RESEARCH ARTICLE

EPHA2 Is a Mediator of Vemurafenib Resistance and a Novel Therapeutic Target in Melanoma

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Published OnlineFirst December 26, 2014; DOI: 10.1158/2159-8290.CD-14-0295
INTRODUCTION

Cutaneous melanoma is the most aggressive form of skin cancer. For patients with metastatic disease, only about 10% are expected to survive to 5 years (1). The identification of activating BRAF mutations in melanomas (2) followed by the directed pursuit of selective BRAF\(^{V600E}\) inhibitors (BRAFi) has led to a campaign of highly successful clinical trials in metastatic melanoma (3–5). At the current time, two BRAFi—vemurafenib and dabrafenib—have been approved by the FDA. Despite the rapid and early control achieved with these compounds, response to the BRAFi can be transient and eventual relapse is the general rule (3–5). The addition of a selective MEK inhibitor (MEKi) to a BRAFi improves efficacy (6), but emergence of resistance to both BRAF and MEK inhibitors remains a problem for which there is no current treatment. Moreover, an effective molecularly targeted therapy has yet to be established for the half of the metastatic population without activating BRAF mutations \((\text{BRAF}^{\text{WT}};\text{ ref. 7})\). In balance, these findings underscore the power of targeted approaches and the unmet need to identify novel “druggable” targets that can be exploited in the setting of therapeutically intractable melanomas \((\text{e.g.}, \text{BRAF}^{\text{WT}}\text{ and therapy-resistant BRAF}^{\text{V600E}}\text{ tumors})\).

Activated receptor tyrosine kinases (RTK) have also come into view as both key drivers of melanoma tumorigenesis and central mediators of resistance of selective BRAF\(^{V600E}\) inhibitors. Copy-number and sequence alterations at the \(\text{KIT}\) locus have specifically been associated with acral and mucosal subtypes of melanoma (8), whereas mutations in \(\text{ERBB}4\) and multiple \(\text{EPH}\) family receptors have also been reported (9). Some RTKs, such as \(\text{EPHA}2\) (10) and \(\text{MERTK}\) (11), have been functionally shown to be crucial mediators of melanoma cell survival and tumorigenesis. Activation of RTKs, such as \(\text{PDGFR}\(\alpha\) (12), \(\text{PDGFR}\(\beta\) (13), insulin-like growth factor 1 receptor \(\text{IGF}1\text{R}\); ref. 14), hepatocyte growth factor \(\text{HGF})–\text{MET}\) (15), \(\text{FGFR}\(3\) (16), and \(\text{EGFR}\) (17, 18), have also been shown to mediate resistance to BRAF inhibition. Thus, RTK signaling appears to be a central determinant of melanoma progression and drug responsiveness.

We recently showed that one RTK, \(\text{EPHA}2\), is highly expressed in both BRAF\(^{V600E}\) and BRAF\(^{WT}\) melanomas and that depletion of this molecule leads to dramatic loss of cellular viability, heightened apoptosis \textit{in vitro}, and abrogation of tumor growth \textit{in vivo} (10). \(\text{EPHA}2\) is a member of the \(\text{EPH}\) family of RTKs and is known to contribute to mammary gland, brain, and limb development, and patterning of the visual system (17–25). Furthermore, \(\text{EPHA}2\) overexpression has been observed in numerous cancer types including melanoma (10, 26–30) and in breast cancer cell lines exhibiting resistance to trastuzumab (31). Signaling by the \(\text{EPH}\) system is complex, impinging on both \(\text{RAS}\)–\(\text{PI3K}\)–\(\text{AKT}\) and \(\text{RAS}\)–\(\text{MAPK}\) pathways, and is layered in a network of signaling cross-talk (32). Nevertheless, given its integral role in melanoma survival (10), its role in growth factor signaling (33–36), and its contribution to therapeutic resistance in breast cancer (31), \(\text{EPHA}2\) is a strong candidate for mediating selective BRAFi inhibitor resistance in melanoma. To explore this hypothesis, we generated vemurafenib-resistant melanoma lines \textit{in vitro} and observed...
a dramatic upregulation of EPHA2 in the resistant lines. Moreover, examination of tumors from patients with acquired resistance to BRAFi also revealed evidence of increased EPHA2 expression. Depletion of EPHA2 preferentially induced apoptosis in vemurafenib-resistant cells and partially restored vemurafenib sensitivity. In addition, we developed first-generation ATP-competitive inhibitors against EPHA2 and demonstrated significant activity in overcoming vemurafenib resistance both in vitro and in vivo.

RESULTS

EPHA2 Upregulation Occurs during the Course of Acquired Resistance to BRAF Inhibitors

