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Resistance to vemurafenib resulting from a novel mutation in the BRAFV600E kinase domain

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Summary

Resistance to the BRAF inhibitor vemurafenib poses a significant problem for the treatment of BRAFV600E-positive melanomas. It is therefore critical to prospectively identify all vemurafenib-resistance mechanisms prior to their emergence in the clinic. The vemurafenib-resistance mechanisms described to date do not result from secondary mutations within BRAFV600E. To search for possible mutations within BRAFV600E that can confer drug resistance, we developed a systematic experimental approach involving targeted saturation mutagenesis, selection of drug-resistant variants, and deep sequencing. We identified a single nucleotide substitution (T1514A, encoding L505H) that greatly increased drug resistance in cultured cells and mouse xenografts. The kinase activity of BRAFV600E/L505H was higher than that of BRAFV600E, resulting in cross-resistance to a MEK inhibitor. However, BRAFV600E/L505H was less resistant to several other BRAF inhibitors whose binding sites were further from L505 than that of PLX4720. Our results identify a novel vemurafenib-resistant mutant and provide insights into the treatment of melanomas bearing this mutation.

Keywords

BRAFV600E; deep sequencing; drug resistance; saturation mutagenesis; vemurafenib

Introduction

BRAF is a serine-threonine kinase that functions as an immediate downstream effector of RAS (reviewed in Dhomen and Marais, 2007). BRAF activates the MAP kinase extracellular signal regulated kinase (MEK), which in turn phosphorylates and activates extracellular signal-regulated kinases 1 and 2 (ERK1 and ERK2). Oncogenic BRAF mutations are found in a significant number of human cancers, with a particularly high frequency (50–70%) occurring in melanomas (Davies et al., 2002). The most frequent

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oncogenic mutation occurs within the BRAF kinase domain and is the substitution of a valine for glutamic acid at amino acid 600 (V600E). The mutation leads to unchecked kinase activity and constitutive activation of the downstream MEK and ERK kinases.

Vemurafenib (also called PLX4032) is a selective inhibitor of BRAFV600E that can elicit marked melanoma tumor regression, resulting in improved progression-free and overall survival in patients with metastatic disease (Bollag et al., 2010; Bollag et al., 2012). However, the durability of the vemurafenib response is limited by acquired drug resistance (Dummer and Flaherty, 2012). Thus, elucidating the basis of resistance to vemurafenib, and other BRAF inhibitors, is essential to developing more effective therapies for the treatment of melanoma.

A common mechanism of resistance to small molecule protein kinase inhibitors is the acquisition of a second-site mutation that interferes with drug binding (Sierra et al., 2010). Such drug-resistant variants, isolated from patients or in cell- or animal-based experiments, typically arise from a single, non-synonymous nucleotide mutation within the protein kinase domain. Several vemurafenib-resistance mechanisms have been described and, in most cases, are due to alternative activation of MEK-ERK signaling (Dummer and Flaherty, 2012). Other vemurafenib-resistance mechanisms include amplification of the BRAFT1799A allele (encoding BRAFV600E) (Shi et al., 2012) and generation of aberrantly spliced BRAFV600E variants (Poulikakos et al., 2011). Surprisingly, however, drug-resistant amino acid substitution mutants within the BRAFV600E protein-coding region have not been isolated from vemurafenib-resistant melanomas or melanoma cell lines, suggesting that they are either impossible or improbable relative to other resistant pathways. Here we describe a systematic, structure-based saturation mutagenesis approach to identify single, second-site nucleotide substitutions within BRAFV600E that can confer vemurafenib resistance.

Results

A structure-based, targeted, saturation mutagenesis screen identifies PLX4720-resistant BRAFV600E mutants

Our experimental strategy is summarized in Figure 1A and discussed below. Guided by the structure of BRAFV600E bound to PLX4720 (PDB:3C4C; Tsai et al., 2008 and Figure 1B), a tool compound for vemurafenib that elicits comparable actions to its clinical-grade counterpart (Tsai et al., 2008), we performed targeted saturation mutagenesis of 77 amino acids surrounding the PLX4720-binding site. Eight mutant pools were generated—corresponding to amino acids 458–466, 467–476, 477–486, 501–510, 511–520, 527–536, 579–587 and 588–596—in which each amino acid was mutated to all possible 64 codons. The mutant pools were transferred into a retroviral vector containing BRAFV600E and then stably transduced into the human melanoma cell line A375, which is homozygous for BRAFV600E and highly sensitive to PLX4720 (Mitsiades et al., 2011). Cells were cultured for 3 weeks in the presence of 10 μ M PLX4720, at which point resistant clones emerged for six of the eight pools (Figure 1C). Pools encoding mutants corresponding to amino acids 458–466 and 467–476 did not result in any detectable PLX4720-resistant colonies above background.

