical or preclinical models, as conferring resistance to BRAF inhibitors such as PLX4032, PLX4720, or SB590885.

GSK2118436 is a potent, ATP-competitive inhibitor of mutant BRAF, wild-type BRAF, and wild-type CRAF enzymes (14). GSK1120212 (trametinib) is a selective, allosteric inhibitor of MEK1 and MEK2 (15). *In vitro*, treatment with GSK2118436 or GSK1120212 inhibits the growth of most BRAF<sup>V600E</sup> melanoma cell lines (ref. 15; King and colleagues; submitted). Both compounds are in phase III clinical trials for the treatment of metastatic melanoma as single agents (NCT01227889 and NCT01245062). Because acquired resistance to BRAF inhibitors can lead to sustained mitogen-activated protein/extracellular signal-regulated kinase (MEK) activation in the presence of compound, the combination of BRAF and MEK inhibitors may enhance growth inhibition. This combination may also deter the outgrowth of resistant melanoma cells by inhibiting the pathway at 2 separate points. As such, the combination of GSK2118436 and GSK1120212 is being tested in phase II clinical trials (16).

In addition to increased activation of BRAF and MEK, members of the phosphoinositide 3-kinases (PI3K) pathway are elevated in metastatic melanoma (17), and inhibition of PI3K/mTOR reduces the growth of tumors in melanoma model systems (18). GSK2126458 is a potent ATP-competitive inhibitor of the PI3 kinase family as well as the mTOR kinase (19). The combination of GSK2126458 and GSK1120212 is currently in a phase I clinical trial (NCT01248858) in patients with solid tumors. Because PI3K pathway activation through signal transduction feedback may mediate BRAF resistance and the RAF-MEK-ERK pathway contributes to mTOR activity, the combination of BRAF or MEK inhibitors with a PI3K/mTOR inhibitor may overcome acquired BRAF inhibitor resistance.

Although data on resistance factors associated with vemurafenib have emerged, limited data exist for GSK2118436. Efforts are ongoing to evaluate clinical samples collected before and after tumor progression upon GSK2118436 treatment (20). In parallel with these efforts, we established clones that proliferate in the presence of, and acquired resistance to, GSK2118436. Findings from this system will guide evaluation of samples obtained from clinical trials as well as provide scientific insights into the potential effectiveness of BRAF, MEK, and PI3K/mTOR inhibitors in BRAF<sup>V600</sup> mutant tumors.

## Materials and Methods

### Chemicals

GSK2118436A, GSK1120212B, GSK2126458A, and PLX4032 (21) were synthesized by GlaxoSmithKline Research Labs and the structures are shown in Fig. 1A.

### Cell lines

A375PF11 was derived from a clonal isolate of the A375 cell line (obtained from American Type Culture Collection) and was 96% similar to Cancer Genome Project A375 reference obtained from the Wellcome Trust Sanger Institute by single-nucleotide polymorphism (SNP) analysis. YUSIT1 (22) was obtained from Yale Dermatology Cell Culture Facility and was characterized by that facility by exome sequencing. This cell line was cultured for less than 6 months at GSK.

A375PF11 cells (referred to henceforth as A375) were exposed to increasing concentrations of GSK2118436 and maintained in a final concentration of 1.2 or 1.6  $\mu\text{mol/L}$ . Similarly, YUSIT1 cells were exposed to 0.1  $\mu\text{mol/L}$  of GSK2118436. Single-cell clones were isolated by limiting dilution from these populations. A375 clones were 95% similar to the parental A375 cells as determined by SNP chip analysis. A representative resistant YUSIT1 clones shared 100% of the parental YUSIT1 SNPs as determined by exome sequencing. All cells were grown in RPMI-1640 medium containing 10% FBS without GSK2118436 for at least one passage before an experiment.

### DNA analysis

DNA extraction, Sanger sequencing methods, SNP analysis, and exome sequencing methods are described in the Supplementary Methods.

### Western blot analysis

Proteins were extracted with cell lysis buffer (Cell Signaling). Fifty micrograms of soluble protein was analyzed by Western blot using indicated antibody and appropriate secondary antibody listed in the Supplementary Methods with the Odyssey Infrared Imaging System (LI-COR Biosciences).

### Cell growth inhibition and apoptosis assays

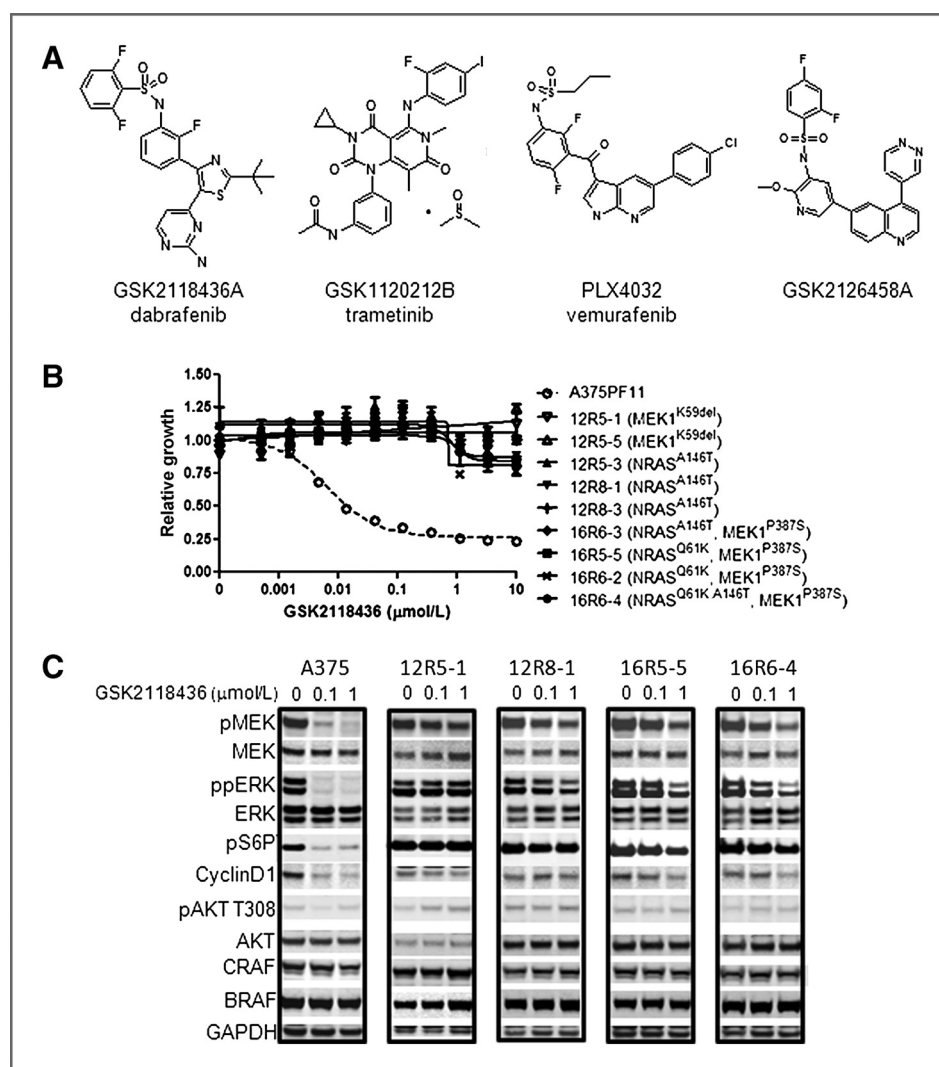
Inhibition of cell growth was estimated after 3-day treatment with compound or combination of compounds using CellTiter-Glo (Promega) as described previously (23). Caspase-3/7 activity was determined via Caspase-Glo 3/7 assay (Promega) 24 hours after similar treatment with compounds. Before 3-day growth assay, cells were transfected or transduced with short interfering RNA (siRNA), short hairpin RNA (shRNA), or expression constructs as described in the Supplementary Methods.

For longer term proliferation assays, cells were plated and treated with compound or combination of compounds in RPMI-1640 containing 10% FBS for 12 days. Compound treatments were replaced at least once during the assay. After 12 days, cells were stained with 0.5% methylene blue in 50% ethanol. Images were captured using flatbed scanner.

