Relief of Feedback Inhibition of HER3 Transcription by RAF and MEK Inhibitors Attenuates Their Antitumor Effects in BRAF-Mutant Thyroid Carcinomas

Cristina Montero-Conde, Sergio Ruiz-Llorente, Jose M. Dominguez, Jeffrey A. Knauf, Agnes Viale, Eric J. Sherman, Mabel Ryder, Ronald A. Ghossein, Neal Rosen, and James A. Fagin
ABSTRACT

The RAF inhibitor vemurafenib (PLX4032) increases survival in patients with BRAF-mutant metastatic melanoma, but has limited efficacy in patients with colorectal cancers. Thyroid cancer cells are also comparatively refractory to RAF inhibitors. In contrast to melanomas, inhibition of mitogen-activated protein kinase (MAPK) signaling by PLX4032 is transient in thyroid and colorectal cancer cells. The rebound in extracellular signal-regulated kinase (ERK) in thyroid cells is accompanied by increased HER3 signaling caused by induction of ERBB3 (HER3) transcription through decreased promoter occupancy by the transcriptional repressors C-terminal binding protein 1 and 2 and by autocrine secretion of neuregulin-1 (NRG1). The HER kinase inhibitor lapatinib prevents MAPK rebound and sensitizes BRAF-mutant thyroid cancer cells to RAF or MAP-ERK kinase inhibitors. This provides a rationale for combining ERK pathway antagonists with inhibitors of feedback-reactivated HER signaling in this disease. The determinants of primary resistance to MAPK inhibitors vary between cancer types, due to preferential upregulation of specific receptor tyrosine kinases, and the abundance of their respective ligands.

SIGNIFICANCE: Thyroid cancer cell lines with mutant BRAF are resistant to PLX4032. RAF inhibitors transiently inhibit the ERK pathway and de-repress HER3 transcription. In the context of constitutive NRG1 secretion, this results in an ERK and AKT rebound that diminishes the antitumor effects of RAF inhibitors, which is overcome by combination with lapatinib. Cancer Discov; 3(5):520–33. ©2013 AACR.

See related commentary by Girotti and Marais, p. 487.

INTRODUCTION

Mutations in BRAF are present in approximately 50% of metastatic melanomas, 35% to 60% of advanced thyroid cancers, and in a lower proportion of colorectal, ovarian, and lung carcinomas (1–4). The small-molecule RAF inhibitor vemurafenib (PLX4032, RG7204) has high specificity for the BRAF oncoprotein, and is a potent inhibitor of mitogen-activated protein kinase (MAPK) signaling and growth of BRAF-mutant melanoma cell lines, but not of cancer cell lines with wild-type BRAF (5, 6). PLX4032 increases overall survival and progression-free survival in patients with BRAF-mutant metastatic melanoma. Although the clinical responses are remarkable, the beneficial effects are not durable, as the median time to progression is 5.3 months (7).

Several mechanisms may account for secondary resistance to PLX4032 in melanomas; for example, acquisition of RAS mutations, overexpression of PDGFRβ, expression of a drug-resistant splice variant of BRAFV600E with enhanced dimerization properties, and overexpression of MAP3K8 (COT), among others (8–11). In addition, exposure to hepatocyte growth factor from the stromal microenvironment can promote some degree of intrinsic resistance to RAF inhibitors in melanoma cell lines (12, 13). In contrast to the high response rate seen in patients with metastatic melanomas, PLX4032 has limited efficacy as a single agent in patients with BRAF-mutant colorectal cancers (14). The decreased sensitivity of many colorectal cancer cell lines to growth inhibition by PLX4032 has recently been ascribed to activation of EGFR receptor (EGFR) signaling (15, 16). This was proposed to be due to feedback-induced relaxation of the activity of CDC25C, a putative EGFR phosphatase (15).

Metastatic thyroid cancers that are refractory to radioactive iodine therapy have a particularly high prevalence of BRAF mutations (17). The MAP-extracellular signal-regulated kinase (ERK) kinase (MEK) inhibitor selumetinib (AZD6244, ARRY-142886) showed minimal activity in a phase II study of thyroid cancer (18). A trial with PLX4032 for this disease is now in progress. Here, we report that the majority of BRAF-mutant thyroid cancer cell lines are insensitive to the growth inhibitory effects of PLX4032, and that this is largely due to a feedback-induced ligand-dependent activation of HER2/HER3 signaling. Hence, the early response of BRAF-mutant cancers to selective MAPK pathway inhibitors is marked by the relaxation of oncoprotein-driven negative feedback events, which differ between tumors of various lineages and which predict a requirement for distinct therapeutic strategies.

RESULTS

Lineage-Specific Differences in Effects of PLX4032 on MAPK Signaling and Cell Growth

BRAF-mutant melanoma cell lines were uniformly sensitive to growth inhibition by PLX4032 (IC50 < 100 nmol/L), whereas most thyroid (5/6) and colorectal lines (3/4) were comparatively refractory (IC50 > 1,000 nmol/L; Fig. 1A). PLX4032 (2 μmol/L) evoked a sustained inhibition in pMEK and pERK in melanoma cell lines through 72 hours. In contrast, the inhibition of RAF effectors

Authors’ Affiliations: 1Human Oncology and Pathogenesis Program, 2Sloan Kettering Institute, Departments of 3Medicine and 4Pathology, and 5Molecular Pharmacology and Chemistry Program, Memorial Sloan-Kettering Cancer Center, New York, New York.

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S. Ruiz-Llorente and J.M. Dominguez contributed equally to this work.