To identify the mutated amino acid(s) that conferred PLX4720 resistance, cells from the resistant cell populations were pooled, genomic DNA was isolated, and BRAFV600E variants were identified by massively parallel sequencing. We anticipated that, following drug selection, the sequences of PLX4720-resistant BRAFV600E mutants would be enriched relative to PLX4720-sensitive BRAFV600E variants. To control for possible unequal representation of the variants in the starting population, deep sequencing was also performed on genomic DNA isolated from transduced cells prior to drug selection. Figure 1D displays the relative enrichment of BRAFV600E variants following PLX4720 selection from two independent biological replicates. The use of replicate experiments enabled us to distinguish between reproducible enrichment, resulting from a drug-resistant mutation, and non-reproducible enrichment, resulting from a random variation in the sequence pool. For example, silent substitutions that did not alter the BRAFV600E protein sequence were occasionally enriched in one replicate, but rarely in both (Figure S1). Mutations that were enriched in both replicates across all measured synonymous codons with a net false discovery rate of less than 1% were considered statistically significant. The results, summarized in Table S1, identified 25 different amino acids encompassing 55 variants, 18 of which could arise by a single base substitution; mutants that were enriched >5 fold are shown in Figure 1E. Figure 1F displays the positions of the 10 amino acids, whose substitutions were enriched >5-fold, on the BRAF-PLX4720 structure.

Sensitivity of the BRAFV600E/L505H mutant to BRAF and MEK inhibitors

We analyzed the 12 most enriched mutants, and a subset of less enriched mutants, by stable expression in A375 cells followed by determination of the cellular PLX4720 median inhibitory concentration (IC₅₀). Figure 2A shows that the strongest PLX4720 resistance was associated with substitutions at amino acids L485, L505 and F516. Notably, there was an excellent correlation between the normalized enrichment obtained from deep sequencing and the relative IC₅₀.

We elected to further characterize BRAFV600E/L505H, which was the single nucleotide substitution mutant most resistant to PLX4720 (see Table S1). For comparison, we also analyzed the most PLX4720-resistant mutant identified in our screen, BRAFV600E/F516G (Figure 2A), which arises from multiple nucleotide substitutions, and BRAFV600E/T529N, a previously characterized mutant derived by directed mutagenesis of the gatekeeper residue (Whittaker et al., 2010). As expected, the cellular proliferation assay of Figure S2 shows that A375 cells expressing BRAFV600E/L505H, BRAFV600E/F516G or BRAFV600E/T529N were all more resistant to PLX4720 compared to A375 cells expressing BRAFV600E. As expected, in the absence of PLX4720, proliferation of A375 cells expressing BRAFV600E was comparable to that of A375 cells expressing BRAFV600E/L505H, BRAFV600E/F516G or BRAFV600E/T529N (Figure S2). We next confirmed the relative sensitivity of the various BRAFV600E mutants to PLX4720 by monitoring phosphorylation of the downstream signaling components, MEK and ERK1/2. Figure 2B shows, as expected, that in A375 cells PLX4720 potently inhibited BRAFV600E with marked reduction of phosphorylated MEK and ERK1/2 (phospho-MEK and phospho-ERK1/2, respectively) by 0.8 μ M PLX4720. By contrast, BRAFV600E/L505H and BRAFV600E/F516G required a ~100–150-fold higher concentration of PLX4720 to obtain similar reduction of phospho-

MEK and phospho-ERK1/2 (Figure 2B and Figure S3A). Notably, the PLX4720 sensitivity of BRAFV600E/T529N was comparable to that of BRAFV600E, which likely explains why this mutant was not isolated in our screen. By comparison to the results with PLX4720, the BRAFV600E/L505H mutant had only a modest (5–7-fold) effect on resistance to two other BRAF inhibitors, SB590885 (Takle et al., 2006) and RAF265 (Amiri et al., 2006) (Figure 2C, D and Figure S3B, C).

A possible approach for treatment of vemurafenib-resistant melanomas is the use of a MEK inhibitor (Dummer and Flaherty, 2012; Smalley et al., 2006). We therefore analyzed the sensitivity of BRAFV600E/L505H to the MEK inhibitor U0126 (Favata et al., 1998). Notably, BRAFV600E/L505H exhibited increased resistance to U0126 compared to BRAFV600E, as evidenced by elevated phospho-ERK levels (Figure 2E and Figure S3D). By contrast, relative to BRAFV600E, the BRAFV600E/F516G mutant was comparably sensitive and the BRAFV600E/T529N mutant was actually more sensitive to U0126 (Figure 2E and Figure S3D).