To determine the role of EPHA2 in vemurafenib response, we performed three lines of investigation. First, we subjected seven melanoma cell lines (A375, SK-mel-28, UACC903, MGH-MC-1, K4, WM239, and WM1158) to escalating doses of vemurafenib, which resulted in significant increases in vemurafenib GI\textsubscript{50} values (Fig. 1A; Supplementary Fig. S1). Because EPHA2 has been shown to be a critical melanoma survival factor (10) and because RTKs are known to mediate BRAFi resistance (12, 14, 37), we hypothesized that EPHA2 may also contribute to acquired vemurafenib insensitivity. As shown in Fig. 1B, there was a clear upregulation of both total EPHA2 and Ser897-phosphorylated EPHA2 (p-EPHA2(Ser897)) in 4 of 7 vemurafenib-resistant lines (A375-P, SK-mel-28-P, MGH-MC-1-P, and K4-P) compared with their matched parental controls (A375, SK-mel-28, MGH-MC-1, and K4), though 3 of 7 pairs exhibited little difference in EPHA2 expression. Second, we used data available for 40 melanoma lines in the Cancer Cell Line Encyclopedia (CCLE) to delineate any relationship between EPHA2 and intrinsic resistance. As shown in Fig. 1C, there was a significant negative correlation between PLX4720 sensitivity (i.e., higher PLX activity area) and EPHA2 RNA levels (P = 0.05). Third, we assessed EPHA2 levels in clinical specimens from patients undergoing clinical trials with vemurafenib or dabrafenib/trametinib (patient information in Supplementary Table S1). In 6 patients with available matched pretreatment/postrelapse tumor RNA, four postrelapse samples showed significantly higher EPHA2 RNA levels compared with the pretreatment specimens (Fig 1D). In 2 patients with only pathology specimens, both postrelapse specimens demonstrated a notable increase in EPHA2 protein levels (Fig 1E). Taken together, these data suggest that EPHA2 may contribute to primary and secondary BRAFi ± MEKi resistance both in vitro and in clinical settings.

The Resistance Phenotype Is Dependent on EPHA2

We next set out to determine whether EPHA2 is functionally mediating vemurafenib resistance. EPHA2 was first over-expressed in WM164 and G-mel melanoma cells (both with low EPHA2 levels) and found to confer a moderate but consistent gain in vemurafenib GI\textsubscript{50} values from 66.39 nmol/L (WM164\textsubscript{con}, 183.65 nmol/L (WM164\textsubscript{EPHA2}) and 2.73 μmol/L (G-mel\textsubscript{con}) to 7.69 μmol/L (G-mel\textsubscript{EPHA2}, Fig. 2A and B). We also depleted EPHA2 in one naive (UACC903) and two vemurafenib-resistant lines (K4-P and MGH-MC-1-P) using shRNA. In each case, there was a reduction in vemurafenib GI\textsubscript{50}–3.35 μmol/L (UACC903\textsubscript{shControl}) to 0.56 μmol/L (UACC903\textsubscript{shEPHA2}), 4.64 μmol/L (K4-P\textsubscript{shControl}) to 1.15 μmol/L (K4-P\textsubscript{shEPHA2}), and 3.32 μmol/L (MGH-MC-1-P\textsubscript{shControl}) to 1.27 μmol/L (MGH-MC-1-P\textsubscript{shEPHA2}, Fig. 2C–E). As Ser897 phosphorylation was associated with vemurafenib resistance (Fig. 1B) and known to become dephosphorylated upon ephrin-A1 binding (38), we next investigated the effects of ephrin-A1 on drug response. We treated a vemurafenib-sensitive (MGH-MC-1) line and a vemurafenib-resistant (K4-P) line with 100 ng/mL ephrin-A1/Fc and observed a decrease in both EPHA2/Ser897 phosphorylation and vemurafenib resistance (Fig. 2F and G). In contrast, ephrin-B1, a non-EPHA2 ligand, was ineffective in abrogating Ser897 phosphorylation or altering vemurafenib sensitivity. These findings suggest that EPHA2 and it's concomitant phosphorylation at Ser897 contribute, at least in part, to the development of vemurafenib resistance.

With the heightened levels of EPHA2 protein and phosphorylation, one could speculate that vemurafenib-resistant cells may adopt a greater phenotypic dependence on EPHA2. One known function of EPHA2 is to trigger cell migration (38). As shown in Fig. 3A and B, EPHA2-enriched–resistant cell lines (A375-P, K4-P, and MGH-MC-1-P) all demonstrated increased transwell migration compared with their matched parental cells. To evaluate the functional involvement of EPHA2 in this phenomenon, we depleted EPHA2 using shRNAs in these lines and observed a strong and selective attenuation of cell migration in the vemurafenib-resistant, but not vemurafenib-sensitive, cells (Fig. 3C–E). Furthermore, we also found that resistant cells were more dependent on EPHA2 compared with sensitive cells in their colony-forming capacities (Fig. 3F and G). These data support the idea that the upregulation of EPHA2 in vemurafenib-resistant lines renders these cells more dependent on this RTK compared with their sensitive counterparts. It also raises the possibility that pharmacologic inhibition of EPHA2 could be an effective approach to overcome resistance to BRAFi and perhaps melanoma growth in general.

First-in-Class EPHA2 Inhibitors Effectively Suppress Vemurafenib-Resistant Melanoma Cells