Corresponding Author: James A. Fagin, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, NY 10065. Phone: 646-888-2136; Fax: 646-422-0890; E-mail: faginj@mskcc.org

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in BRAF-mutant thyroid and colorectal cell lines was transient, with a rebound beginning 6 hours after addition of the drug in 5 of 6 thyroid and 3 of 4 colorectal cancer cell lines (Fig. 1B). The excursions in pERK were consistent with the gene expression kinetics of the ERK phosphatase DUSP5, a component of the transcriptional output driven by MAPK activation (Supplementary Fig. S1A; ref. 19). The rebound in pERK was not due to rapid drug metabolism, as readdition of 2 μmol/L PLX4032 72 hours after initial exposure did not reinhibit the pathway (Fig. 1C), whereas addition of the MEK inhibitor AZD6244 had a potent effect (Supplementary Fig. S1B). The rebound in MAPK signaling seen after treatment with PLX4032 likely contributes to attenuate the biologic response to RAF inhibition.

**Treatment of Thyroid Cancer Cell Lines with RAF Inhibitors Is Associated with Ras Activation and Increased Expression and Phosphorylation of Receptor Tyrosine Kinases (RTKs)**

In cancer cells with mutant BRAF, signaling inputs upstream of the oncoprotein are inhibited by negative feedback (20). As shown in Fig. 1D, treatment of the thyroid cancer cell line SW1736 with PLX4032 led to a time-dependent increase in GTP-bound Ras, consistent with relaxation of the negative feedback upstream of RAF, which was of a much greater magnitude in thyroid cells as compared with the SK-MEL-28 melanoma cells. The increase in Ras activity is potentially significant, as enforced Ras activation can overcome the PLX4032-induced block of MAPK in mutant BRAF melanoma cells (8, 21). We used 2 different screens to identify potential mediators of these effects. We first obtained gene expression profiles at 0, 1, 6, and 48 hours after addition of PLX4032 to SW1736 and SK-MEL-28 cells, and identified several gene clusters with significantly different expression kinetics between the thyroid and melanoma lines (Supplementary Fig. S2A). Functional enrichment analysis against the Kyoto Encyclopedia of Genes and Genomes (KEGG) database revealed overrepresentation of the following terms in SW1736 compared with SK-MEL-28 cells: ERBB signaling pathway, insulin signaling pathway, cytokine–cytokine receptor pathway, and MAPK signaling pathway (Supplementary Fig. S2B).
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Figure 2. Phospho-ERK inhibition promotes expression and activation of RTKs in BRAF-mutant thyroid cancer cells. A, SW1736 cells were left untreated or exposed for 72 hours to 2 μmol/L PLX4032 and lysates incubated with phospho-RTK arrays. Spots are in duplicate, with each pair corresponding to a specific pRTK. The pair spots in the corners are positive controls. Comparison between treated and untreated cells shows increased phosphorylation of several RTKs by PLX4032, with pHER3 being the most prominently induced. Normalized data from densitometry analysis of the arrays are listed in the table. B, Western blots of SW1736 cells treated with 2 μmol/L PLX4032 and collected at the indicated times. Rebound in phospho-ERK and pAKT is associated with induction of total and pHER3, and total HER2. C, a panel of 6 thyroid cancer, 3 melanoma, and 4 colorectal cancer cell lines with BRAF<sup>V600E</sup> mutation were treated with or without PLX4032 for 72 hours. Immunoblots show an increase of pHER3 in 5 of 6 thyroid cancer cell lines (SW1736, Hth104, 8505C, BCPAP, and T235, see boxes). In contrast, EGFR phosphorylation was lower in 4 of 6 thyroid cell lines, and unchanged in the others. No comparable induction of pHER3 was observed in melanoma or colorectal cancer cell lines. Lysates of SW1736 were used as an interblot control (*). D, Western immunoblots of thyroid cancer tissue lysates of TPO-Cre/LSL-Braf<sup>V600E</sup> mice treated with a single 25 mg/kg dose of the MEK inhibitor PD0325901 for 6 hours. Each lane corresponds to lysates from one mouse thyroid cancer tissue.

Supplementary Fig. S3A). Four of 6 thyroid cancer cell lines showed decreased pEGFR 72 hours after PLX4032, whereas there was no change in colorectal lines (Fig. 2C and Supplementary Fig. S3A).

HER2 and HER3 expression and activation were also markedly increased by the allosteric MEK inhibitor PD0325901 6 hours after treatment in thyroid cancers of TPO-Cre/LSL-Braf<sup>V600E</sup> mice, a genetically accurate model of thyroid tumorigenesis induced by endogenous expression of the oncoprotein (ref. 22; Fig. 2D).

PLX4032 Induces the Expression and Activation of HER2/HER3 Heterodimers in Thyroid Cancer Cells

Thus, following treatment of BRAF-mutant thyroid cancer cells with PLX4032, there is a relief of feedback that results in increased expression of the RTKs HER2 and HER3, and this is associated with RAS activation. HER3 is a kinase-impaired member of the HER family, which is phosphorylated and activated by heterodimerization with one of the
other family members (HER2, EGFR, or HER4). To identify the HER3 dimer partner, we depleted the expression of EGFR or HER2 by RNA interference in 8505C thyroid cells (Fig. 3A). PLX4032-induced HER3 phosphorylation was inhibited by knockdown of HER2 but not of EGFR. Moreover, communoprecipitation of either HER3 or HER2 resulted in pulldown of the reciprocal partner, confirming the induction of HER2/HER3 complexes by 2 μmol/L PLX4032 in both cell lines (Fig. 3B). Of the various HER dimers, the HER2/HER3 heterodimer is considered the most potent signaling unit (23). The C-terminal residues of these receptors provide docking sites for the adaptor protein GRB2 (HER2>HER3) and the p85 regulatory subunit of phosphoinositide-3 kinase (PI3K; HER3>HER2). These molecules couple the heterodimer to the RAS/RAF/MAPK and PI3K signaling pathways, respectively (24). Immunoprecipitation of HER3 confirmed the recruitment of p85 after PLX4032 treatment (Fig. 3B). Similarly, immunoprecipitation of either HER3 or HER2 after PLX4032 treatment was associated with increased recruitment of GRB2, likely accounting for the induction of RAS signaling (Fig. 3C). Moreover, treatment of 8505C cells with the HER kinase inhibitor lapatinib abrogated the PLX4032-induced phosphorylation of HER3, recruitment of p85 to HER3, and the increase in RAS-GTP levels (Fig. 3D and E). The addition of lapatinib also largely prevented the activation of pAKT and pERK in SW1736 and 8505C cells exposed to the RAF inhibitor (Fig. 3F).