To determine whether the differences in MEK/ERK signaling were biologically relevant, we performed two additional experiments. First, we monitored expression of three representative ERK target genes (*FOSL1*, *SPRY2* and *DUSP6*), a biologically relevant output of the BRAF-MEK-ERK signaling pathway (Pratilas et al., 2009). Gene expression was measured by quantitative RT-PCR (qRT-PCR) following treatment of A375 cells with a drug concentration that differentially affected the various BRAFV600E mutants. Consistent with the results of Figure 2B, at a PLX4720 concentration of 20 μ M, expression of *FOSL1*, *SPRY2* and *DUSP6* was greatly reduced in cells expressing BRAFV600E or BRAFV600E/T529N compared to cells expressing BRAFV600E/L505H or BRAFV600E/F516G (Figure S4). Similarly, treatment with SB590885 (0.8 μ M) or U0126 (4 μ M) reduced *FOSL1*, *SPRY2* and *DUSP6* expression to a greater extent in cells expressing BRAFV600E or BRAFV600E/T529N relative to cells expressing BRAFV600E/L505H or BRAFV600E/F516G. Finally, treatment with RAF265 (2.4 μ M) reduced ERK target gene expression to a greater extent in cells expressing BRAFV600E, BRAFV600E/F516G or BRAFV600E/T529N relative to cells expressing BRAFV600E/L505H.

In a second set of experiments, we measured the relative drug resistance of A375 cells expressing the various BRAFV600E mutants. Figure S5A–D shows that cells expressing either BRAFV600E/L505H or BRAFV600E/F516G were relatively more resistant to PLX4720, SB590885, RAF265 and U0126 than cells expressing BRAFV600E or BRAFV600E/T529N. Collectively, these results indicate that the differences in MEK-ERK signaling (Figure 2B) correlated well with both ERK target gene expression (Figure S4) and relative drug resistance (Figure S5A–D) of cells expressing the mutants.

Finally, we also confirmed the PLX4720 resistance of the BRAFV600E/L505H mutant in an additional BRAFV600E-positive human melanoma cells line, MALME-3M. The results show that MALME-3M cells expressing BRAFV600E/L505H were substantially more resistant to PLX4720 than cells expressing BRAFV600E (Figure S5E).

Characterization of the BRAFV600E/L505H mutant in 293T cells and Ba/F3 cells

As described above, the initial characterization of BRAFV600E/L505H was performed in the A375 cell line. However, we found that A375 cells transduced with BRAFV600E were approximately 6-fold more resistant to PLX4720 compared to parental A375 cells (Figure S6). Consistent with our results, previous reports have shown that BRAFV600E amplification leads to vemurafenib resistance (Shi et al., 2012). We therefore considered that the elevated levels of endogenous BRAFV600E in A375 cells might confound an accurate determination of the resistance conferred by PLX4720-resistant alleles, and elected to analyze the BRAFV600E/L505H mutant in two other cell lines that lacked BRAFV600E.

First, we transiently expressed BRAFV600E/L505H in human embryonic kidney 293T cells, which contain wild-type BRAF and have relatively low levels of phospho-MEK and phospho-ERK1/2. As expected, expression of BRAFV600E resulted in activation of MEK-ERK signaling, as evidenced by increased levels of phospho-MEK and phospho-ERK1/2 (Figure 3A). Interestingly, 293T cells expressing BRAFV600E/L505H had substantially higher levels of phospho-MEK and phospho-ERK1/2 compared to 293T cells expressing BRAFV600E, despite similar levels of BRAF protein, indicating that the L505H substitution increases BRAFV600E kinase activity. Even in the absence of the V600E mutation, the L505H substitution (BRAFL505H) led to elevated levels of phospho-MEK (Figure 3A).

Treatment of 293T cells expressing BRAFV600E with PLX4720 resulted in dose-dependent inhibition of MEK phosphorylation, with phospho-MEK levels being nearly undetectable by 2 μ M PLX4720 (Figure 3B). By comparison, 293T cells expressing BRAFV600E/L505H displayed persistent phospho-MEK levels even at a PLX4720 concentration of 50 μ M (see also Figure S7A). Finally, consistent with the results in A375 cells, BRAFV600E/L505H was substantially more resistant to U0126 compared to BRAFV600E (Figure 3C and Figure S7B).