### GSK1120212 MEK1 model construction

GSK1120212 was modeled into crystal structure 3EQG (24) as described in the Supplementary Methods.

**Figure 1.** Acquired resistance of A375 clones to GSK2118436. **A**, structures of the BRAF inhibitors GSK2118436 and PLX4032, MEK inhibitor GSK1120212, and PI3K/mTOR inhibitor GSK2126458. **B**, relative growth of A375- and A375-resistant clones after 3-day treatment with GSK2118436. **C**, Western blot analysis of A375 and selected A375-resistant clones after 24-hour treatment with GSK2118436. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.



### Affymetrix expression analyses

16R6-4 was selected for comparison with A375 after compound treatment with GSK2118436 and GSK1120212 alone and in combination with each other for 24 hours. Data analysis was done as described in the Supplementary Methods. The microarray data were deposited in NCBI's Gene Expression Omnibus (GEO, <http://www.ncbi.nlm.nih.gov/geo/>) and are accessible through GEO Series accession number GSE35230.

## Results

### Derivation of A375 and YUSIT1 clones that acquired resistance to GSK2118436

To establish models of resistance to GSK2118436, 9 single-cell clones were isolated from A375 BRAF<sup>V600E</sup> melanoma cells selected to grow in the presence of 1.2 or 1.6  $\mu\text{mol/L}$  GSK2118436. Similarly, multiple YUSIT1 clones were selected to proliferate in the presence of 0.1  $\mu\text{mol/L}$  GSK2118436. Cell growth assays showed

that the A375 clones were resistant to GSK2118436 ( $\text{IC}_{50} > 10 \mu\text{mol/L}$ ; Fig. 1B). The A375 clones were also resistant to PLX4032 ( $\text{IC}_{50} > 10 \mu\text{mol/L}$ , data not shown), and displayed greater than 10-fold reduced sensitivity to the MEK inhibitor GSK1120212 compared with parental A375 cells (Table 1). The YUSIT1 clones showed 38-fold or more reduced sensitivity to GSK2118436 ( $\text{IC}_{50} > 0.5 \mu\text{mol/L}$ ) and 3- to 7-fold reduced sensitivity to GSK1120212 ( $\text{IC}_{50} = 0.002\text{--}0.005 \mu\text{mol/L}$ ) compared with the parental YUSIT1 cells (Supplementary Table S1).

### NRAS and MEK1 mutations identified in clones that acquired resistance to GSK2118436

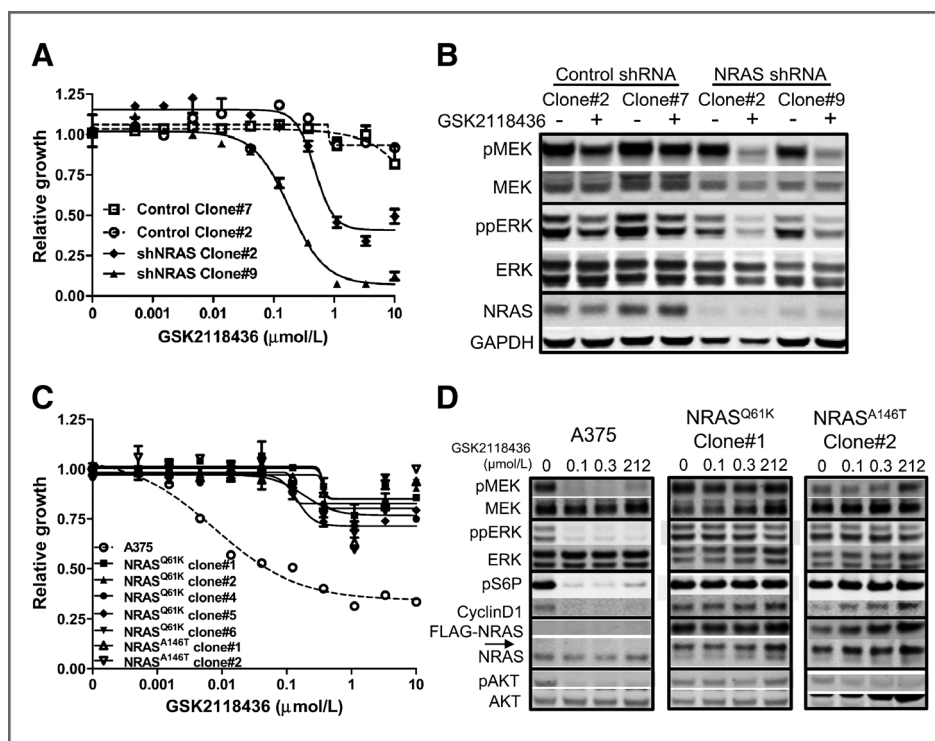
Genetic characterization by Sanger sequencing showed 7 of the 9 A375-derived clones had heterozygous mutations in NRAS at Q61 (Q61K) and/or A146 (A146T) with and without a concomitant MEK1 mutation at P387 (P387S; Table 1). The remaining 2 clones contained an in-frame deletion of K59 (K59del) in MEK1. Sequencing

**Table 1.** Inhibition of cell growth by GSK2118436, GSK1120212, GSK2126458, and their combination in A375 clones with acquired resistance to GSK2118436

Clone	NRAS	MEK1	Single agent		GSK2118436 + GSK1120212		GSK2118436 + GSK2126458		GSK2118436 + GSK1120212 + GSK2126458		GSK1120212 + GSK2126458		GSK1120212 + GSK2126458	
			GSK2118436	GSK1120212	GSK2126458	IC <sub>50</sub> <sup>a</sup>	EOHSA <sup>b</sup>	IC <sub>50</sub> <sup>c</sup>	EOHSA	IC <sub>50</sub> <sup>d</sup>	EOHSA	IC <sub>50</sub> <sup>e</sup>	EOHSA	CI <sup>f</sup>
A375 <sup>f</sup>	WT	WT	0.028 ± 0.016	0.005 ± 0.003	0.050 ± 0.017	0.007 ± 0.005	19 ± 1	0.025 ± 0.018	13 ± 3	0.003 ± 0.001	14 ± 3	0.45 ± 0.13		
12R5-3	A146T	WT	>10	0.107 ± 0.046	0.130 ± 0.106	0.252 ± 0.070	28 ± 5	0.505 ± 0.085	12 ± 2	0.027 ± 0.010	31 ± 6	0.50 ± 0.08		
12R8-1	A146T	WT	>10	0.062 ± 0.031	0.128 ± 0.037	0.266 ± 0.136	29 ± 8	0.984 ± 0.372	14 ± 6	0.022 ± 0.004	24 ± 12	0.65 ± 0.15		
12R8-3	A146T	WT	>10	0.082 ± 0.030	0.167 ± 0.041	0.178 ± 0.182	27 ± 1	0.612 ± 0.008	23 ± 4	0.045 ± 0.021	25 ± 21	0.75 ± 0.31		
16R6-3	A146T	P387S	>10	0.147 ± 0.064	0.131 ± 0.075	0.280 ± 0.123	36 ± 9	0.731 ± 0.204	3 ± 3	0.019 ± 0.005	31 ± 6	0.33 ± 0.09		
16R5-5	Q61K	P387S	>10	0.121 ± 0.042	0.142 ± 0.093	0.268 ± 0.072	36 ± 3	0.833 ± 0.372	23 ± 7	0.021 ± 0.002	33 ± 6	0.45 ± 0.08		
16R6-2	Q61K	P387S	>10	0.123 ± 0.065	0.065 ± 0.047	0.242 ± 0.057	28 ± 8	0.304 ± 0.103	8 ± 1	0.011 ± 0.003	33 ± 3	0.28 ± 0.11		
16R6-4	Q61K, A146T	P387S	>10	>0.9	0.161 ± 0.140	0.380 ± 0.169	>50	0.495 ± 0.123	21 ± 13	0.047 ± 0.032	30 ± 3	0.31 ± 0.08		
12R5-1	WT	K59del	>10	>1	0.177 ± 0.099	0.392 ± 0.149	19 ± 1	0.736 ± 0.169	30 ± 5	0.037 ± 0.007	25 ± 21	N/A <sup>g</sup>		
12R5-5	WT	K59del	>10	>0.9	0.162 ± 0.083	0.383 ± 0.101	46 ± 12	0.640 ± 0.033	35 ± 13	0.024 ± 0.007	33 ± 6	0.24 ± 0.06		