**PLX4032-Induced HER2/HER3 Activation Is Dependent on Autocrine Neuregulin-1 Expression and Is Augmented by Exogenous Addition of the Ligand.**

Neuregulin-1 (NRG1) is the major HER3 ligand, which promotes its engagement with HER2 kinase and the subsequent transphosphorylation of HER3 (25). To investigate whether PLX4032-induced activation of HER2/HER3 is mediated by NRG1, we deprived 8505C cells of serum for 24 hours in the absence or presence of 2 μmol/L PLX4032. Serum starvation did not entirely shut down the HER3 phosphorylation induced by PLX4032 (Fig. 4A, lane 5 vs. 2), pointing to the existence of either a basal level of ligand-independent HER3 activation, or an autocrine production of NRG1. Knockdown of NRG1 markedly inhibited HER3 phosphorylation in cells incubated in serum-free medium, consistent with autocrine secretion of the ligand (Fig. 4B). The addition of exogenous NRG1 further stimulated HER3 phosphorylation, even in the absence of PLX4032 (Fig. 4A, lanes 3 vs. 4), suggesting that basal coexpression of HER3 and HER2, although low, was sufficient to be efficiently activated by NRG1, and to transduce signaling via AKT and ERK effectors. The addition of NRG1 to PLX4032-pretreated cells superactivated HER3, PI3K, and MAPK signaling (lane 6) and this was blocked by 1 μmol/L lapatinib (lane 7). The HER2 antagonist pertuzumab also inhibited signaling in thyroid cancer cell lines exposed to exogenous NRG1 (Fig. 4C).

Interestingly, expression of NRG1 was consistently higher in BRAF-mutant thyroid cell lines than in melanoma or colorectal cell lines (Fig. 4D and E). Some melanoma cell lines, such as SK-MEL-28, do show some increase in total HER3 after exposure to PLX4032 (Supplementary Fig. S4A), but no HER3 phosphorylation because they do not secrete NRG1. Treatment of SK-MEL-28 cells with exogenous ligand induced HER2/HER3 signaling (Supplementary Fig. S4B) and attenuated the growth-inhibitory effects of PLX4032 (Supplementary Fig. S4C).

**Inhibition of Oncogenic BRAF Increases HER3 Gene Transcription.**

We next examined the mechanisms accounting for the increase in HER3 by MAPK pathway inhibitors in BRAF-mutant thyroid cell lines. Upregulation of HER3 has been found to mediate resistance to PI3K/AKT (26) or HER2 (27) inhibitors in HER2-amplified breast cancer cell lines, which is caused in part through a FOXO3A-dependent induction of HER3 gene transcription. As shown in Fig. 5A, PLX4032 treatment increased HER3 and HER2 mRNAs in all 6 BRAF-mutant thyroid cancer cell lines tested. Similar results were found following treatment with the MEK inhibitor AZD6244 (not shown). The effects of the MEK inhibitor on total HER2, HER3 protein, and on pHER3 were dose dependent and inversely associated with the degree of inhibition of pERK (Fig. 5B). RAF or MEK inhibitors induced luciferase activity of a HER3 promoter construct spanning approximately 1 kb upstream of the transcriptional start site in 8505C cells. Serial deletions identified a minimal HER3 promoter retaining transcriptional response to PLX4032 and AZD6244, which was located between −401 and −42 bp (Fig. 5C). This region does not contain any predicted FOXO-binding sites. Moreover, PLX4032 led to an increase in phosphorylation of FOXO1/3A between 4 and 10 hours after addition of compound (not shown), which is known to promote its dissociation from DNA, and likely discards involvement of these factors as transcriptional regulators of HER3 in response to MAPK pathway inhibition. The minimal HER3 promoter region regulated by MAPK inhibitors overlaps with sequences previously described to be immunoprecipitated using antibodies against the ZFN217 transcription factor and C-terminal binding protein 1 and 2 (CTBP1/CTBP2) corepressors (28–30). CTBPs have also been described to negatively regulate transcriptional activity of the HER3 promoter in breast carcinoma cell lines (30). Silencing of CTBP1, and to a lesser extent CTBP2, increased basal HER3 in 8505C cells, and markedly potentiated the effects of PLX4032 (Fig. 5D and E). Knockdown of these factors modestly increased basal and PLX4032-induced HER2 levels, which likely contributes to the remarkable increase in pHER3 we observed (Fig. 5D and E). Finally, CTBPs have also been described to negatively regulate transcriptional activity of the HER3 promoter in breast carcinoma cell lines (30). Silencing of CTBP1, and to a lesser extent CTBP2, increased basal HER3 in 8505C cells, and markedly potentiated the effects of PLX4032 (Fig. 5D and E). Knockdown of these factors modestly increased basal and PLX4032-induced HER2 levels, which likely contributes to the remarkable increase in pHER3 we observed (Fig. 5D and E). Finally, CTBPs have also been described to negatively regulate transcriptional activity of the HER3 promoter in breast carcinoma cell lines (30).