Previous studies have shown that stable expression of BRAFV600E renders Ba/F3 cells, a BRAF-wild type, interleukin-3 (IL-3)-dependent pro-B cell line, dependent on BRAF-MEK-ERK signaling following IL-3 deprivation (Whittaker et al., 2010). To examine the effects of PLX4720 on cellular proliferation, we stably expressed BRAFV600E and BRAFV600E/L505H in Ba/F3 cells, to generate Ba/F3-BRAFV600E and Ba/F3-BRAFV600E/L505H cells, respectively. As expected, expression of either BRAFV600E or BRAFV600E/L505H led to robust activation of MEK-ERK signaling (Figure 4A). Interestingly, total levels of BRAF were reduced in Ba/F3-BRAFV600E/L505H cells compared to Ba/F3-BRAFV600E cells, perhaps due to cytotoxicity of the BRAFV600E/L505H mutant. Nonetheless, MEK-ERK signaling was comparable or higher in Ba/F3-BRAFV600E/L505H cells compared to Ba/F3-BRAFV600E cells, again indicating that the BRAFV600E/L505H mutant has elevated kinase activity. As in 293T cells, even in the absence of the V600E mutation, the L505H substitution (BRAFL505H) led to elevated levels of phospho-MEK (Figure 4B). Treatment of Ba/F3-BRAFV600E cells with PLX4720 resulted in a dose-dependent reduction in phospho-MEK and phospho-ERK1/2 levels (Figure 4C). Notably, Ba/F3-BRAFV600E/L505H cells required a ~20–25-fold higher concentration of PLX4720 to achieve similar reduction of phospho-MEK and phospho-ERK1/2 compared to Ba/F3-BRAFV600E cells (Figure 4C and Figure S8).

Consistent with previous studies (Whittaker et al., 2010), stable expression of BRAFV600E or BRAFV600E/L505H in Ba/F3 cells conferred IL-3-independent proliferation (Figure 4D), enabling determination of the effect of PLX4720 on cellular proliferation. Notably, the cellular PLX4720 IC₅₀ was 40-fold higher for Ba/F3-BRAFV600E/L505H cells compared to Ba/F3-BRAFV600E cells (Figure 4E). As expected, similar resistance was observed when vemurafenib was used instead of PLX4720 (Figure 4F).

Sensitivity of the BRAFV600E/L505H mutant to PLX4720 in mouse xenografts, and to other BRAF inhibitors in cell culture

To determine whether the BRAFV600E/L505H mutant also conferred resistance to PLX4720 in vivo we performed mouse xenograft experiments. Ba/F3-BRAFV600E/L505H cells or Ba/F3-BRAFV600E cells were injected into the flanks of immunocompromised mice and PLX4720 or control vehicle was administered intraperitoneally. PLX4720 markedly reduced growth of Ba/F3-BRAFV600E tumors, as expected, whereas Ba/F3-BRAFV600E/L505H tumors were resistant to PLX4720 (Figure 5A, B). Notably, even in the absence of PLX4720 the growth of Ba/F3-BRAFV600E/L505H tumors was faster than that of Ba/F3-BRAFV600E tumors (Figure 5A), which is likely due to the elevated kinase activity of BRAFV600E/L505H.

Finally, the BRAFV600E/L505H mutant conferred a much lower level of resistance to several other BRAF inhibitors in Ba/F3 cells. For example, the cellular IC₅₀s for SB590885 and GDC0879 were only 4-fold higher (Figure 6A, B) and the cellular IC₅₀ for RAF265 was only 3-fold higher (Figure 6C) in Ba/F3-BRAFV600E/L505H cells compared to Ba/F3-BRAFV600E cells.

DISCUSSION

The comprehensive identification of drug-resistant mutations in cancers is an important step towards the development of improved therapeutic strategies. Here we describe a systematic, structure-based experimental strategy to identify drug-resistant mutants within the oncogenic protein kinase BRAFV600E and its inhibitor vemurafenib. This method can be broadly applied for evaluating resistance to inhibitors of other proteins and has several important advantages over previous experimental approaches. Traditional drug-resistance studies generate mutants by either: (i) random methods (e.g., error prone PCR) that produce undesired mutants with multiple amino acid substitutions and do not necessarily generate all relevant single mutants (because of sampling limitations and biases in random mutagenesis), or (ii) directed mutagenesis based on results from related targets (e.g., gatekeeper mutations in protein kinases) that do not broadly sample sequence space (reviewed in Warmuth et al., 2007). By using systematic, structure-based saturation mutagenesis, we experimentally generate all relevant mutants without complications from undesired multiple amino acid substitutions. Furthermore, coupling the mutagenesis with a comprehensive deep sequencing readout directly quantifies the enrichment of each mutant in response to drug treatment. Thus, our systematic approach not only broadly samples sequence space but also quantifies the contribution of each mutant to drug resistance.