<sup>a</sup>IC<sub>50</sub> for GSK2118436 dosed in a 10:1 molar combination with GSK1120212 expressed in μmol/L.<sup>b</sup>EOHSA, excess over single agent expressed as percentage points.<sup>c</sup>IC<sub>50</sub> for GSK2118436 dosed in a 10:1 molar combination with GSK2126458 expressed in μmol/L.<sup>d</sup>IC<sub>50</sub> for GSK1120212 dosed in a 1:1 molar combination with GSK2126458 expressed in μmol/L.<sup>e</sup>CI, combination index for GSK1120212 and GSK2126458.<sup>f</sup>Combination index for GSK2118436 + GSK1120212 = 0.49 ± 0.28, GSK2118436 + GSK2126458 = 0.43 ± 0.20.<sup>g</sup>N/A, not applicable.

**Figure 2.** NRAS<sup>Q61K</sup> and NRAS<sup>A146T</sup> promote resistance to GSK2118436. **A**, relative cell growth after 3-day treatment with GSK2118436 in stable clones from 16R6-4 transduced with control shRNA (control #2 and control #7) or shRNA targeting NRAS (clone #2 and clone #9). **B**, the stable clones described above were treated with 0.3  $\mu\text{mol/L}$  GSK2118436 for 24 hours before Western blot analysis with indicated antibodies. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. **C**, relative cell growth of A375, 5 A375 clones that express FLAG-NRAS<sup>Q61K</sup> and 2 A375 clones that express FLAG-NRAS<sup>A146T</sup> after 3-day treatment with GSK2118436. **D**, Western blot analysis with indicated antibodies of A375, A375 clone #1 transfected with NRAS<sup>Q61K</sup> and A375 clone #2 transfected with NRAS<sup>A146T</sup> treated with GSK2118436 or 0.01  $\mu\text{mol/L}$  GSK1120212 (212) for 24 hours. Arrow, expected position of FLAG-NRAS.



confirmed retention of the homozygous BRAF<sup>V600E</sup> mutation in these clones with no additional BRAF mutation. In addition, no mutations in KRAS, HRAS, ARAF, CRAF, PTEN, or PIK3CA were identified. All A375 clones retained a synonymous MEK2 polymorphism at the codon for I220. Resistant clones derived from YUSIT1, likewise, acquired NRAS<sup>Q61K</sup> in all 3 of the clones tested. These clones retained BRAF<sup>V600K</sup> and MEK2<sup>E27K</sup>.

### Sustained MAP kinase signaling in acquired resistance clones

Four representative A375-resistant clones were treated with GSK2118436 and compared with the parental cells (Fig. 1C). In the absence of GSK2118436, both A375 and the resistant clones showed high levels of phosphorylation of MEK, ERK, and S6 ribosomal protein (S6P), and low levels of phosphorylated AKT at T308. ARAF, BRAF, and CRAF protein levels were similar between the resistant clones and the parental cells (Fig. 1B and data not shown). In A375 cells, GSK2118436 potentially inhibited phosphorylation of MEK, ERK, and S6P by more than 75% and decreased cyclin D1 protein levels by more than 65% (Fig. 1C). These effects were attenuated in the resistant clones treated with GSK2118436. MEK phosphorylation was reduced 30% to 70%, whereas ERK and S6P phosphorylation were reduced by less than 50% and 25%, respectively. Unlike the parental line, cyclin D1 protein levels were relatively unchanged in the resistant clones following GSK2118436 treatment. Similar results for MEK and ERK phosphorylation in a subset of resistant clones were observed with GSK2118436 treatment for 4 hours (data not shown).

### NRAS mutations mediate resistance to GSK2118436

Next, we evaluated the effect of the NRAS mutations on GSK2118436 and GSK1120212 sensitivity in 3 representative resistant clones with NRAS<sup>A146T</sup> (12R5-3 and 12R8-1) or both NRAS<sup>Q61K</sup> and NRAS<sup>A146T</sup> (16R6-4). Activated RAS levels were increased 2.5- to 7-fold in these clones over the parental line (Supplementary Fig. S1). GSK2118436 increased the coprecipitation of CRAF with BRAF<sup>V600E</sup> in both 12R5-3 and 16R6-4 (Supplementary Fig. S2). In 16R6-4 clones, stable reduction of NRAS by shRNA partially restored the sensitivity to GSK2118436 (Fig. 2A) and GSK1120212 (Supplementary Fig. S3) and the ability of GSK2118436 to decrease phosphorylation of MEK and ERK (Fig. 2B). Partial restoration of sensitivity to GSK2118436 and GSK1120212 was also observed in clones 12R5-3 and 12R8-1 following stable knockdown of NRAS (data not shown).

To further confirm that NRAS<sup>Q61K</sup> or NRAS<sup>A146T</sup> mutations promote resistance to GSK2118436, we transfected plasmids encoding these mutations and the neomycin resistance gene into A375 cells. Selection with G418 disulfate, which selects for cells transfected with the neomycin resistance gene, isolated clones that did not express mutant NRAS (data not shown). However, selection with 0.1  $\mu\text{mol/L}$  GSK2118436 resulted in clones expressing mutant NRAS. Mock or wild-type NRAS transfections failed to form colonies when selected with GSK2118436. The mutant NRAS expressing clones were insensitive to cell growth inhibition by GSK2118436 (Fig. 2C) and GSK1120212 (Supplementary Fig. S4). GSK2118436 treatment failed to reduce