**The HER Kinase Inhibitor Lapatinib Cooperates with RAF or MEK Inhibitors to Inhibit Thyroid Cancer Cell Growth In Vitro.**

To examine whether HER2/HER3 activation mediates resistance to PLX4032, we treated SW1736, 8505C, and Hh104 cells for 4 days with increasing concentrations of the RAF inhibitor with or without 1 μmol/L lapatinib, or with 2 μmol/L PLX4032 combined with increasing doses of lapatinib. As shown in Fig. 6A, lapatinib alone markedly
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Figure 3. PLX4032 induces HER2/HER3 heterodimers, recruitment of p85, and activation of RAS. A, 8505C cells were transfected with siRNAs against EGFR or HER2, or with a scrambled siRNA control. After 16 hours, cells were treated with 2 μmol/L PLX4032 for 72 hours. Western blotting with the indicated antibodies shows that knockdown of HER2, but not EGFR, reduces PLX4032-induced HER3 phosphorylation. As HER4 was not expressed, RNAi experiments for this RTK were not done.

B, SW1736 and 8505C cells were treated with 2 μmol/L PLX4032 and collected at 24 and 48 hours after treatment. Lysates were immunoprecipitated with either anti-HER2 or anti-HER3 antibodies and Western blotted for HER3, HER2 or p85.

C, 8505C cells were treated with 2 μmol/L PLX4032 and collected 72 hours after treatment. Lysates were immunoprecipitated with either anti-HER3 or anti-HER2 antibodies and Western blotted for HER3, HER2, pHER2, or GRB2. PLX4032-treated cells show increased association of GRB2 to the HER2/HER3 immunocomplex.

D, 8505C cells were treated with PLX4032 for 22 hours and then treated with or without 1 μmol/L lapatinib for 2 hours. Lysates were immunoprecipitated with anti-HER3 and blotted with the indicated antibodies. E, 8505C cells were treated with PLX4032 for 70 hours and then incubated with or without 1 μmol/L lapatinib for 2 hours. Lysates were immunoprecipitated with CRAF-RBD and blotted with the indicated antibodies. PLX4032 treatment induced RAS activation that is blocked by lapatinib. Immunoblotting of the input lysates shows inhibition of pHER3 and pERK1/2 by lapatinib.

F, Western blot analysis of lysates of SW1736 and 8505C cells treated with 2 μmol/L PLX4032 with or without 1 μmol/L lapatinib for the indicated times. Lapatinib blocked the PLX4032-induced HER3 and AKT phosphorylation and the pERK1/2 rebound. EGFR phosphorylation, which was not induced by PLX4032, was also blocked by lapatinib.
Figure 4. PLX4032-induced HER2/HER3 activation is dependent on autocrine secretion of NRG1. A, 8505C cells were grown in 10% FBS for 72 hours, or for 48 hours followed by 24 hours in serum-free medium, and treated with or without 2 μmol/L PLX4032 for 72 hours. Cells in lane 7 were also incubated with 1 μmol/L lapatinib for 24 hours. Starved cells were stimulated with the HER3 ligand NRG1 for 5 minutes. B, Western blot analysis of lysates or concentrated media of 8505C cells incubated with control or NRG1 siRNA for 60 hours and with 2 μmol/L PLX4032 for the final 48 hours. Knockdown of NRG1 inhibited the PLX4032-induced HER3 activation. C, effects of short-term incubation with pertuzumab on PLX4032-induced signaling in 8505C cells. Addition of 10 μg/mL pertuzumab for 2 hours decreased pHER3, pMEK, and pERK, with more subtle effects on pAKT T308. Similar findings were seen in SW1736 and Hth104 cells. D, endogenous expression of NRG1 in protein lysates of the indicated thyroid cancer and melanoma cell lines. E, Western immunoblotting analysis for NRG1 of concentrated serum-free media conditioned by the indicated cell lines.