Using this method we have shown that vemurafenib resistance can occur by a novel second-site mutation, T1514A (encoding L505H), within the BRAFV600E kinase domain. A series of cell proliferation and biochemical assays were used to demonstrate the PLX4720 resistance of the BRAFV600E/L505H mutant in three human melanoma cell lines, human 293T cells and mouse Ba/F3 cells. The similarity of the results obtained in diverse cell lines indicates that the PLX4720 resistance of the BRAFV600E/L505H mutant, which is the major focus of our study, is independent of cell type. The L505H mutant increased the kinase activity of both wild-type BRAF and BRAFV600E. Interestingly, a search of the Catalogue of Somatic Mutations in Cancer (COSMIC) database (Forbes et al., 2011) revealed that the T1514A mutation was recently found in an individual with prostate cancer (Barbieri et al., 2012), suggesting that the elevated kinase activity of BRAFL505H may contribute to cancer development.

We found that BRAFV600E/L505H was relatively resistant to MEK inhibition, which is likely due its increased kinase activity. By contrast, the BRAFV600E/L505H mutant had a lesser effect on sensitivity to several other BRAF inhibitors, such as SB590885, RAF265 and GDC0879. Inspection of the crystal structure of the BRAFV600E kinase domain bound to PLX4720 reveals that the propyl group of the sulfonamide moiety of the drug extends toward L505 in the BRAF α C helix (Figure 1E and Figure 6D, E). By contrast, the crystal structure of BRAFV600E bound to SB590885 (PDB:2FB8; King et al., 2006) (Figure 6D) or an analogue of GDC0879 (PDB:3D4Q; Hansen et al., 2008) (Figure 6E) reveals that neither drug is proximal to L505. Thus, resistance of the BRAFV600E/L505H mutant to PLX4720, but not SB590885 or GDC0879, can be explained by differences in steric clash imposed by the L505H substitution. Collectively, these results indicate that such BRAFV600E/L505H-containing melanomas will be more responsive to other BRAF inhibitors than to a MEK inhibitor.

It is important to prospectively identify the various mechanisms that can result in vemurafenib resistance prior to their emergence in the clinic. This information is essential both to accurately assess the growing population of treated patients that will become drug-resistant, and to develop better drugs or drug combinations to overcome diverse resistance mechanisms. Based upon our results we predict that the L505H mutant will be found in some vemurafenib-resistant melanomas particularly if the BRAFV600E-independent resistance pathways can be inhibited. Consistent with this prediction, Tian Xu and colleagues have recently described the isolation of the BRAFV600E/L505H mutant in a melanoma cell line derived from an individual with vemurafenib-resistant melanoma (Choi et al., submitted). These findings clearly demonstrate the power of our experimental approach for the prospective identification of drug-resistant mutants.

Methods

Cell culture

A375 and Ba/F3 cells were cultured in RPMI-1640 (Sigma) supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals), 1 mM sodium pyruvate and Pen/Strep (Sigma). Media for Ba/F3 cells was additionally supplemented with 10 ng/ml murine IL-3 (Peprotech). The identity of the Ba/F3 cell line was confirmed by IL-3 withdrawal

experiments and fluorescence-activated cell sorting (FACS) analysis (Figure S9). 293T cells were cultured in DMEM (Sigma) with 10% FBS, 1 mM sodium pyruvate and Pen/Strep.

Transient and stable transfections

Retroviruses expressing BRAFV600E (Addgene) and all variants were generated by transient transfection of 293T cells using Effectene (Qiagen). Retroviral supernatants were collected 48 h post-transfection, filtered and supplemented with 5 or 10 µg/ml polybrene for infection of Ba/F3 or A375 cells, respectively. For infection of Ba/F3 cells, cells were centrifuged for 90 min at $1,000 \times g$. At 24 h post-infection cells were selected with puromycin at 2 µg/ml. Transient transfections of 293T cells were performed using Effectene.

Mutagenesis screen

Saturation mutagenesis was used to generate point mutant libraries for individual amino acids. To facilitate mutagenesis, a portion of BRAFV600E (nucleotides 1270–1900) was cloned into pRNDM (Hietpas et al., 2012), a minimal cloning plasmid, bracketed by SacI and SphI restriction sites. Cassette mutagenesis was performed on this plasmid as previously described (Hietpas et al., 2012; Hietpas et al., 2011). The mutant pools were then transferred into the retroviral vector pBABE-Puro-BRAFV600E (Addgene #15269) by sequence and ligation-independent cloning (SLIC) (Li and Elledge, 2007). Briefly, the mutagenized cassette was excised using SacI and SphI, treated with T4 DNA polymerase to create 5' single-stranded overhangs (~30 nt), and then purified on a silica column. A pBABE-Puro-BRAFV600E SLIC vector was created by replacing BRAF nucleotides 1300–1870 with an ApaI restriction site. The construct was linearized and treated with T4 DNA polymerase as described above. The treated mutagenized cassette library and pBABE SLIC vector were annealed, and the entire reaction transformed into bacteria. Based on plating of a small fraction of the bacteria, this procedure routinely resulted in greater than 30,000 transformants, which was sufficient to transfer our libraries without compromising diversity. Focused deep sequencing was used to determine the representation of each mutation in the pBABE plasmid libraries and indicated that all mutations were present well above noise level. Individual mutations were generated by the same method and confirmed by Sanger sequencing.