Given EPHA2’s potential role in sustaining growth (10) and in mediating vemurafenib resistance, a small-molecule inhibitor of EPHA2 would serve as both a pharmacologic means to interrogate the dependence on EPHA2 kinase activity and a starting point for a potential drug-discovery campaign. To this end, we developed a library of ATP-competitive inhibitors that was designed to bind to the inactive, so-called “Asp-Phe-Gly (DFG)-out” conformation of kinases and broadly screened this library against a panel of several hundred kinases including EPHA2. Two compounds that emerged from this screen included ALW-II-41-27 and HG-6-64-1 (Fig. 4A and B). As shown in Fig. 4C, both compounds strongly inhibited EPHA2 activity with biochemical IC\textsubscript{50} of 11.0 nmol/L and 77.1 nmol/L for ALW-II-41-27 and HG-6-64-1, respectively. Both compounds were then subjected to kinase-selectivity profiling using the KinomeScan. As expected, both compounds inhibited a number of additional kinases, with ALW-II-41-27 and HG-6-64-1 returning S(1)-scores of 0.10 and 0.06 (Supplementary Fig. S2A and S2B), respectively, which are comparable to other clinically approved kinase inhibitors (39).
Figure 1. EPHA2 upregulation occurs during the course of induced resistance to BRAF inhibitors both in vitro and in vivo. A, a set of 7 melanoma cell lines were induced into vemurafenib (VEM) resistance by sequential exposure to escalating doses of vemurafenib. Cell viability assay was performed, and the cellular GI50 values for vemurafenib in matched sensitive and resistant lines are shown. Error bars, mean viability ± SEM (n = 3). **, P < 0.001 by the Student t test. B, increased total EPHA2 and phosphorylated EPHA2 (Ser897) in matched vemurafenib-sensitive and vemurafenib-resistant lines were assessed by Western blotting. *, apparent upregulation of EPHA2 in vemurafenib-resistant lines compared with control lines. C, the CCLE database was used to examine PLX sensitivity (PLX activity area) and relative EPHA2 expression. There is a negative correlation between PLX activity area and EPHA2 levels (P = 0.05). D, EPHA2 mRNA levels are elevated in postrelapse tumor samples compared with patient-matched pretreatment specimens during the course of treatment with BRAF ± MEK inhibitors. Of note, a BRAF splice product has also been detected in the postrelapse specimen for patient #22. The BRAF splice variant may explain resistance to dabrafenib but not necessarily trametinib. RNA levels were measured using qPCR assay (in triplicate) with GUSB as control. Error bars, mean normalized levels ± SEM (n = 3). *, P < 0.05 by the Student t test. E, immunohistochemistry showed increased EPHA2 levels in postrelapse tumor samples compared with pretreatment tumor specimens from patients #26 and #34. H&E, hematoxylin and eosin.
Figure 2. EPHA2 mediates resistance to vemurafenib. EPHA2 overexpression in WM164 (A) and G-mel (B) cells leads to vemurafenib (VEM) resistance, whereas EPHA2 depletion in UACC903 (C), K4-P (D), and MGH-MC-1-P (E) cells by EPHA2 shRNA partially restores vemurafenib sensitivity. Ephrin-A1 (100 ng/mL), but not ephrin-B1 (100 ng/mL), suppresses EPHA2 Ser897 phosphorylation and partially reverses vemurafenib resistance in K4-P (F) and MGH-MC-1 (G) cells. All error bars, mean viability ± SEM (n = 3).

To establish EPHA2 as an intracellular target for these compounds, we first examined whether the inhibitory activities of ALW-II-41-27 and HG-6-64-1 correlated with EPHA2 protein levels. Both ALW-II-41-27 and HG-6-64-1 were more effective in CHL-1 cells (high EPHA2) compared with SK-mel-119 (low EphA2; Supplementary Fig. S3A–S3C). There was also a consistent decrease in the GI50 values for both compounds when EPHA2 was overexpressed in SK-mel-119 and WM164 cells (Supplementary Fig. S3D–S3I). In contrast, depletion of EPHA2 in UACC903 and CHL-1 cells by shRNA partially attenuated the inhibitory activity of both EPHA2 inhibitors (Supplementary Fig. S3J–S3O). Moreover, a weaker EPHA2 inhibitor NG-25 with a biochemical IC50 of 773 nmol/L also exhibited less potent cellular inhibition compared with more active ALW-II-41-27 (Supplementary Fig. S3P–S3R). These results show a positive correlation between EPHA2 levels and inhibitor sensitivity, thereby providing supportive evidence that EPHA2 is a functionally relevant target of these compounds in melanoma cells.

In the context of vemurafenib resistance, both EPHA2 inhibitors showed similar, if not greater, activity against vemurafenib-resistant lines (i.e., lines with higher EPHA2) compared with the sensitive parental lines (i.e., lines with lower EPHA2; Fig. 4D and E; Supplementary Fig. S4A and S4B). Even under conditions of vemurafenib resistance, EPHA2 appears to be the target of ALW-II-41-27 and HG-6-64-1, as depletion of EPHA2 in MGH-MC-1-P led to increased resistance to both EPHA2 compounds (Fig. 4F and G). Morphologically, EPHA2 inhibition resulted in cell rounding, loss of adhesion, and cell death. In contrast, vemurafenib caused no significant cell death in BRAF WT and resistant lines (Supplementary Fig. S4C).

Beyond single-agent efficacy, ALW-II-41-27 also demonstrated significant synergism with vemurafenib in both sensitive A375 cells [Supplementary Table S2; lowest combination index (CI) = 0.12, “strong” synergism] and resistant A375-P cells (lowest CI = 0.22, “strong” synergism). Overall, 11 of 16 ALW-II-41-27 + vemurafenib dose points exhibited at least “moderate” synergism (i.e., CI < 0.85). Furthermore, when
The MEK inhibitor AZD6244 was tested in combination with ALW-II-41-27 or HG-6-64-1, 15 of 16 and 16 of 16 dose points, respectively, exhibited at least “moderate” synergism. Lastly, we also found that pretreatment with ALW-II-41-27 and HG-6-64-1 restored vemurafenib sensitivity in resistant A375 (A375-P) and SK-mel-28 (SK-mel-28-P) cells (Fig. 4H and I).