sensitized the 3 cell lines to dose-dependent inhibition by the RAF inhibitor. The IC50 for PLX4032 shifted from ≥1.5 μmol/L in the 3 cell lines to 0.19, 0.97, and 0.49 μmol/L, respectively. Accordingly, the fraction of cells in G1 phase was significantly higher when 2 μmol/L PLX4032 was combined with 1 μmol/L lapatinib (Fig. 6B). The percentage of cells in sub-G1 phase was greater in cells treated with the combination; however, at levels that are not likely to be biologically significant (not shown). Knockdown of HER3 by short hairpin RNA (shRNA) also increased the sensitivity to PLX4032 in SW1736 cells (Supplementary Fig. S6A). Treatment of SW1736 cells with the MEK inhibitor AZD6244 also results in robust activation of HER3 signaling (Supplementary Fig. S6B). Accordingly, lapatinib shifted the IC50 for the MEK inhibitor (18–34 nmol/L and 110–330 nmol/L in SW1736 and 8505C cells, respectively; Supplementary Fig. S6C), and significantly increased the fraction of cells in G1 phase (Fig. 6B). We next tested the effects of lapatinib, the RAF
Figure 5. CTBPs modulate PLX4032-mediated induction of HER3 gene transcription. A, a panel of BRAF-mutant thyroid cells was treated with 2 μmol/L PLX4032 for 1, 6, or 48 hours and cell lysates analyzed for expression of HER3 and HER2 by quantitative (q)RT-PCR. Points represent fold-change of HER/GAPDH qRT-PCR values of triplicate assays (mean ± SD) over untreated controls. B, 8505C cells were treated with increasing concentrations of AZD6244. Lysates were extracted at 24 hours after treatment and immunoblotted with the indicated antibodies. C, luciferase assays of 8505C cells transfected with plasmids containing HER3 promoter-reporter constructs (−992/+63, −730/+63, −401/+63, or −42/+63, relative to transcriptional start site) and pRenilla-CMV, used as transfection normalizing control plasmid. Twelve hours after transfection, complete media containing 2 μmol/L PLX4032 or 0.5 μmol/L AZD6244 was added to cells. Lysates were obtained at different time points after treatment (0, 6, 24, and 48 hours), and luciferase activity measured. Promoter activity was determined as the ratio between luciferase and Renilla, relative to untreated cells. The results shown are the mean ± SD of triplicate samples. ∗, P < 0.05; ∗∗, P < 0.01; ∗∗∗, P < 0.001. D, 8505C cells were transfected with control, CTBP1, or CTBP2 siRNAs and treated with or without 2 μmol/L PLX4032 for 24 hours. Lysates were analyzed for expression of HER3 and HER2 by RT-PCR. Bars represent mean ± SD of triplicate assays of HER/GAPDH RT-PCR values relative to untreated controls. E, panels show protein levels of HER3, HER2, CTBP1, CTBP2, and p85 (loading control) in cells transfected with control, CTBP1, or CTBP2 siRNAs and grown in either the absence or presence of 2 μmol/L PLX4032 for 24 hours. F, CTBP1 and CTBP2 chromatin immunoprecipitation assays were conducted in 8505C cells treated with or without 2 μmol/L PLX4032 for 24 (left) or 48 hours (right). A fragment (−246 to −162) of the HER3 promoter that includes known interacting sites for CTBP proteins was amplified by means of RT-PCR for both conditions. Graph shows normalized RT-PCR data of HER3 fragment of the immunoprecipitated complex compared with input lysate. Data represent mean ± SD of 2 independent biologic replicates conducted in quadruplicate.
Figure 6. Lapatinib cooperates with PLX4032 to inhibit BRAF-mutant thyroid cancer cell growth. A, bars represent growth inhibition of 8505C, SW1736, and Hth104 cells by lapatinib, PLX4032, or their combination. Growth was measured 4 days after addition of the indicated concentration of PLX4032 in the absence or presence of 1 μmol/L lapatinib, or after addition of the indicated concentration of lapatinib in the absence or presence of 2 μmol/L PLX4032. Bars represent percent change (mean ± SD) in cell count of triplicate wells compared with untreated cells. B, SW1736 and 8505C cells were treated with 2 μmol/L PLX4032, 0.1 μmol/L AZD6244, or 1 μmol/L lapatinib alone or in combination for 48 hours. FACS shows a significant increase in G1 phase in cells exposed to PLX4032 or AZD6244 when combined with lapatinib. C, thyroid volumes of TPO-Cre/LSL-BrafV600E mice treated with lapatinib (150 mg/kg 5 times a week), PLX4720-impregnated chow, or their combination at days 0 and 21 of treatment. The fold-change of thyroid volume was significantly lower in mice treated with the combination compared with vehicle (P = 0.02), and PLX4720 alone (P = 0.02). D, bars represent average number of Ki67-positive cells per high-power field in mice treated as described above for 2 weeks, except that lapatinib was given 3 times a week alone or in combination with PLX4720. At least 8 higher-powered fields/section on 4 separate sections were counted for each mouse (2–3 mice per treatment group). **, P < 0.01.
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inhibitor PLX4720 or their combination in thyroid cancers of Tpo-Cre/LSL-Braf mice (22). The combination resulted in a greater inhibition of mitotic rate than PLX4720 alone and in a significant reduction of thyroid volume (Fig. 6C and D).

Role of Other RTKs in the Intrinsic Resistance of Thyroid Cancers to RAF Inhibitors

Expression profiling (Supplementary Fig. S2C) and the RTK phosphoarray (Fig. 2A) show that in addition to Her3, there was upregulation of expression and/or phosphorylation of ephrins and of PDGFRβ in response to PLX4032. This is of interest because activation of PDGFRβ has been proposed as a mechanism of acquired resistance to PLX4032 in patients with metastatic melanoma (8). Western blots showed a marked induction of PDGFRβ in SW1736 cells by the RAF inhibitor. PDGFRβ was induced to a much lesser extent in RKO-1 and T235 cells, and was constitutively high in Hth104 cells (Supplementary Fig. S7A). Despite the remarkable increase in PDGFRβ in SW1736 cells after PLX4032 treatment, stimulation with PDGF-BB only activated pAKT and pERK modestly at a time when the receptor was maximally expressed (Supplementary Fig. S7B). In contrast, stimulation with NRG1 under the same conditions induced a far greater activation of signaling, indicating that the HER2/HER3 dimer is dominant under these conditions. Moreover, treatment with the PDGFR inhibitor imatinib failed to sensitize SW1736 cells to growth suppression by PLX4032 (Supplementary Fig. S7C).

As EGFR has been implicated in resistance to PLX4032 in colorectal cancer and melanoma cell lines (15, 16, 31), we further investigated the possible contribution of this receptor to the insensitivity to RAF inhibition in thyroid cancer cells. Of note, pEGFR decreased 24 and 48 hours after PLX4032 treatment, corresponding to the peak of pHER3 and pERK (Fig. 3F). Knockdown of EGFR did not sensitize 8505C cells to PLX4032 (Supplementary Fig. S7D), and indeed paradoxically decreased sensitivity to the compound. In contrast, knockdown of HER2 significantly augmented the growth inhibitory effects of PLX4032 (Supplementary Fig. S7D). Treatment with cetuximab also failed to sensitize 8505C and Hth104 cells to RAF kinase inhibition (not shown).