Retroviral particles were generated by transient transfection of 293T cells using Effectene as described above. A375 cells (3×10^6) were transduced at a multiplicity of infection of 3 with the retroviral BRAFV600E mutant pools in 100 mm plates, selected for stable integration with puromycin and cultured in the presence of 10 µM PLX4720 for 3 weeks. Drug-resistant colonies were isolated and pooled, and genomic DNA was extracted.

The library version of BRAF was PCR amplified from genomic DNA and prepared for Illumina sequencing as described (Hietpas et al., 2012). Briefly, PCR was used to introduce an MmeI site immediately 5' to randomized regions and a 3' Illumina primer binding site 250 bases downstream. This product was digested with MmeI, and ligated to adapters that included a barcode and a 5' Illumina primer binding site. This product was PCR amplified with Illumina Sequencing Primers, gel purified, and submitted for short single-read (36 base) analysis using a Solexa-Illumina GA Massively Parallel Deep Sequencer. Raw

sequencing files in fastq format were processed essentially as described (Hietpas et al., 2012). All sequences were subjected to quality filtering requiring a PHRED score of 20 at all read positions. In addition, sequence reads where more than one codon differed from the parental construct were eliminated from analysis. The remaining sequences were analyzed to determine the number of reads for each point mutant in the plasmid library, as well as in transduced cells prior to and following treatment with drug. Mutations that created internal MmeI sites or that were severely under-represented in cells (100-fold relative to median wild-type synonym) without drug were omitted from analysis. Of the 1160 possible protein point mutations (58*20), all but five (478A, 486D, 486E, 511W, and 580W) were successfully analyzed. The ratio of each codon substitution from cells after and before treatment with drug was used as a metric of drug resistance. The median enrichment of silent substitutions was used to normalize the data (resulting in the median synonymous substitution having an enrichment score of 1). Based on the distribution of enrichments for synonymous substitutions, we set a false discovery threshold for each codon observation. Amino acid substitutions were considered enriched in drug if all observed synonymous substitutions were above a statistical cutoff such that the net p-value was <0.01.

Structural images

Images of BRAF bound to PLX4720 were generated from Protein Data Bank (PDB) entry 3C4C (Tsai et al., 2008) using the Pymol software package (Delano Inc.)

Drug treatment

PLX4720 (Selleckchem), SB590885 (Tocris), RAF265 (Selleckchem), U0126 (Cell Signalling), GDC0879 (Selleckchem) and vemurafenib/PLX4032 (Selleckchem) were prepared in DMSO at 20 mM. 1×10^3 A375 or 1.5×10^4 Ba/F3 cells were plated per well of a 96 well plate in 100 μ L volume, and 24 h later 50 μ L of drug was added to the cells.

Cellular and pMEK IC₅₀ determination

Cells expressing BRAFV600E or BRAFV600E mutants were cultured in the presence of serially diluted drug for 72 h, and viability was measured by Alamar Blue assay (Invitrogen). In Figure 2A, Trp and Met mutants were excluded from consideration. For Figures 2F, 4E, 4F and 6A–C, data were plotted in GraphPad Prism and a dose response curve was fit with nonlinear regression. To determine p-MEK IC₅₀, the phospho-MEK immunoblots were quantified in ImageJ (NIH) and the densitometry used to plot the data.

Immunoblotting

Cells were lysed with cell lysis buffer [1%NP-40, 10% glycerol, 150 mM NaCl, 50 mM Tris-HCl (pH 7.4), 1 mM EDTA] supplemented with complete protease inhibitor tablet (Roche) and phosphatase inhibitor cocktails 2 and 3 (Sigma). Cell extract was quantified with bicinchoninic acid (Pierce) and equal amounts of protein were loaded in each lane. Blots were probed with the following primary antibodies: phosphorylated-MEK (S217/221), total-MEK, phosphorylated-ERK1/2 (T202/Y204), total ERK1/2, myc (all from Cell Signaling Technology), and tubulin.