**EPHA2 Inhibition Abolishes AKT and ERK Phosphorylation and Induces Apoptosis**

The physiologic effects of ALW-II-41-27 and HG-6-64-1 on EPHA2-AKT and EPHA2-MEK-ERK signaling were then investigated. At 2 μmol/L, both agents effectively inhibited EPHA2<sup>Ser897</sup> and AKT<sup>Ser473</sup> phosphorylation; at 10 μmol/L,
Figure 4. EPHA2 inhibitors are highly effective in vemurafenib-resistant cell lines. Structures of ALW-II-41-27 (A) and HG-6-64-1 (B). C, both ALW-II-41-27 and HG-6-64-1 strongly inhibited EPHA2 activity in a cell-free system using the SelectScreen Kinase Profiling Service. Vemurafenib (VEM)-resistant cells (MGH-MC-1-P and SK-mel-28-P) are more sensitive to ALW-II-41-27 (D) and HG-6-64-1 (E) than their vemurafenib-sensitive parental lines (MGH-MC-1 and SK-mel-28). Depletion of EPHA2 by shRNA (Fig. 3C, Western blot) increased resistance to ALW-II-41-27 (F) and HG-6-64-1 (G). Pretreatment (2 μmol/L for 2 hours) with ALW-II-41-27 and HG-6-64-1 restored vemurafenib sensitivity in EPHA2-upregulated vemurafenib-resistant A375-P (H) and SK-mel-28-P (I) cells. *, P < 0.01 by the Student t test.
efficient inhibition of MEK and partial inhibition of ERK were also achieved. ALW-II-41-27 and HG-6-64-1 extinguished MAPK and AKT signaling in both vemurafenib-sensitive (A375) and vemurafenib-resistant (A375-P) lines, whereas vemurafenib abrogated only MAPK, but not AKT, signaling and then only in the sensitive cells. Vemurafenib also inhibited p-EPHA2Ser897 in the sensitive A375 line (Fig. 5A and B).

Because both EPHA2–AKT and EPHA2–MEK–ERK pathways play critical roles in promoting proliferation and inhibiting apoptosis, we next assessed the impact of the EPHA2 compounds on the cell cycle and on the induction of apoptosis. FACs analysis showed that treatment with either ALW-II-41-27 or HG-6-64-1 caused cell-cycle arrest at G0–G1 and induced apoptosis at higher doses in both vemurafenib-sensitive and vemurafenib-resistant cell lines (Fig. 5C and D); morphologically, the nuclei of cells treated with either ALW-II-41-27 or HG-6-64-1 exhibited a condensed and fragmented morphology, characteristic of apoptosis (Fig. 5E-G). In contrast, vemurafenib was effective in triggering G0–G1 arrest but not apoptosis and only in sensitive cells (Fig. 5C and D). These data suggest that EPHA2 blockade abolishes EPHA2–AKT as well as EPHA2–MEK–ERK signaling and induces apoptosis independent of vemurafenib responsiveness.

**EPHA2 Inhibitors Suppress In Vivo Tumor Growth of Both Vemurafenib-Sensitive and Vemurafenib-Resistant Melanomas**

Lastly, we evaluated the efficacy of ALW-II-41-27 against A375 xenografts in mice. ALW-II-41-27 dosed at 30 mg/kg by i.p. significantly suppressed growth of both vemurafenib-sensitive A375 and vemurafenib-resistant A375-P tumors in vivo compared with the vehicle control (P < 0.001). In contrast, PLX4720 inhibited the growth of only the parental A375 (P < 0.001) tumors but not the resistant A375-P (P = N.S.) tumors in vivo (Fig. 6A and B; Supplementary Fig. S5A and S5B). Compared with control groups, ALW-II-41-27 reduced A375 and A375-P tumor volumes by 79.7% and 81.6%, respectively, whereas PLX4720 reduced the tumor volumes by 78.6% and 16.9%, respectively (Supplementary Fig. SSC). Administration of ALW-II-41-27 or PLX4720 was well tolerated by healthy mice without significant signs of overt toxicity or weight loss (P > 0.05; Supplementary Fig. SSD and SSE).

Consistent with the *in vitro* data, analysis of both A375 and A375-P tumors from ALW-II-41-27-treated and PLX4720-treated animals showed widespread apoptosis as indicated by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining, nuclear fragmentation (Fig. 6C), and both apoptosis and necrosis on hematoxylin/eosin staining (Fig. 6D). PLX4720 induced significant apoptotic changes only in A375 tumors (Fig. 6C and D). Notably, ALW-II-41-27 caused more dramatic apoptosis in the vemurafenib-resistant A375-P tumors compared with the A375 tumor (Fig. 6C and D), further supporting a stronger EPHA2 dependency for the vemurafenib-resistant tumor cells. Consistent with the *in vitro* observations, administration of ALW-II-41-27 caused significant suppression of EPHA2–MEK–ERK signaling in both A375 and A375-P tumors, whereas vemurafenib suppressed EPHA2–MEK–ERK signaling only in the parental A375 line (Fig. 6E-G). Moreover, because EPHA2 influences migration and invasion, we assessed for tumor metastasis in our animal experiments and failed to observe any gross metastatic deposits in the visceral organs (data not shown). Taken together, these experiments provide *in vivo* evidence of feasibility for EPHA2 inhibition in the setting of therapeutic resistance.