DISCUSSION

Oncogenic drivers reprogram the signaling network of cancer cells, in part, by inducing negative feedback events (32). Targeted therapies may relieve those feedbacks and reengage signaling loops that limit the antitumor response. What is under-appreciated, and is shown in this report, is that the receptors that are reactivated are to a significant extent lineage specific and dependent on the availability of their cognate ligands. Moreover, not all reactivated pathways drive resistance to therapy, emphasizing the need to explore their functional consequences in detail. The corollary to this is that tumors of different cell types harboring the same activated oncoprotein respond in a different way to selective inhibitors, through mechanisms that are tractable and that may in part be anticipated on the basis of the cell of origin.

Activation of MAPK signaling in normal cells occurs in response to growth factors, cytokines, and stress signals. BRAF-transformed cells hyperactivate MAPK independently of upstream inputs, to which cells become unresponsive through induction of ERK-dependent negative feedback loops (32). Accordingly, Ras-GTP levels are depleted in BRAF-mutant tumor cells regardless of their cell type and, as shown in this article, the activation state of RTKs is low. When exposed to RAF or MEK inhibitors, BRAF-mutant melanoma and thyroid cells show markedly different MAPK signaling kinetics. Although thyroid cancer cells are primed to reactivate the pathway shortly after exposure to the kinase inhibitors, melanoma cells are not. Thyroid cells show rapid increases in Ras-GTP in response to RAF kinase inhibition, consistent with the activation of upstream signals. The MAPK rebound was not affected by the readdition of PLX4032, likely because of its relative selectivity for monomeric mutant BRAF (21). In contrast, the MEK inhibitor AZD6244 suppressed the RAF inhibitor-induced ERK rebound, presumably by interrupting upstream signals mediated by wild-type RAF proteins. There was a more pronounced rebound in ERK compared with MEK phosphorylation after PLX4032, which may be due to transient downregulation of ERK phosphatases (e.g., DUSP5), in particular at early time points after exposure to the drug.

Two different screening approaches pointed to an increase in the expression and activation of RTKs as primary candidates in mediating the rebound in MAPK activity after treatment with RAF or MEK inhibitors. We focused on HER3 because it showed the greatest magnitude change in phosphorylation in the index line, and because its activation was the most prevalent across all other thyroid lines we tested. Indeed, both HER3 and HER2 mRNA levels increased ubiquitously in thyroid cancer cells treated with PLX4032. HER3 is a kinase inactive receptor that requires heterodimerization to transduce signaling. Communoprecipitation assays and transient knockdown of possible dimer partners led us to identify HER2 as the principal HER3 partner. We did not detect any significant increase in EGFR phosphorylation in response to PLX4032 in any of the thyroid cell lines we tested. EGFR activation has been recently linked to unresponsiveness to MAPK inhibition in BRAF-mutant melanoma and colorectal cancer cells (15, 31). In the latter study, the authors also proposed that the same mechanism likely accounted for the unresponsiveness of thyroid cancer cell lines to RAF inhibitors. This conclusion was based in part on the demonstration that gefitinib enhanced the growth-inhibitory effects of PLX4032 in 8505C and BHT101 thyroid cancer cells; however, the EGFR-specific monoclonal antibody cetuximab had no effect. In our hands, cetuximab or EGFR knockdown also failed to sensitize thyroid cancer cells to PLX4032, whereas HER3 or HER2 knockdown had significant growth-inhibitory effects. However, HER3 interactions with other ERBB family kinases can be promiscuous, suggesting that cotargeting of HER2 and EGFR with kinase inhibitors may be a productive approach to sensitize cells to MAPK pathway inhibition.

HER2/HER3 signaling is controlled by HER2 overexpression or ligand binding. As HER2 has no known ligand, the heterodimer is primarily induced by the HER3 ligand NRG1 (24), which can be produced by tumor cells or by tumor stroma. The importance of tumor production of NRG1 was...
recently shown in non-HER2-amplified cancer cells, which were found to be dependent on a NRG1-mediated autocrine loop (33, 34). These cell lines, mainly from head and neck tumors, showed HER3 expression and phosphorylation and sensitivity to lapatinib (34). All thyroid cancer cell lines we tested expressed NRG1, and knockdown of NRG1 markedly inhibited HER3 phosphorylation, suggesting that the HER3 activation by MAPK pathway blockade is dependent on this autocrine loop. Interestingly, 2 of the 4 melanoma cell lines we tested showed an induction of HER3 expression by PLX4032 treatment, but no evident HER3 activation. In contrast to thyroid cells, basal expression of NRG1 was not detectable in melanoma cells. When ligand was added to their media, HER2/HER3 signaling was activated, leading to an attenuation of the growth-inhibitory effects of PLX4032. Colorectal cancer cell lines express low levels of HER3 constitutively, but no NRG1, and hence HER3 activation does not ensue. Hence, the peculiar resistance to RAF and MEK inhibitors of BRAF-mutant thyroid cancers is due to a combination of factors: that is, the induction of HER3 expression by MAPK inhibitors and the constitutive autocrine production of its ligand (Fig. 7).

The increase in expression of HER3 after MAPK inhibition is due to the activation of gene transcription, which was associated with a reduction of binding of the transcriptional repressors CTBP1 and CTBP2 to the HER3 gene promoter. These corepressors have been previously linked to inhibition of HER3 transcription through promoter regions that show overlapping occupancy with ZNF217, a transcription factor also involved in HER3 regulation (30). Accordingly, knockdown of CTBPs acutely induced HER3 expression and phosphorylation in thyroid cancer cells. MAPK inhibition may dictate a chromatin redistribution of these repressors and thus activate HER3 transcription. The biochemical mechanisms involved in delocalization of CTBPs by MAPK inhibition have not been explored, but posttranslational modifications are known to regulate the repressive activity of CTBPs either by translocation to the cytoplasm or by targeting them for degradation (35, 36).