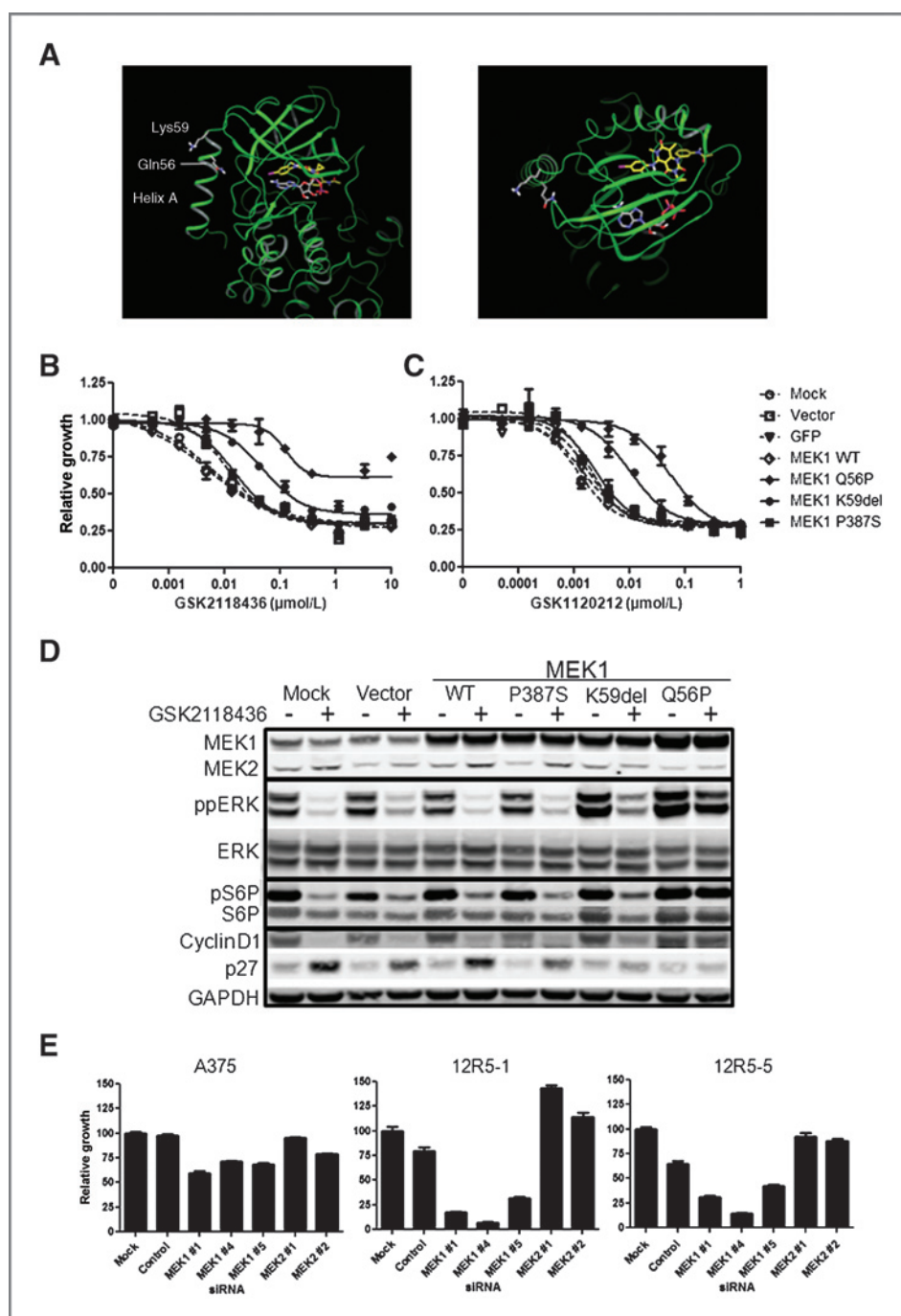


Figure 3. MEK mutation reduces sensitivity in A375 cells. A, front and top views of a MEK1 model showing relative positions of GSK1120212 (yellow), ADP, helix A, Gln56, and Lys59. B and C, relative growth of A375 cells not transduced (Mock) or transduced with BacMAM viruses containing Vector, GFP, MEK1 wild-type (WT), MEK1<sup>Q56P</sup>, MEK1<sup>K59del</sup>, MEK1<sup>P387S</sup> for 24 hours before a 3-day growth assay in the presence of GSK2118436 (B) or GSK1120212 (C). D, A375 cells were transduced as above for 24 hours then treated with 0.1 μmol/L GSK2118436 for 24 hours. Cell lysates were analyzed by Western blot with indicated antibodies. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. E, proliferation compared with mock transfected cells determined 3 days after siRNA-mediated knockdown of MEK1 or MEK2 as described in the Supplementary Methods in the A375 or acquired resistant clones 12R5-1 and 12R5-5 that contain MEK1<sup>K59del</sup>.

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MEK, ERK, and S6P phosphorylation, and cyclin D1 protein levels in these NRAS expressing clones (Fig. 2D).

**MEK mutations confer resistance to GSK2118436**

In a similar manner, we tested whether the MEK mutations K59del and P387S found in the GSK2118436-resistant clones activate MEK and promote resistance to GSK2118436 and GSK1120212. Amino acid residue K59 is located adjacent to the negative regulatory helix preceding the core kinase domain of MEK. Helix A has been

hypothesized to stabilize an inactive conformation of MEK1. This conformation is the same as, or similar to, the conformation that binds allosteric inhibitors such as GSK1120212 (Fig. 3A). Mutation of MEK1 at Q56 within this regulatory helix has been shown to activate MEK1 and decrease sensitivity to single-agent BRAF inhibitor PLX4720 and MEK inhibitor AZD6244 (25). P387 is located in the largely disordered C-terminus of MEK1 adjacent to T386, which is involved in negative regulation of MEK1 (24).

As shown in Fig. 3B, expression of MEK1<sup>K59del</sup> and MEK1<sup>Q56P</sup> in A375 cells decreased the ability of GSK2118436 to inhibit proliferation ( $IC_{50} = 0.126$  and  $>10 \mu\text{mol/L}$ , respectively). MEK1<sup>P387S</sup> sensitivity to GSK2118436 was similar to controls and wild-type MEK1 ( $IC_{50} \sim 0.024 \mu\text{mol/L}$ ). Expression of MEK1<sup>K59del</sup> or MEK1<sup>Q56P</sup>, but not MEK1<sup>P387S</sup>, also reduced sensitivity to GSK1120212 (Fig. 3C). Consistent with the proliferation data, MEK1<sup>Q56P</sup> and MEK1<sup>K59del</sup> displayed higher ERK phosphorylation following treatment with GSK2118436 compared with wild-type MEK1 or MEK1<sup>P387S</sup> (Fig. 3D). Likewise, p27<sup>kip1</sup> induction was minimal in cells expressing MEK1<sup>Q56P</sup> and MEK1<sup>K59del</sup>. Retention of S6P phosphorylation after treatment with GSK2118436 was most profound in MEK1<sup>Q56P</sup>, but still greater in MEK1<sup>K59del</sup> compared with wild-type MEK1 or MEK1<sup>P387S</sup>. Both MEK1<sup>Q56P</sup> and MEK1<sup>K59del</sup> displayed increased basal phosphorylation of ERK.

To confirm that MEK mutations contribute to GSK2118436 resistance in our A375 clones, we used siRNA to knock down MEK1 in clones 12R5-1 and 12R5-5. In the absence of compound, these clones were very sensitive to MEK1 downregulation, as cell proliferation was reduced by more than 75% in 12R5-1 and by more than 60% in 12R5-5, suggesting MEK1 dependence for proliferation (Fig. 3E, Supplementary Fig. S5). MEK2 knockdown had no effect on proliferation.

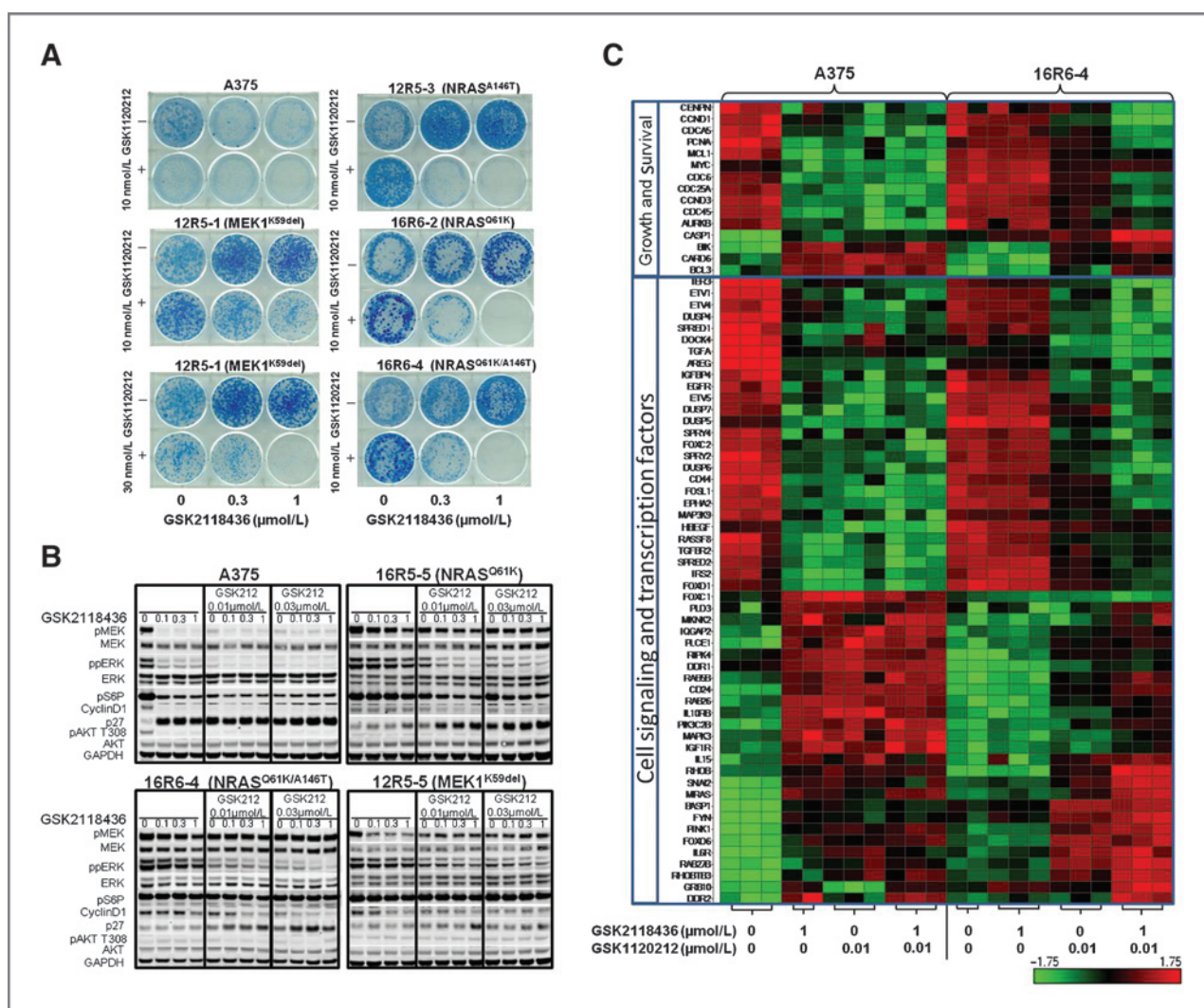
#### Melanoma cells with acquired resistance to GSK2118436 are sensitive to the combination of GSK2118436 and GSK1120212