Cell growth assays

Parental Ba/F3 cells or Ba/F3 cells stably transduced with BRAFV600E or BRAFV600E/L505H and cultured in the presence or absence of 10 ng/ml IL-3 were assessed for growth by counting.

qRT-PCR analysis

Total RNA was isolated from A375 cells treated in the presence or absence of drug using TriPure Isolation Reagent (Roche). Reverse transcription was performed using SuperScript II Reverse Transcriptase (Invitrogen) followed by qPCR using Fast SYBR Green Master Mix (Applied Biosystems) using the following primers: *FOSIL* forward (5'-CACTCCAAGCGGAGACAGAC-3') and reverse (5'-AGGTCATCAGGGATCTTGCAG-3'); *SPRY2* forward (5'-ATGGCATAATCCGGGTGCAA-3') and reverse (5'-TGTCGCAGATCCAGTCTGATG-3'); and *DUSP6* forward (5'-AGCTCAATCTGTCTGATGAACG-3') and reverse (5'-GCGTCCTCTCGAAGTCCAG-3'). The expression level of each gene was normalized to that of three internal control genes, *B2M* forward (5'-CGCTCCGTGGCCTTAGC-3') and reverse (5'-AATCTTTGGAGTACGCTGGATAGC-3'); *TBP* forward (5'-CACAGGAGCCAAGAGTGAAG-3') and reverse (5'-CAAGGCCTTCTAACCTTATAGG-3') and *GUSB* forward (5'-TTGAGCAAGACTGATACCACCTG-3') and reverse (5'-TCTGGTCTGCCGTGAACAGT-3'). The qRT-PCR data was normalized to the three internal control genes individually, and then combined.

Tumor formation assays

Balb/c nu/nu mice (NCI; n=5 per group) were injected subcutaneously with 6×10^6 Ba/F3 cells transduced with BRAFV600E or BRAFV600E/L505H. Tumors were allowed to form for 14 days at which point animals received daily intraperitoneal injections of vehicle (water + 5% DMSO) or PLX4720 (50 mg/kg). Tumor dimensions were measured every 3–4 days and tumor volume was calculated using the formula $\pi/6 \times (\text{length}) \times (\text{width})^2$. After the final dose, animals were sacrificed and tumors were excised and weighed. For statistical analysis, Wilcoxon rank sum test was performed in R (www.r-project.org). Animal experiments were performed in accordance with the Institutional Animal Care and Use Committee (IACUC) guidelines.

Probability determinations

The probabilities of mutation types for idealized libraries were calculated based on independent probabilities (Bayesian theory). For random mutagenesis, these probability estimates assume ideal conditions (a mutation rate of 1 base per gene). For a protein of N amino acids (3N nucleotides), the probability of a mutation at any base (p) is $1/3N$, the probability of any position being wild-type (q) is $1-p$, and the probability of all positions being wild-type is q^{3N} . The dependence of this equation on protein length is negligible for proteins > 100 amino acids. The probability of having only one base mutated is $p \cdot q^{(3N-1)}$. The probability of having two or more mutations is $1 - \text{wild-type probability} - \text{single mutant probability}$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Significance

The oncogenic BRAF kinase mutation BRAFV600E is found at high frequency in melanomas. Vemurafenib, a BRAF kinase inhibitor, has shown remarkable clinical efficacy in the treatment of BRAFV600E-positive melanoma. Inevitably, however, resistance emerges. Therefore, prospectively identifying possible vemurafenib-resistance mechanisms is critical for developing more effective therapeutic approaches. Toward this end, we developed a systematic saturation mutagenesis approach to search for second-site mutations within BRAFV600E that could confer drug resistance. Using this method, we identify and characterize a novel vemurafenib-resistant BRAFV600E mutant, which arises by a single nucleotide substitution, and provide insights into the potential treatment of melanomas bearing this mutation.

Figure 1.

A structure-based, targeted, saturation mutagenesis screen identifies PLX4720-resistant BRAFV600E mutants. (A) Schematic summary of the PLX4720 resistance screen. (B) Structure of BRAF complexed with PLX4720 (yellow); amino acids surrounding the drug-binding site are colored magenta. (C) Images of A375 cells transduced with retroviruses expressing BRAFV600E or pools of mutagenized BRAFV600E and grown in media containing PLX4720. Cells were fixed and stained with crystal violet. (D) Scatter plot showing enriched BRAFV600E mutants after PLX4720 selection from two independent biological replicates. Mutants that occur due to single nucleotide substitution are indicated by a red dot. (E) Amino acid sequence of the mutagenized region of human BRAFV600E showing the PLX4720-resistant mutants isolated from the screen showing a >5-fold enrichment. Mutants that occur by single nucleotide substitution are indicated by a red dot. (F) Location of the amino acids whose mutants are enriched >5-fold (shown in pink, except for L505 in red) in the BRAF–PLX4720 structure.