Besides the activation of HER3, there was either induction or high basal levels of expression of other RTKs in thyroid cancer cell lines exposed to MAPK pathway inhibitors, such as PDGFRβ, previously implicated in acquired resistance to PLX4032 in patients with metastatic melanoma (8). However, treatment with the PDGFR kinase inhibitor imatinib did not sensitize thyroid cancer cells to the growth-inhibitory effects of the RAF inhibitor. These findings have potentially significant clinical ramifications. A commonly advocated approach to identify mechanisms of intrinsic or acquired resistance to kinase inhibitors such as PLX4032 in clinical trials is to biopsy tumor sites while patients are on therapy to assay for expression and phosphorylation of RTKs or other signaling
proteins. The findings described here show that this could be misleading, as simple demonstration of RTK overexpression or phosphorylation is not likely to point to the functionally relevant signaling unit. Instead, a deeper understanding of lineage-specific determinants of response to MAPK inhibition is more likely to be rewarding and to guide selection of appropriate combination therapies.

**METHODS**

**Reagents**

PLX4032 and PLX4720 were from Plexxikon, and AZD6244 was from AstraZeneca. Lapatinib and imatinib were purchased from Selleck Chemicals. All drugs were dissolved in dimethyl sulfoxide (DMSO) for in vitro studies. For in vivo studies, PD0352901 and lapatinib were dissolved in 0.5% hydroxypropylmethylcellulose/0.2% Tween 80 (Sigma) and 0.5% hydroxypropylmethylcellulose/0.1% Tween 80 (Sigma), respectively, and administered by oral gavage. Recombinant human NRG1 (#5218SC) and hPDGF-BB (#8912SC) were a gift from D.B. Solit (Memorial Sloan-Kettering Cancer Center, NY). All thyroid cancer cell lines used in this study were authenticated and sources is included in Supplementary Experimental Procedures.

**Cell Culture**

Cancer cell lines were maintained at 37°C and 5% CO₂ in humidified atmosphere and grown in RPMI-1640 or Dulbecco’s modified Eagle’s medium growth media supplemented with 10% of FBS, 2 mmol/L glutamine, 50 μg/mL penicillin (GIBCO), and 50 μg/mL streptomycin. BHT101 cells were grown in minimum essential medium (MEM) supplemented with 20% of FBS. Melanoma and colorectal cancer cell lines were a gift from D.B. Solit (Memorial Sloan-Kettering Cancer Center, NY). All thyroid cancer cell lines used in this study were authenticated using short tandem repeat and single nucleotide polymorphism array analysis (37). For cell growth assays, cells were plated in triplicate into 6-well plates at 5 x 10⁴ cells per well, and treated with DMSO or the indicated drug. After 48 hours, adherent and floating cells were harvested and stained with ethidium bromide (38). Flow cytometric analysis was conducted in triplicate.

**Immunoblotting**

Cells were harvested by trypsinization, washed twice with cold PBS, and lysed in NP-40 buffer (containing 25 μL of 1 mol/L NaCl, 20 μL of 1 mol/L NaF, 5 μL of NP-40, and 435 μL of distilled water). Protein concentration was determined using the BCA kit (Thermo Scientific). Western blots were conducted on 25 μg protein separated by SDS-7.5–10% PAGE using the indicated antibodies and sources is included in Supplementary Experimental Procedures.

**Cell-Cycle Analyses**

Cells were plated in triplicate into 60-mm dishes at a density of 1 to 2.5 x 10⁶ cells per well. Next day, cells were treated with DMSO or the indicated drug. After 48 hours, adherent and floating cells were harvested and stained with ethidium bromide (38). Flow cytometric analysis was used for quantification of cell-cycle distribution.

**RNA Interference**

Cells were plated at 20% of confluence in medium without antibiotics and transfected with 5 mmol/L siRNA and Lipofectamine RNAiMAX Transfection Reagent (Invitrogen) as indicated in the manufacturer’s protocol. siRNAs for EGFR (#s565), HER2 (#s611), HER3 (#s4779 and #s4780), NRG1 (#s194522), CTBP1 (#s3699), CTBP2 (#s3702), and negative control (#AM4611) were purchased from Ambion.

**Phospho-RTK Arrays**

Human Phospho-RTK arrays were used according to the manufacturer’s instructions (R & D Systems). Briefly, cells were washed with cold PBS and lysed in NP40 lysis buffer with 10% glycerol, and 100 μg of lysates was incubated with blocked membranes overnight. Membranes were then washed and exposed to chemiluminescent reagent and then to X-ray film. Quantification of pixels was conducted by densitometry using Kodak MI software.

**Semi-quantitative PCR**

Total RNA was extracted using TRIZOL Reagent (Invitrogen) and subjected to DNase I Amplification Grade (Invitrogen). For quantitation RT-PCR, mRNA was retrotranscribed using SuperScript III First-Strand Synthesis Super Mix (Invitrogen). Real-time PCR was conducted using standard conditions and Power SYBR Green PCR master mix (Applied Biosystems) with AB-7700 System and analyzed with Q-Genie Core Module file (39). Primer sequences are shown in the Supplemental Experimental Procedures.