Because the resistant clones showed sustained ERK phosphorylation following treatment with GSK2118436, we tested whether combined inhibition of BRAF and MEK would be beneficial. The combination of GSK2118436 and GSK1120212 at a constant molar to molar ratio of 10:1 enhanced growth inhibition in all clones with acquired resistance, irrespective of *NRAS* or *MEK1* mutation (Table 1). Although a combination index (CI) could not be calculated because of the lack of GSK2118436 single-agent activity, the excess over highest single-agent parameter (EOSHA) showed 19% to 50% increase of growth inhibition at the  $IC_{50}$ , when compared with GSK1120212 alone (Table 1 and Supplementary Fig. S6). A similar benefit with the combination of GSK2118436 and GSK1120212 was observed in A375 cells that stably expressed *NRAS*<sup>Q61K</sup> and *NRAS*<sup>A146T</sup> (Supplementary Fig. S7) or were transduced with MEK1<sup>K59del</sup> and MEK1<sup>Q56P</sup> (Supplementary Fig. S8). One micromolar GSK2118436 restored most of the sensitivity to GSK1120212 in 12R5-3 (*NRAS*<sup>A146T</sup>; Supplementary Fig. S9). However, similar concentrations only partially restored sensitivity to GSK1120212 in 16R6-4 (*NRAS*<sup>Q61K</sup> and *NRAS*<sup>A146T</sup>) and 12R5-5 (MEK1<sup>K59del</sup>) clones. In extended proliferation assays, the combination of 1  $\mu\text{mol/L}$  GSK2118436 with 0.01  $\mu\text{mol/L}$  GSK1120212 inhibited more than 90% of cell growth in the *NRAS* mutant clones (Fig. 4A). Growth inhibition by the combination in *NRAS* mutant clones was

more pronounced than in the MEK1<sup>K59del</sup> clone, which required 0.03  $\mu\text{mol/L}$  GSK1120212 to observe a similar effect. In the YUSIT1-resistant clones, the combination of GSK2118436 and GSK1120212 was effective and slightly synergistic to nearly additive (CI = 0.8–0.9), with a modest increase in potency compared with GSK1120212 alone (Supplementary Table S1).

To characterize the combination of GSK2118436 and GSK1120212 further, we examined the effect of this combination on RAF–MEK–ERK signaling. As shown in Fig. 4B, single-agent GSK2118436 or GSK1120212 effectively reduced ERK and S6P phosphorylation in A375. These inhibitors were sufficient to reduce cyclin D1 and increase p27<sup>kip1</sup>. In combination, ERK and S6P phosphorylation were reduced further. In a representative subset of resistant clones, GSK2118436 alone had little effect on ERK or S6P phosphorylation or cyclin D1 and p27<sup>kip1</sup> protein levels. GSK1120212 alone reduced phosphorylation of ERK. In combination, ERK phosphorylation levels in the *NRAS* mutant clones were reduced to a similar extent as that observed in A375 treated with GSK2118436. The effect on ERK phosphorylation with combination treatment was attenuated in the MEK1<sup>K59del</sup> clones. In addition, this combination decreased cyclin D1 and increased p27<sup>kip1</sup>, although not to the same extent as A375 treated with GSK2118436. S6P phosphorylation was reduced to varying degrees, or unchanged, in the clones treated with the combination. This combination slightly reduced AKT phosphorylation and did not increase apoptosis as determined by caspase-3/7 activity in both A375 and the resistant clones (data not shown).

Because the combination of GSK2118436 and GSK1120212 reduced MEK–ERK signaling and proliferation of resistant clones, we compared the effect of this combination on global gene regulation in A375 and 16R6-4 (*NRAS*<sup>Q61K/A146T</sup>, MEK1<sup>P387S</sup>). Treatment of A375 cells with GSK2118436 or GSK1120212 as single agents altered expression of a select subset of genes related to growth, survival, signal transduction, and transcription (Fig. 4C). Consistent with the observed resistance in 16R6-4, GSK2118436 alone did not alter these transcripts, whereas a modest effect was observed with GSK1120212. The combination of GSK1120212 and GSK2118436 in the resistant clone downregulated genes involved in cell proliferation and survival (e.g., *CCND1*, *CDC25A*, *PCNA*, *MYC*, and *MCL1*) in a manner similar to treatment of the parental cells with either GSK2118436 or GSK1120212 alone. Transcripts associated with apoptosis (e.g., *BIK*, *CASP1*, and *CARD6*) were upregulated in 16R6-4 following combined treatment, but not to the same extent as A375 treated with either single agent. Combination treatment in both 16R6-4 and A375 decreased transcripts dependent on MEK–ERK signaling (e.g., *DUSP4*, *DUSP5*, *DUSP7*, *ETV1*, *ETV4*, *FOXC2*, and *SPRY2*) and upregulated transcripts related to alternative RAS signaling pathways (e.g., *RAB26*, *RHOB*) or epithelial–mesenchymal transition (*SNAI2*). The data showed that the combination of



**Figure 4.** The combination of GSK2118436 and GSK1120212 is effective in the A375-resistant clones. **A**, relative growth of A375 and representative clones treated with GSK2118436 and GSK1120212 for 12 days before staining with methylene blue. **B**, Western blot analysis with indicated antibodies on protein extracted from A375, 16R6-2, 16R6-4, and 12R5-1 treated with GSK2118436 and GSK1120212 (GSK212) for 24 hours. The single-agent response to GSK1120212 is presented in lanes 5 and 9. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. **C**, heatmap of normalized Affymetrix probe sets representing 70 genes that were differentially expressed greater than 2-fold in A375 and 16R6-4 treated for 24 hours with the combination of 1  $\mu\text{mol/L}$  GSK2118436 and 0.01  $\mu\text{mol/L}$  GSK1120212 compared with vehicle.

GSK2118436 and GSK1120212 in a BRAF inhibitor acquired resistant clone nearly restored the gene expression profile seen following single-agent RAF or MEK inhibition in the parental line.