**DISCUSSION**

EPHA2 is overexpressed in many human malignancies including melanomas, is associated with a worse prognosis especially among glioblastomas and esophageal carcinomas, and is a promoter of proliferation and invasiveness in various cancer cell lines (10, 19, 21–30). Previous studies from our laboratory showed that EPHA2 is an essential survival factor in melanoma (10). In this article, we provide several lines of evidence to suggest that EPHA2 is also a novel mediator of vemurafenib resistance and potential pharmacologic target in melanoma. First, both total and phosphorylated EPHA2 are upregulated in vemurafenib-resistant cells compared with vemurafenib-sensitive cells, while EPHA2 levels are also increased in relapse specimens compared with pretreatment specimens from clinical trials. Next, overexpression of EPHA2 in vemurafenib-sensitive cells significantly increased vemurafenib resistance, whereas depletion of EPHA2 in both naive and vemurafenib-resistant cells can dramatically confer or restore vemurafenib sensitivity. Third, we found that the natural EPHA2-selective ligand, ephrin-A1, reduced EPHA2 signaling and enhanced vemurafenib responsiveness. Lastly, vemurafenib-resistant cells appear to be more “addicted” to EPHA2 in their survival and migration phenotype compared with their vemurafenib-sensitive counterparts.

EPHA2 joins the canon of RTKs known to be upregulated with BRAFi resistance—these include PDGFRβ (12), PDGFRα (13), IGF1R (14), FGFR3 (15), and EGFR (17, 18). Like other RTKs, higher EPHA2Ser897 correlates with a resistant phenotype. It is interesting to note that the vemurafenib-resistant cells appear to have acquired a greater dependence on EPHA2 in terms of migration and colony-formation capacity (Fig. 3). This could also explain why EPHA2 inhibitors are effective as single agents in the resistant cells even in the absence of additional vemurafenib, though there is clear synergism between anti-EPHA2 and anti-MAPK compounds. In the animal studies, vemurafenib-resistant A375-P tumors exhibited greater levels of apoptosis compared with vemurafenib-sensitive A375 tumors when treated with ALW-II-41-27. Thus, secondary vemurafenib resistance may come with a heightened “addiction” to EPHA2, which could reflect biologic changes induced by the increased EPHA2 or a fundamental change in the nature of the resistant cells or both. Unlike other receptor systems that are activated by soluble ligands, interaction between ephrin-A1 and EPHA2 suppresses EPHA2Ser897 phosphorylation and sensitizes cells to vemurafenib. Thus, the mechanism by which EPHA2 and other RTK signaling are sustained in the setting of acquired resistance is not known and is a focus of ongoing investigation.

With the myriad of resistance lesions reported, it is in fact possible that drug selection recruits multiple simultaneous mechanisms to bypass growth blockade. These can be current in the same cell or scattered across clones within...
Figure 5. EPHA2 inhibitors inhibit phosphorylation of AKT and ERK, arrest cell cycle at G0–G1, and induce apoptosis in both vemurafenib (VEM)-sensitive and VEM-resistant cells. ALW-II-41-27 and HG-6-64-1 abrogated EPHA2–AKT and EPHA2–MAPK signaling in both vemurafenib-sensitive A375 (A) and A375-P (B) cells, whereas vemurafenib inhibited only the MAPK pathway and only in the parental A375 line. Similarly, ALW-II-41-27 and HG-6-64-1 caused both G0–G1 arrest and sub-G1 fractionation in both vemurafenib-sensitive A375 (C) and A375-P (D) cells. However, vemurafenib only induced G0–G1 arrest and exclusively in the A375-sensitive line. Error bars, percentage of cells ± SEM (n = 2). E, ALW-II-41-27 and HG-6-64-1 caused nuclear condensation and fragmentation in both A375 (vemurafenib-sensitive) and CHL-1 (vemurafenib-resistant) cells. The nuclei were stained with Hoechst, and analyzed using a fluorescent microscope (the condensed/fragmented nuclei are indicated by arrowheads). F and G, quantification results for the nuclei staining in E.
Figure 6. EPHA2 inhibitor ALW-II-41-27 suppresses in vivo tumor growth of both vemurafenib-sensitive and vemurafenib-resistant melanomas. Administration of ALW-II-41-27 inhibited growth of both A375 and A375-P tumors in vivo. After inoculation of 4 x 10^7 A375 (A) or A375-P (B) cells, ALW-II-41-27 was administered twice a day by intraperitoneal injection (30 mg/kg) whereas PLX-4720 was administered once a day by intraperitoneal injection (30 mg/kg). The tumor volumes were measured every day by caliper. Error bars, mean tumor volume ± SEM (n = 6 animals each arm). ALW-II-41-27 administration induced significant apoptosis of both A375 and A375-P tumor cells, which was readily apparent with the TUNEL assay (green in C) and hematoxylin/eosin staining (D). E, ALW-II-41-27 suppressed phosphorylation of EPHA2 and ERK in both A375 and A375-P tumors as demonstrated by Western blot analysis. Immunohistochemistry showed that both ALW-II-41-27 and PLX4720 abrogated phosphorylation of EPHA2 (F) and ERK1/2 (G) in the A375 tumors, whereas only ALW-II-41-27 suppressed these signaling events in the A375-P tumors.
a single population. We have observed a modest increase in pTyr-AXL in two of the EPHA2-upregulated-resistant lines, though functional studies suggest that the increased AXL signaling is not significantly contributing to vemurafenib resistance (manuscript submitted by Zhenyu Ji, et al.). Furthermore, Wagle and colleagues (40) recently performed whole-exome sequencing of paired pretreatment and post-relapse specimens and delineated a constellation of genetic changes associated with gain of resistance to combination dabrafenib + trametinib. However, it is not yet possible to parse out the contribution of individual changes to the overall pharmacophenotype. In fact, a BRAF splice variant in one of the EPHA2-augmented relapse tumor specimens (patient #22) was identified in that study (40), thereby supporting the idea that long-term drug selection may elicit multiple concurrent resistance lesions.