**Cloning of HER3 Promoter Constructs and Luciferase Assays**

PCR-amplified fragments of the human HER3 promoter (~992/+3730/+3901/+363 and ~42/+463, relative to transcription start site) were cloned using BglII and KpnI restriction sites of pGL3b reporter plasmid (BglII-HER3-992:5′-CGGGGTACC TAGCACGCTGCGGAGCAAGCAA-3′, BglII-HER3-730: 5′-CGGGTTACCCCACTGGTCGTGGAGG-3′, BglII-HER3-401: 5′-CGGGGTACCTCCCCCTCAAAAACAC-3′, BglII-HER3-42: 5′-CGGGGTACCTCCCCCTTCCTGTTCCCT-3′ and KpnI-HER3+63: 5′-GGAGAATCTAACCATGCGAGGCGG-3′). 8505C cells were transiently transfected using Lipofectamine 2000 (Invitrogen), 1 μg of reporter construct, and 60 ng of pRL-CMV. Twelve hours after transfection, 2 μmol/L PLX4032 or 0.5 μmol/L AZD6244 were added to 8505C in complete media and cells harvested at the indicated time points, lysed, and analyzed for luciferase and Renilla activities. The promoter activity was determined as the ratio between luciferase and Renilla, relative to the ratio obtained in nontreated cells. The results shown are the mean ± SD of 2 independent experiments, each conducted in triplicate.

**Chromatin Immunoprecipitation**

Chromatin immunoprecipitation (ChIP) samples were prepared from 8505C cells as follows: cultures of 25 x 10⁶ cells were treated with or without 2 μmol/L PLX4032 during 24 hours. Then, cross-linking was conducted with 1% formaldehyde for 10 minutes at room temperature. Cross-linking was stopped by the addition of glycine to a final concentration of 125 mmol/L, and cells were washed twice with PBS. The cell pellet was resuspended consecutively in ChIP lysis buffers (40) and sonicated using the Bioruptor sonicator (Diagenode) to produce chromatin fragments of 200–500 bp on average. Sheared chromatin was incubated with CTBP1 (BD Biosciences, #612042) or CTBP2 (BD Biosciences, #612044) mouse antibody-coated magnetic beads. To prepare the coated beads, 75 μL Dynabeads Protein G (Invitrogen) were incubated overnight with 7.5 μg of the above-mentioned antibodies. The following day, the beads were rinsed and added to the sheared chromatin and incubated overnight at 4°C. Samples were...
then rinsed 8 times with radioimmunoprecipitation assay buffer, and the antibody was captured with Protein A and Proteinase K, and DNA was extracted using Qiaquick PCR Purification kit (Qiagen Sciences). Controls included an input condition obtained before DNA protein immunoprecipitation. Semi-quantitative PCR reactions to confirm enrichment of HER3 promoter sequences were conducted as described. ChiP ratio was calculated as enrichment over noise normalized to the input.

**Animal Studies**

Three-week-old TPO-Cre/LSL-Braf<sup>W600E</sup> mice were treated with a single dose of PD352901 (25 mg/kg). Thyroids were collected before and 6 hours after administering PD352901 and snap frozen in liquid N<sub>2</sub>. Proteins were extracted and Western blots conducted as described. To test the effects of PLX4720 alone or in combination with lapatinib on tumor growth, 4- to 6-week-old TPO-Cre/LSL-Braf<sup>W600E</sup> mice were treated for 3 weeks with lapatinib (150 mg/kg 5 times a week), PLX4720-impregnated chow, or their combination. Thyroid imaging was conducted at day 0 and day 21 with a Vevo 2100 micro-ultrasound system and volumes calculated with the Vevo 2100 imaging software version 1.3.0. To determine effects on mitotic rate, mice were treated for 2 weeks as above except that lapatinib was administered 3 times a week. Thyroids were collected 6 hours after the final dose of lapatinib, fixed in 4% paraformaldehyde, and representative sections stained with Ki67 antibody (Vector Laboratories; 0.05 μg/mL). All procedures were conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committees at the Memorial Sloan-Kettering Cancer Center.

**Statistical Analysis**

Statistical significance of differences between the results was assessed using a standard 2-tailed t test and Mann-Whitney test for in vivo data, conducted using Prism v5.04 (GraphPad Software). P < 0.05 was considered statistically significant.

**Data Deposition**

The microarray data reported in this article have been deposited in NCBI's Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession no. GSE37441).

**Disclosure of Potential Conflicts of Interest**

N. Rosen is on the Scientific Advisory Board for Chugai and AstraZeneca, has received commercial research grants from Chugai, AstraZeneca, and Merck, and is a consultant/advisory board member for Novartis and GSK. J.A. Fagin has received a commercial research grant from AstraZeneca and is a consultant/advisory board member for AstraZeneca.

**Authors’ Contributions**

Conception and design: C. Montero-Conde, S. Ruiz-Llorente, J.M. Dominguez, J.A. Fagin


Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J.M. Dominguez, A. Viale, E.J. Sherman, M. Blyler, R.A. Ghossein, J.A. Fagin

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): C. Montero-Conde, S. Ruiz-Llorente, J.M. Dominguez, N. Rosen, J.A. Fagin

Writing, review, and/or revision of the manuscript: C. Montero-Conde, S. Ruiz-Llorente, J.M. Dominguez, E.J. Sherman, N. Rosen, J.A. Fagin

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): C. Montero-Conde, J.M. Dominguez, J.A. Knauf

Study supervision: J.A. Knauf, J.A. Fagin

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Resistance to MAPK Inhibitors via NRG1-HER3/HER2


Relief of Feedback Inhibition of HER3 Transcription by RAF and MEK Inhibitors Attenuates Their Antitumor Effects in BRAF-Mutant Thyroid Carcinomas

Cristina Montero-Conde, Sergio Ruiz-Llorente, Jose M. Dominguez, et al.


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