**Inhibition of MAPK and PI3K/mTOR pathways enhances cell growth inhibition in the acquired resistant clones**

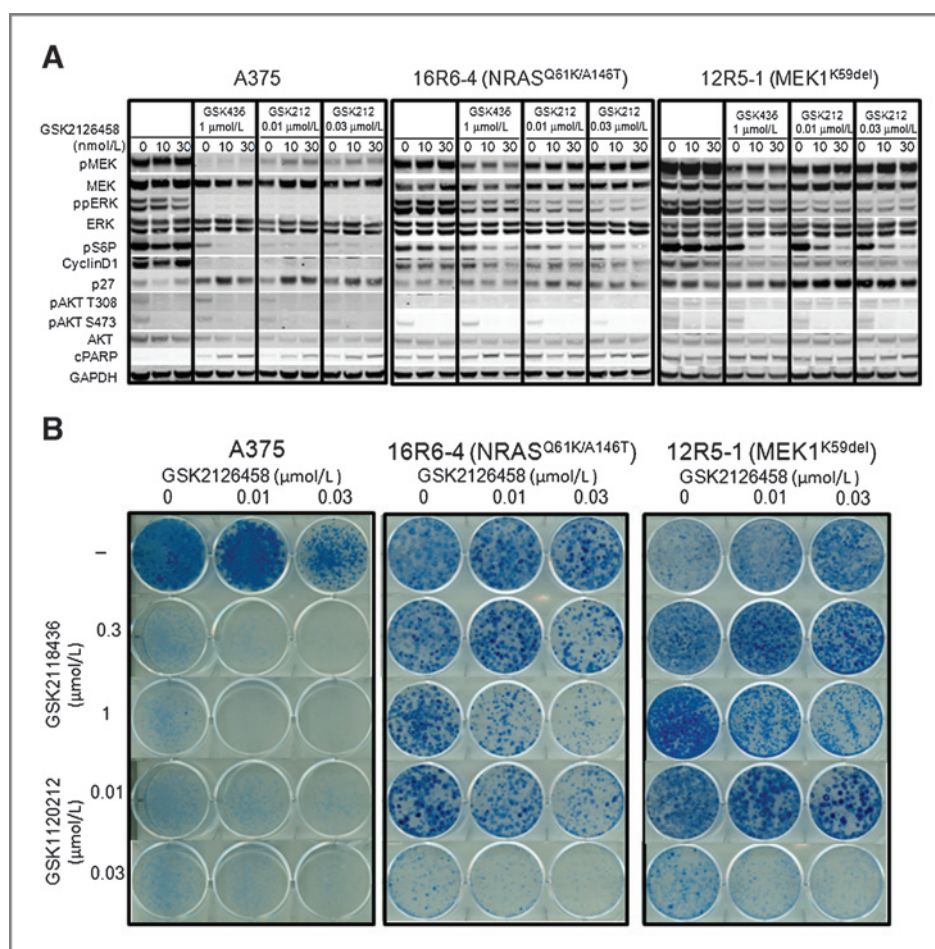
Although the combination of GSK2118436 and GSK1120212 profoundly inhibited proliferation of the resistant clones, phosphorylation of S6P was not inhibited completely. Because S6P phosphorylation can be induced by activation of the PI3K/mTOR pathway, we evaluated the combination of GSK2118436 or GSK1120212 with GSK2126458 (PI3K/mTOR inhibitor). All clones displayed modest sensitivity cell growth inhibition by

GSK2126458 (Table 1). GSK2126458 treatment of representative resistant clones decreased AKT phosphorylation and had minimal effect on S6P phosphorylation (Fig. 5A). GSK2126458 in combination with GSK2118436 or GSK1120212 reduced S6P phosphorylation in the resistant clones, whereas GSK2118436 or GSK1120212 alone was sufficient to reduce S6P phosphorylation in A375. The reduction in pS6P with either GSK2118436 or GSK1120212 in combination with GSK2126458 was greater than the reduction observed with the GSK2118436 and GSK1120212 combination. MEK and ERK phosphorylation was similar to treatment with GSK2118436 or GSK1120212 alone. Cleaved PARP and caspase-3/7 activity, indicators of apoptosis, were increased slightly by GSK2126458 in combination with either GSK2118436 or GSK1120212 in clones 16R6-4 and 16R6-2, although basal

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**Figure 5.** A375 resistant clones respond to GSK2126458 in combination with GSK2118436 or GSK1120212. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. A, Western blot analysis after treatment of A375, 16R6-4, and 12R5-1 with GSK2126458 in combination with GSK2118436 (GSK436) or GSK1120212 (GSK212) for 24 hours. B, relative growth of A375, 16R6-4, and 12R5-1 treated with GSK2126458 in combination with GSK2118436 or GSK1120212 for approximately 12 days before staining with methylene blue.



apoptosis levels were higher in all of the resistant clones compared with A375 (Fig. 5A and data not shown). The addition of GSK2118436 to GSK2126458 enhanced cell growth inhibition (EOSHA >10 ppts) in 5/7 clones with *NRAS* mutations and the 2 clones harboring *MEK1*<sup>K59del</sup> (Table 1). The combination of GSK1120212 and GSK2126458 was synergistic (CI <0.8) in 8/9 clones and enhanced cell growth inhibition (EOSHA >20 ppts) in all 9 clones regardless of *NRAS* or *MEK1* mutation (Table 1). Long-term proliferation assays confirmed the enhancement of growth inhibition by the combination of GSK2118436 or GSK1120212 with GSK2126458 (Fig. 5B). In general, the resistant clones were more sensitive to cell growth inhibition with the combination of GSK2126458 and GSK1120212 at the concentrations used; however, appreciable activity was also observed with 1 μmol/L GSK2118436 and 0.03 μmol/L GSK2126458. The anti-proliferative effect of GSK2118436 in combination with GSK2126458 was not as potent as that observed with the GSK2118436 and GSK1120212 combination, or the GSK1120212 and GSK2126458 combination. Benefits were observed for these combinations in the YUSIT1 GSK2118436-resistant clones, although the combination of GSK2126458 with GSK2118436 or GSK1120212 was

moderately synergistic to nearly additive in these clones (Supplementary Table S1).

## Discussion

Our results suggest that mutation of *NRAS* or *MEK1*, in a *BRAF*<sup>V600</sup> background, confers resistance to GSK2118436 or PLX4032. In our resistant clones, GSK2118436 reduced phosphorylation of MEK; however, it did not decrease phosphorylation of either ERK or S6P, or the levels of cyclin D1 protein. Thus, mutational activation of *NRAS* or *MEK* was sufficient to maintain ERK signaling even in the presence of a *BRAF* inhibitor. Moreover, we show that combined inhibition of both *BRAF* and *MEK* with GSK2118436 and GSK1120212 effectively suppresses signaling and gene expression related to an activated *RAF*-*MEK*-*ERK* pathway, thereby reducing cell proliferation in a similar manner as observed in GSK2118436-treated parental cells. These resistant clones also were responsive to the combination of GSK2126458, a *PI3K*/*mTOR* inhibitor, with GSK2118436 or GSK1120212.

*NRAS* is mutated in 18% of melanoma patient samples (26). The common substitution of Q61 maintains an active state by impairing GTP hydrolysis. Mutations at

*NRAS*<sup>A146</sup> have been reported in lymphoblastic leukemia (27). In the related RAS isoform, *KRAS*, mutation of A146 activates the RAF–MEK–ERK pathway and promotes transformation of 3T3 cells, although to a lesser extent than the more common *KRAS*<sup>G12</sup> mutation (28). Reports indicate that mutation of *KRAS* or *NRAS* in a *BRAF* wild-type background promotes heterodimerization of BRAF and CRAF, thereby activating ERK signaling in the presence of a RAF inhibitor (29–31). Our data in a *BRAF*<sup>V600</sup> mutant background suggest that activating mutations of *NRAS* could also promote a mutant BRAF and CRAF complex in the presence of a BRAF inhibitor, leading to sustained ERK signaling. Similar observations of increased RAS activation have been reported in other *BRAF*<sup>V600E</sup> models of acquired resistance (32). A co-occurring *NRAS*<sup>Q61K</sup> mutation with *BRAF*<sup>V600E</sup> was identified in a patient sample after progression on PLX4032 (9). Furthermore, we also identified *NRAS* mutations associated with acquired resistance in a *BRAF*<sup>V600K</sup> mutant melanoma cell line. Thus, *NRAS* mutation in a *BRAF*<sup>V600</sup> mutant background is a potential mechanism of resistance for BRAF inhibitors in general.