Earlier studies have employed cotargeting of both MAPK and PI3K-AKT pathways to overcome RTK-mediated vemurafenib resistance (12, 13). However, we set out to demonstrate that EPHA2 can be directly antagonized by generating first-in-class small-molecule inhibitors of EPHA2. These agents proved efficacious in overcoming vemurafenib resistance both in vitro and in vivo. There have been previous inroads made into anti-EPHA2 therapy (41). The early use of soluble EPHA2-Fc molecules demonstrated significant antitumor activity in animal studies (42, 43). In 2008, two 2,5-dimethylpyrrolyl benzoic acid derivatives were shown to competitively inhibit the ephrin–EPH interaction by interacting with the FGF receptor. However, none of these approaches to date hold the same promise as a small-molecule kinase inhibitor that is selective against EPHA2. ALW-II-41-27 and HG-6-64-1 were discovered using a rationally designed kinase-directed library and are believed to bind to the “DFG-out” inactive kinase (type II) conformation based on similarity to structurally related compounds that have been crystallized with EPHA3 and EPHA7. Like other type II inhibitors that are clinically approved, such as imatinib, nilotinib, and sorafenib, ALW-II-41-27 and HG-6-64-1 possess a spectrum of kinase targets in addition to EPHA2 (Supplementary Fig. S2; manuscript submitted by Jianming Zhang, et al.). Although the biologic effects are undoubtedly related to the entire constellation of intracellular targets, our molecular and pharmacologic results support the idea that EPHA2 is a critical target for both ALW-II-41-27 and HG-6-64-1 and that EPHA2 may be successfully leveraged as a novel pharmacologic target in vemurafenib-resistant melanomas.

Although there are important findings revealed by these studies, there are also limitations. First, a phenotypic selection in vitro, such as induction of vemurafenib resistance, is likely to identify compensatory mechanisms based in part by the nutrient and growth factor conditions in which these experiments were performed. Moreover, pharmacologic selection, either in vitro or in vivo, may galvanize multiple resistance mediators as alluded to above. This could explain why vemurafenib responsiveness is not fully reproduced or restored despite significant alterations in EPHA2 levels. We are attempting to improve the gene/drug response specification by isolating individual subclones for analysis. Second, although ALW-II-41-27 and HG-6-64-1 potently inhibit EPHA2 biochemically and in cells, both compounds possess additional targets which most likely contribute to their pharmacology. Efforts are under way to further refine the structure to enhance EPHA2 specificity and to identify mutations in EPHA2 that confer resistance to the inhibitors. Thus, future iterations of these compounds may in fact demonstrate even greater potency. Third, our studies do not address the mechanisms by which EPHA2, or possibly other RTKs, becomes activated. Given our current understanding of HGF–MET in vemurafenib resistance (15), autocrine and paracrine loops are likely to play a role. On the other hand, these RTKs are clearly vulnerable to pharmacologic targeting, as evidenced by our results.

In summary, we have identified EPHA2 induction as a mechanism of vemurafenib resistance and successfully provided a first-pass pharmacologic solution to inhibit this RTK. Our results confirm that small-molecule inhibition of EPHA2 can be an effective means to neutralize vemurafenib resistance. These studies thus offer a new strategy to treat patients with melanomas that are either primarily insensitive to vemurafenib or those that have developed resistance against BRAF inhibition.

**METHODS**

**Materials**

PLX4032/vemurafenib, PLX4720, and AZD6244 were purchased from Chembridge. ALW-II-41-27 and HG-6-64-1 were provided by Dr. Nathanael Gray’s laboratory at Dana-Farber Cancer Institute, Harvard Medical School (Boston, MA). The purity of all compounds was >95%. shRNAs targeting EPHA2 were purchased from Open Biosystems. The recombinant ephrin-A1 and ephrin-B1 were purchased from R&D systems. Mouse polyclonal anti-EPHA2 (C-20) antibody was purchased from Santa Cruz Biotechnology. Rabbit anti–phospho-EPHA2 (Ser897), anti–phospho-ERK (Thr202/Tyr204), and anti–phospho-AKT (Ser473) antibodies were obtained from Cell Signaling Technologies. All other reagents and chemicals were purchased from Sigma.

**Cells and Cell Culture**

Human melanoma cell lines (A375, SK-mel-28, UACC903, MGH-MC-1, K4, WM239, WM1158, WM164, G-mel, CHL-1, SK-mel-119) were developed in-house, purchased from the American Type Culture Collection, or gifts from Meenhard Herlyn (Wistar Institute, Philadelphia, PA). Vemurafenib-resistant melanoma cell lines (A375-P, SK-mel-28-P, UACC903-P, MGH-MC-1-P, K4-P, WM239-P, and WM1158-P) were obtained by sequentially exposing cells to escalating doses of vemurafenib in 2014. DMEM medium with 10% FBS, supplemented with 100 units/mL penicillin, 100 µg/mL streptomycin, and 2 mmol/L glutamine, was used for routine culturing of cells; vemurafenib-resistant cells were cultured in vemurafenib-free media for 10 to 14 days before retesting vemurafenib sensitivity; this drug holiday period had no effect on the vemurafenib resistance (data not shown). All cells were incubated in a humidified atmosphere of 95% air and 5% CO2 at 37°C. Tumor specimens from patients were obtained before treatment with BRAFi (vemurafenib) or BRAFi + MEKi (dabrafenib + trametinib) and after relapse as indicated in Supplementary Table S1. Acquisition of tissue was covered under a protocol approved by the Dana-Farber/Harvard Cancer Center (legacy #11-181) in accordance with the Declaration of Helsinki.
Cell Line Authentication

Cell lines were verified in 2014 by matching mutational profiles and/or copy-number variants obtained in our laboratory with those independently reported in the Catalogue of Somatic Mutations in Cancer (A375, G-mel, SK-mel-28, CHL-1) or with those published by at least two independent laboratories (WM1158, SK-mel-119, WM239, UACC903).