Figure 2.

Sensitivity of the BRAFV600E/L505H mutant to BRAF and MEK inhibitors. (A) Relative cellular IC₅₀ of A375 cells transduced with a subset of candidate PLX4720-resistant mutants plotted against their relative deep sequencing enrichment. (B-E) Immunoblots showing levels of phospho-MEK (p-MEK), total MEK (t-MEK), phospho-ERK1/2 (p-ERK1/2) and total ERK1/2 (t-ERK1/2) in A375 cells transduced with a retrovirus expressing BRAFV600E, BRAFV600E/L505H, BRAFV600E/F516G or BRAFV600E/T529N and treated with increasing doses of PLX4720 (B), SB590885 (C), RAF265 (D) or U0126 (E). α tubulin (TUBA) was monitored as a loading control.

Figure 3.

Characterization of the BRAFV600E/L505H mutant in 293T cells. (A) Immunoblots showing levels of p- and t-MEK, p- and t-ERK1/2 and myc-tagged BRAF in 293T cells transfected with empty vector, wild-type BRAF, BRAFL505H, BRAFV600E or BRAFV600E/L505H. (B, C) Immunoblots showing levels of p- and t-MEK, p- and t-ERK1/2 and myc-tagged BRAF in 293T cells transiently transfected with BRAFV600E or BRAFV600E/L505H and treated with PLX4720 (B) or U0126 (C).

Figure 4.

Characterization of the BRAFV600E/L505H mutant in Ba/F3 cells. (A) Immunoblots showing levels of p- and t-MEK, p- and t-ERK1/2 and myc-tagged BRAF in parental Ba/F3 cells [(–); cultured with IL-3] or Ba/F3 cells stably expressing BRAFV600E or BRAFV600E/L505H (cultured without IL-3). (B) Immunoblots showing levels of p- and t-MEK, p- and t-ERK1/2 and myc-tagged BRAF in Ba/F3 cells stably expressing empty vector, wild-type BRAF or BRAFL505H (cultured with IL-3). (C) Immunoblots showing levels of p- and t-MEK, p- and t-ERK1/2 and myc-tagged BRAF in Ba/F3 cells cultured without IL-3, stably expressing BRAFV600E or BRAFV600E/L505H and treated with PLX4720 (D) Growth of parental Ba/F3 cells (–) or Ba/F3 cells stably expressing BRAFV600E or BRAFL505H/V600E and cultured with (+) or without (–) IL-3. (E, F) Cellular IC₅₀ of parental Ba/F3 cells [(–); cultured with IL-3] and Ba/F3 cells stably transduced with

BRAFV600E or BRAFV600E/L505H (cultured without IL-3) and treated with PLX4720 (E) or vemurafenib (F). Error bars indicate SEM.

Figure 5.

Sensitivity of the BRAFV600E/L505H mutant to PLX4720 in mouse xenografts, and to other BRAF inhibitors in cell culture. (A) Tumor growth in mice subcutaneously injected with Ba/F3-BRAFV600E or Ba/F3-BRAFV600E/L505H cells and then intraperitoneally injected daily with either vehicle or PLX4720. For BRAFV600E $\pm$ PLX4720, $P < 0.05$; for BRAFV600E/L505H $\pm$ PLX4720, $P > 0.05$; for BRAFV600E versus BRAFV600E/L505H (vehicle), $P < 0.05$ at day 27. (B) Tumor weight after the final injection of either vehicle or PLX4720. Results are plotted with group mean, and SEM indicated by the horizontal and vertical lines, respectively. For BRAFV600E $\pm$ PLX4720, $P < 0.05$; for BRAFV600E/L505H $\pm$ PLX4720, $P > 0.05$; for BRAFV600E versus BRAFV600E/L505H (vehicle), $P < 0.05$.

Figure 6.

Sensitivity of the BRAFV600E/L505H mutant to other BRAF inhibitors can be explained by differences in steric clash imposed by the L505H substitution. (A–C) Cellular IC₅₀ of parental Ba/F3 cells [(-); cultured with IL-3] and Ba/F3 cells stably transduced with BRAFV600E or BRAFV600E/L505H (cultured without IL-3) and treated with SB590885 (A), GDC0879 (B) or RAF265 (C). Error bars indicate SEM. (D, E) Position of the α C helix of BRAFV600E bound to SB590885 (blue; D) or the GDC0879 analog (green; E) superimposed on the structure of the BRAFV600E kinase domain (gray) bound to PLX4720 (black). The position of the L505 residue is shown in red.