In addition, our data support the hypothesis that mutations within the regulatory helix of MEK can increase its activity, thereby decreasing sensitivity to BRAF and MEK inhibitors. It is unlikely that *MEK1*<sup>P387S</sup>, which is adjacent to a regulatory site of *MEK1*, is sufficient to confer resistance to GSK2118436 or GSK1120212 (Fig. 3). The clones with both *BRAF*<sup>V600E</sup> and *MEK1*<sup>K59del</sup> mutations were dependent on *MEK1*, even in the absence of GSK2118436, than parental A375 cells. Expression of *MEK1*<sup>K59del</sup> or *MEK1*<sup>Q56P</sup> decreased GSK1120212 activity. Although mutations in the negative regulatory helix are not known to affect the binding site of GSK1120212 directly, these mutations may destabilize this regulatory helix and promote an active confirmation of MEK, for which the compound has decreased potency. For instance, GSK1120212 binds to the unphosphorylated form of MEK with an IC<sub>50</sub> of 0.7 nmol/L, whereas its binding to the activated form of MEK is dramatically shifted (IC<sub>50</sub> = 14.3 nmol/L; ref. 15). In cardio-facio-cutaneous syndrome, *MEK1* and *MEK2* mutations within the negative regulatory helix result in a constitutively activated form of MEK (33–35). In a *MEK1* mutagenesis screen, *MEK1*<sup>Q56P</sup>, among others, conferred resistance to MEK and BRAF inhibitors (25). Cell lines expressing mutant *MEK1*<sup>K57N</sup>, which was identified in 2 lung adenocarcinoma samples, likewise showed a decreased sensitivity to a MEK inhibitor in cell growth assays (36). In addition, a tumor isolated from a patient progressing on PLX4032 was found to express *MEK1*<sup>C121S</sup> (10), suggesting that even those activating MEK mutations outside the negative regulatory helix, may cause resistance to BRAF inhibitors. A similar MEK mutation, *MEK1*<sup>F129L</sup>, displayed enhanced interaction with CRAF, thereby increasing intrinsic kinase activity of CRAF and, thus, circumventing MEK

or BRAF inhibition. However, combination of RAF and MEK inhibitors overcome the observed drug resistance in this model system (37). Similarly, our data show that the combination of GSK2118436 and GSK1120212 effectively inhibits proliferation of cells with *MEK1*<sup>K59del</sup> and *MEK1*<sup>Q56P</sup> expression. Studies are necessary to determine the frequency of MEK mutations following BRAF inhibitor treatment. One such mutation, *MEK2*<sup>Q60P</sup>, was found in a *BRAF*<sup>V600E</sup>-positive, pretreatment melanoma sample from a patient treated in the phase I clinical trial with GSK2118436. This patient had stable disease (20).

Mutations that activate RAS or MEK lead to hyperactive RAS–MEK–ERK pathway that is insufficiently inhibited with BRAF or MEK inhibition alone. However, in our model systems, this pathway is effectively inhibited with the combination of GSK2118436 and GSK1120212. This combination led to a profound growth arrest and restoration of transcriptional output similar to the parental cells treated with a BRAF or MEK inhibitor alone in the clones that acquired resistance to GSK2118436. This transcriptional output included a decrease in genes designated as ERK transcriptional output genes (*DUSP4*, *DUSP6*, *SPRY2*, *ETV5*, and *CCND1*; ref. 38), which were also decreased in PLX4032-treated melanoma cells (39). In our assays, combinations with clinically relevant concentrations of GSK2118436 and GSK1120212 had profound effects on the proliferation of the parent cells and acquired resistance clones. This combination is well tolerated in the clinic. Clinical benefit has been observed with this combination in *BRAF*<sup>V600</sup> mutant melanoma patients including patients who progressed with BRAF inhibitor treatment. A decrease in the incidence of rash and BRAF inhibitor induced hyperproliferative skin lesions has also been seen (16, 40).

In addition, our acquired resistant clones responded to the PI3K/mTOR inhibitor, GSK2126458, albeit at slightly higher concentrations compared with the parental cells. The addition of GSK2126458 to either GSK2118436 or GSK1120212 further reduced cell proliferation and S6P phosphorylation in both resistant clones and parental cells. Combining GSK2126458 with GSK1120212 is more potent and more effective than the combination of GSK2126458 and GSK2118436 in short- and long-term proliferation assays. However, both represent practicable alternatives for clinical evaluation. Combined MEK and PI3K/mTOR inhibition with AZD6244 and BEZ235 more effectively induced apoptosis than inhibition of BRAF with PLX4032 and PI3K/mTOR with BEZ235 in PDGFR-mediated resistance to PLX4032 (41). However, in our model system, enhanced apoptosis as determined by cleaved PARP was similar in the combination of GSK2126458 with either GSK2118436 or GSK1120212.

Ongoing studies are evaluating genetic mutations, including *NRAS* and *MEK*, in patients' tumors that progress on GSK2118436. Clinical trials are in progress to test the effectiveness of the combination of GSK2118436 and GSK1120212 (NCT01072175) in patients that progress

after BRAF inhibitor treatment and in BRAF treatment-naïve patients.

### Disclosure of Potential Conflicts of Interest

All authors are employees and stockholders of GlaxoSmithKline.

### Authors' Contributions

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**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** J.G. Greger, S.D. Eastman, V. Zhang, M.R. Bleam, A.M. Hughes, S.H. Dickerson, S.G. Laquerre, L. Liu, and T.M. Gilmer  
**Writing, review, and/or revision of the manuscript:** J.G. Greger, S.D. Eastman, V. Zhang, M.R. Bleam, K.N. Smitheman, S.H. Dickerson, S.G. Laquerre, L. Liu, and T.M. Gilmer

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**Study supervision:** S.G. Laquerre, L. Liu, and T.M. Gilmer