Data on PLX4720 sensitivity (PLX activity area) and EPHA2 expression for the melanoma lines were obtained through the CCLE website (http://www.broadinstitute.org/ccle/home). Statistical analysis was performed using GraphPad Prism 6 in 2014.

Cell Viability Assay

Melanoma cells were seeded into 96-well white plates at a density of 5 to 10 × 10⁴ cells per well in 100 μL media. The compounds were added at the designated concentrations and incubated for 72 hours. A luminescence-based commercial kit (CellTiter-Glo; Promega) was used to measure cell viability. Briefly, 30 μL of cell lysate/ATP detection reagent was added to each well and incubated on a shaking platform for 10 minutes at room temperature, and the luminescence was measured with a plate reader (Molecular Devices). Cellular GI₅₀ values were determined using CompuSyn software.

Cell Migration Assays

The 24-Transwell Boyden chambers (Costar) with a polycarbonate membrane (6.5 mm diameter, 10 μm thickness, and 8 μm pore size) were used. Melanoma cells were seeded in the upper compartment of the well in serum-free media (5 × 10⁴ cells/well) with or without compounds. The lower compartment was supplied with 600 μL serum-free media supplemented with 20 μg/mL fibronectin. Cells were treated for 8 hours, and then were fixed and stained with 0.1% crystal violet. The nonmigrating cells on the upper surface of the membrane were removed, and the migrated cells on the lower side were photographed with a microscope (Nikon) in five random fields. For quantitation, the migrated crystal violet-stained cells were lysed with 10% acetic acid, and colorimetric determination was made at 595 nm.

Cellular Colony Formation Assay

Melanoma cells were seeded in 6-well plates at a density of 500 to 1,000 cells per well. The media were changed every other day, and the colonies were counted at day 14 after staining with 0.1% crystal violet.

Cell Migration Assays

The 24-Transwell Boyden chambers (Costar) with a polycarbonate membrane (6.5 mm diameter, 10 μm thickness, and 8 μm pore size) were used. Melanoma cells were seeded in the upper compartment of the well in serum-free media (5 × 10⁴ cells/well) with or without compounds. The lower compartment was supplied with 600 μL serum-free media supplemented with 20 μg/mL fibronectin. Cells were treated for 8 hours, and then were fixed and stained with 0.1% crystal violet. The nonmigrating cells on the upper surface of the membrane were removed, and the migrated cells on the lower side were photographed with a microscope (Nikon) in five random fields. For quantitation, the migrated crystal violet-stained cells were lysed with 10% acetic acid, and colorimetric determination was made at 595 nm.

Biochemical Profiling of Compounds for Inhibition of EPHA2

The SelectScreen Kinase Profiling Service (Life Technologies) was used to profile the potency of compounds in vitro. In general, the assay was conducted in 50 mmol/L HEPES (pH 7.5), 0.01% Brij-35, 10 mmol/L MgCl₂, 1 mmol/L EGTA, 2 mmol/L DTT, and 100 μmol/L/ATP for 1 hour. The biochemical IC₅₀ was calculated according to 10-point compound titration (0.5 mmol/L to 10 μmol/L).

Real-Time PCR

RNA was isolated from samples of patients with melanoma using an RNeasy kit (Qiagen). cDNA was generated from 1 μg of RNA using First Strand Ready-to-Go beads (GE Healthcare Life Science). Real-time PCR was performed in triplicate using EPHA2 and GUSB TagMan primers (Invitrogen) and the LightCycler TagMan Master Kit (Roche Applied Sciences) on a Roche LC480 qRT-PCR machine. EPHA2 cDNA levels in human samples were normalized to GUSB, and are presented as relative units to pretreatment levels. Data acquisition and analysis were performed according to the manufacturer’s instructions.

Plasmid Construction and Transfection

The eukaryotic expression plasmid of human EPHA2 gene was generated by cDNA cloning followed by sequence confirmation. The cell lines were transfected with 4 μg of the EPHA2 plasmid or control vector using Lipofectamine (Invitrogen) according to the manufacturer’s protocol. The cells were selected with G418 (1 mg/mL) for stable incorporation. The stable clones were collected, and protein expression levels were confirmed with Western blotting.

EPHA2 Gene Silencing

shRNAs targeting EPHA2 were purchased from Open Biosystems. The shRNA plasmids were mixed with the lentivirus packaging plasmids (Invitrogen) in the ratio according to the manufacturer’s protocol. They were then mixed with Lipofectamine 2000 (Invitrogen) and transfected into HEK 293T cells for 48 hours to generate virus. Then the viral supernatants were filtered and used for target cell infection in the presence of 8 μg/mL Polybrene (American Bioanalytical), followed by selection with puromycin to get stable populations of cells.