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### References

- Davies H, Bignell GR, Cox C, Stephens P, Edkins S, Clegg S, et al. Mutations of the BRAF gene in human cancer. *Nature* 2002;417:949–54.
- Chapman PB, Hauschild A, Robert C, Haanen JB, Ascierto P, Larkin J, et al. Improved survival with vemurafenib in melanoma with BRAF V600E mutation. *N Engl J Med* 2011;364:2507–16.
- Ribas A, Flaherty KT. BRAF targeted therapy changes the treatment paradigm in melanoma. *Nat Rev Clin Oncol* 2011;8:426–33.
- Kefford R, Arkenau H, Brown MP, Millward M, Infante JR, Long GV, et al. Phase I/II study of GSK2118436, a selective inhibitor of oncogenic mutant BRAF kinase, in patients with metastatic melanoma and other solid tumors. *ASCO Meeting Abstracts* 2010;28:8503.
- FDA approves Zelboraf and companion diagnostic test for late-stage skin cancer. [cited 17 August 2011; 9 December 2011 (ref type: Internet Communication)]. Available from: www.fda.gov.
- Sierra JR, Cepero V, Giordano S. Molecular mechanisms of acquired resistance to tyrosine kinase targeted therapy. *Mol Cancer* 2010;9:75.
- Balzano D, Santaguida S, Musacchio A, Villa F. A general framework for inhibitor resistance in protein kinases. *Chem Biol* 2011;18:966–75.
- Whittaker S, Kirk R, Hayward R, Zamboni A, Viroso A, Cantarino N, et al. Gatekeeper mutations mediate resistance to BRAF-targeted therapies. *Sci Transl Med* 2010;2:35ra41.
- Nazarian R, Shi H, Wang Q, Kong X, Koya RC, Lee H, et al. Melanomas acquire resistance to B-RAF(V600E) inhibition by RTK or N-RAS upregulation. *Nature* 2010;468:973–7.
- Wagle N, Emery C, Berger MF, Davis MJ, Sawyer A, Pochanard P, et al. Dissecting therapeutic resistance to RAF inhibition in melanoma by tumor genomic profiling. *J Clin Oncol* 2011;29:3085–96.
- Villanueva J, Vultur A, Lee JT, Somasundaram R, Fukunaga-Kalabis M, Cipolla AK, et al. Acquired resistance to BRAF inhibitors mediated by a RAF kinase switch in melanoma can be overcome by cotargeting MEK and IGF-1R/PI3K. *Cancer Cell* 2010;18:683–95.
- Shao Y, Aplin AE. Akt3-mediated resistance to apoptosis in B-RAF-targeted melanoma cells. *Cancer Res* 2010;70:6670–81.
- Johannessen CM, Boehm JS, Kim SY, Thomas SR, Wardwell L, Johnson LA, et al. COT drives resistance to RAF inhibition through MAP kinase pathway reactivation. *Nature* 2010;468:968–72.
- Laquerre S, Arnone M, Moss K, Yang J, Fisher K, Kane-Carson LS, et al. Abstract B88: A selective Raf kinase inhibitor induces cell death and tumor regression of human cancer cell lines encoding B-RafV600E mutation. *Mol Cancer Ther* 2009;8:B88.
- Gilmartin AG, Bleam MR, Groy A, Moss KG, Minthorn EA, Kulkarni SG, et al. GSK1120212 (JTP-74057) is an inhibitor of MEK activity and activation with favorable pharmacokinetic properties for sustained in vivo pathway inhibition. *Clin Cancer Res* 2011;17:989–1000.
- Infante J, Falchook G, Lawrence D, Weber J, Kefford R, Bendell J, et al. Phase I/II study to assess safety, pharmacokinetics, and efficacy of the oral MEK 1/2 inhibitor GSK1120212 (GSK212) dosed in combination with the oral BRAF inhibitor GSK2118436 (GSK436). *J Clin Oncol* 2011;29:suppl; abstr CRA8503.
- Aziz SA, Davies M, Pick E, Zito C, Jilaveanu L, Camp RL, et al. Phosphatidylinositol-3-kinase as a therapeutic target in melanoma. *Clin Cancer Res* 2009;15:3029–36.
- Aziz SA, Jilaveanu LB, Zito C, Camp RL, Rimm DL, Conrad P, et al. Vertical targeting of the phosphatidylinositol-3 kinase pathway as a strategy for treating melanoma. *Clin Cancer Res* 2010;16:6029–39.
- Knight SD, Adams ND, Burgess JL, Chaudhari AM, Darcy MG, Donatelli CA, et al. Discovery of GSK2126458, a highly potent inhibitor of PI3K and the mammalian target of rapamycin. *ACS Med Chem Lett* 2010;1:39–43.
- Nathanson KL, Martin A, Letrero R, D'Andrea KP, O'Day S, Infante JR, et al. Tumor genetic analyses of patients with metastatic melanoma treated with the BRAF inhibitor GSK2118436 (GSK436). *ASCO Meeting Abstracts* 2011;29:8501.
- Bollag G, Hirth P, Tsai J, Zhang J, Ibrahim PN, Cho H, et al. Clinical efficacy of a RAF inhibitor needs broad target blockade in BRAF-mutant melanoma. *Nature* 2010;467:596–9.
- Halaban R, Zhang W, Bacchiocchi A, Cheng E, Parisi F, Ariyan S, et al. PLX4032, a selective BRAF(V600E) kinase inhibitor, activates the ERK pathway and enhances cell migration and proliferation of BRAF melanoma cells. *Pigment Cell Melanoma Res* 2010;23:190–200.
- Liu L, Shi H, Liu Y, Anderson A, Peterson J, Greger J, et al. Synergistic effects of foretinib with HER-targeted agents in MET and HER1- or HER2-coactivated tumor cells. *Mol Cancer Ther* 2011;10:518–30.
- Fischmann TO, Smith CK, Mayhood TW, Myers JE, Reichert P, Mannarino A, et al. Crystal structures of MEK1 binary and ternary complexes with nucleotides and inhibitors. *Biochem* 2009;48:2661–74.
- Emery CM, Vijayendran KG, Zipser MC, Sawyer AM, Niu L, Kim JJ, et al. MEK1 mutations confer resistance to MEK and B-RAF inhibition. *Proc Natl Acad Sci U S A* 2009;106:20411–6.
- Forbes SA, Bindal N, Bamford S, Cole C, Kok CY, Beare D, et al. COSMIC: mining complete cancer genomes in the catalogue of somatic mutations in cancer. *Nucleic Acids Res* 2011;39:D945–50.
- Smith G, Bounds R, Wolf H, Steele RJ, Carey FA, Wolf CR. Activating K-Ras mutations outwith 'hotspot' codons in sporadic colorectal tumours—implications for personalised cancer medicine. *Br J Cancer* 2010;102:693–703.
- Edkins S, O'Meara S, Parker A, Stevens C, Reis M, Jones S, et al. Recurrent KRAS codon 146 mutations in human colorectal cancer. *Cancer Biol Ther* 2006;5:928–32.

29. Heidorn SJ, Milagre C, Whittaker S, Noury A, Niculescu-Duvas I, Dhomen N, et al. Kinase-dead BRAF and oncogenic RAS cooperate to drive tumor progression through CRAF. *Cell* 2010;140:209–21.
30. Hatzivassiliou G, Song K, Yen I, Brandhuber BJ, Anderson DJ, Alvarado R, et al. RAF inhibitors prime wild-type RAF to activate the MAPK pathway and enhance growth. *Nature* 2010;464:431–5.
31. Kaplan FM, Shao Y, Mayberry MM, Aplin AE. Hyperactivation of MEK-ERK1/2 signaling and resistance to apoptosis induced by the oncogenic B-RAF inhibitor, PLX4720, in mutant N-RAS melanoma cells. *Oncogene* 2011;30:366–71.
32. Tap WD, Gong KW, Dering J, Tseng Y, Ginther C, Pauletti G, et al. Pharmacodynamic characterization of the efficacy signals due to selective BRAF inhibition with PLX4032 in malignant melanoma. *Neoplasia* 2010;12:637–49.
33. Stevenson DA, Schwarz EL, Carey JC, Viskochil DH, Hanson H, Bauer S, et al. Bone resorption in syndromes of the Ras/MAPK pathway. *Clin Genet* 2011;80:566–73.
34. Dentici ML, Sarkozy A, Pantaleoni F, Carta C, Lepri F, Ferese R, et al. Spectrum of MEK1 and MEK2 gene mutations in cardio-facio-cutaneous syndrome and genotype-phenotype correlations. *Eur J Hum Genet* 2009;17:733–40.
35. Senawong T, Phuchareon J, Ohara O, McCormick F, Rauen KA, Tetsu O. Germline mutations of MEK in cardio-facio-cutaneous syndrome are sensitive to MEK and RAF inhibition: implications for therapeutic options. *Hum Mol Genet* 2008;17:419–30.
36. Marks JL, Gong Y, Chitale D, Golas B, McLellan MD, Kasai Y, et al. Novel MEK1 mutation identified by mutational analysis of epidermal growth factor receptor signaling pathway genes in lung adenocarcinoma. *Cancer Res* 2008;68:5524–8.
37. Wang H, Daouti S, Li WH, Wen Y, Rizzo C, Higgins B, et al. Identification of the MEK1(F129L) activating mutation as a potential mechanism of acquired resistance to MEK inhibition in human cancers carrying the B-RafV600E mutation. *Cancer Res* 2011;71:5535–45.
38. Pratilas CA, Taylor BS, Ye Q, Viale A, Sander C, Solit DB, et al. (V600E) BRAF is associated with disabled feedback inhibition of RAF-MEK signaling and elevated transcriptional output of the pathway. *Proc Natl Acad Sci U S A* 2009;106:4519–24.
39. Joseph EW, Pratilas CA, Poulikakos PI, Tadi M, Wang W, Taylor BS, et al. The RAF inhibitor PLX4032 inhibits ERK signaling and tumor cell proliferation in a V600E BRAF-selective manner. *Proc Natl Acad Sci U S A* 2010;107:14903–8.
40. Flaherty KT, Infante JR, Falchook GS, Weber J, Daud A, Hamid O, et al. Phase I/II expansion cohort of BRAF inhibitor GSK2118436 + MEK inhibitor GSK1120212 in patients with BRAF mutant metastatic melanoma who progressed on a prior BRAF inhibitor. *Pigment Cell Melanoma Res* 2011;24:990–1075.
41. Shi H, Kong X, Ribas A, Lo RS. Combinatorial treatments that overcome PDGFR{beta}-driven resistance of melanoma cells to V600E-BRAF inhibition. *Cancer Res* 2011;71:5067–74.