Western Blotting

Cells were lysed in 20 mmol/L HEPES, pH 7.5, 150 mmol/L NaCl, 1% NP-40, 10 mmol/L sodium pyrophosphate, 100 μmol/L NaF, 175 mmol/L 1-glycerophosphate buffer supplemented with Complete Mini Protease Inhibitor Cocktail tablets (Roche Applied Sciences). Samples separated by SDS-PAGE were transferred to nitrocellulose membranes, blocked with 5% BSA (w/v) at room temperature for 1 hour, and incubated with primary antibodies (1:1,000 dilution) at 4°C overnight. After incubation with secondary antibody (1:3,000 dilution) at room temperature for 1 hour, the membranes were developed with enhanced chemiluminescence (ECL) reagent (LumiGold; SigmaGen Laboratories) and exposed to Hyperfilm MP (GE Healthcare Life Science). Tumor samples were lysed in RIPA buffer (50 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 5 mmol/L EDTA, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP-40, supplemented with Complete Mini protease inhibitors), and equal amounts of protein were subjected to Western blotting analysis.

Fluorescent Immunocytochemistry

Cells were seeded on coverslips, followed by treatments with compounds. Then cells were fixed with 4% paraformaldehyde for 30 minutes, permeabilized with 0.1% Triton X-100 for 20 minutes, blocked with 5% normal serum for 30 minutes, and incubated with Hoechst for 10 minutes. All images were obtained using Olympus BX51 microscope.

Flow Cytometry Analysis

Melanoma cells were seeded in 6-well plates (5 × 10⁵ cells/well) with or without compounds treatment. Then cells were harvested, fixed with 70% ethanol, and stained with propidium iodide (PI; 5 μg/mL) in the presence of RNase (1 g/L), 1 g/L sodium citrate, and 0.5% Triton X-100 (v/v) in the dark for 30 minutes. Cells were collected for apoptosis analysis using FACS-Calibur (BD Biosciences). The percentage of hypodiploidy was analyzed using ModFIT LT software (Verity Software).

Immunohistochemistry

Tumor tissues were fixed in phosphate-buffered formalin, embedded in paraffin, cut to 4-μm thickness, and applied to slides. The slides were deparaffinized in xylene using three changes for 5 minutes each, and hydrated gradually through graded alcohols: 100% ethanol twice for 10 minutes each, 95% ethanol twice for 10 minutes each, and then deionized water for 1 minute with stirring. For antigen unmasking, slides were placed in a container, covered with 10 mmol/L sodium citrate buffer, pH 6.0, and heated in a convection steamer for 1 hour. The slides were washed in deionized water three times for 2 minutes each, blocked with 5% normal goat blocking serum for 30 minutes, incubated with primary antibodies for 1 hour, and incubated with a Rhodamine-conjugated secondary antibody for 30 minutes, and then

MARCH 2015 CANCER DISCOVERY | 285
incubated with Hoechst for 10 minutes. The slides were analyzed and photographed using a fluorescent microscope. TUNEL staining was performed using a DeadEnd Fluorometric TUNEL kit according to the manufacturer’s instructions (Promega).

Subcutaneous Xenograft Tumor Growth In Vivo

Nude/nude mice were purchased from in-house colonies at Massachusetts General Hospital (MGH), and housed in a BL2 lab at MGH. All animal experiments were carried out in accordance with protocols approved by the MGH Animal Care and Use committees. A375 and A375-P cells were cultured in vitro in DMEM medium and then resuspended in PBS (4 × 10⁶ cells/mL). The melanoma cells were injected subcutaneously into axillary regions of nude/nude mice (4 × 10⁶ cells/100 μL/mouse). When the tumor volumes reached approximately 60 mm³ (about one week), the mice were randomized to control group and various treatment groups (n = 6 per group), including ALW-II-41-27 (30 mg/kg, twice/day; vehicle: 10% 1-methyl-2-pyrolidinone and 90% PEG 300) and PLX4720 (30 mg/kg; vehicle: DMSO) groups. Tumors were measured every day for approximately 2 weeks with a microlancer. The body weight was measured every day with a scale. The tumor volumes were calculated with the formula: (mm³) = width × width × length × 0.5. After administration with vehicle, ALW-II-41-27, or PLX4720 via intraperitoneal injection for approximately 2 weeks, the mice were euthanized using carbon dioxide (CO₂) and tumors were harvested. Tumor cell apoptosis and EPHA2-related signaling were analyzed using immunohistochemistry and Western blotting.

Statistical Analysis

The Student t test and ANOVA were performed using StatView (SAS Institute). The data shown are representative of at least two independent experiments with similar results, and the data points represent the mean of at least triplicate measurements with error bars corresponding to SD.

Disclosure of Potential Conflicts of Interest

J.A. Wargo has received honoraria from the speakers’ bureau of Dava Oncology. No potential conflicts of interest were disclosed by the other authors.

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Grant Support

This work was supported in part by the NIH (K24 CA149202 to H. Tsao; R01CA163222 to H. Tsao, K.T. Flaherty, and D.T. Frederick; 5T32AR007098 to B. Miao; 5T32CA071345 to Z. Ji; and R01 CA173469 to N.S. Gray), the American Skin Association (to H. Tsao), and the generous donors to the MGH Millennium Melanoma Fund (to H. Tsao).

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Received March 21, 2014; revised December 18, 2014; accepted December 19, 2014; published OnlineFirst December 26, 2014.

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