Overcoming Resistance to Cetuximab with Honokiol, A Small-Molecule Polyphenol

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Abstract

Overexpression and activation of the EGFR have been linked to poor prognosis in several human cancers. Cetuximab is a mAb against EGFR that is used for the treatment in head and neck squamous cell carcinoma (HNSCC) and metastatic colorectal cancer. Unfortunately, most tumors have intrinsic or will acquire resistance to cetuximab during the course of therapy. Honokiol is a natural compound found in the bark and leaves of the Chinese Magnolia tree and is established to have several anticancer properties without appreciable toxicity. In this study, we hypothesized that combining cetuximab and honokiol treatments could overcome acquired resistance to cetuximab. We previously developed a model of acquired resistance to cetuximab in non–small cell lung cancer H226 cell line. Treatment of cetuximab-resistant clones with honokiol and cetuximab resulted in a robust antiproliferative response.

Introduction

The HER family of receptor tyrosine kinases (RTK) includes EGFR, HER2, HER3, and HER4. Ligand binding of these receptors stimulates the kinase domain and allows for hetero- or homodimerization within the family (1). Activation of the HER family of RTKs leads to proliferation of several downstream signaling pathways. Most notably, the HER family activates proliferation via the MAPK and survival by protein kinase B (AKT) pathways (2, 3). Previous research demonstrated that the HER family also impacts metastasis and invasion via other downstream signaling networks (2).

EGFR overexpression is identified in many human cancers including head and neck squamous cell carcinoma (HNSCC; ref. 4), non–small cell lung cancer (NSCLC; ref. 5), colorectal, breast (6), pancreatic (7), and brain cancers (8). The deregulation of EGFR signaling in these cancers leads to poor prognosis in patients. Given the identified role of EGFR signaling in human cancers, several targeted therapies against this receptor have been developed. In particular, a mAb called cetuximab has significant promise against EGFR. Cetuximab binds to the extracellular domain of EGFR and inhibits ligand binding, dimerization, and promotes receptor internalization (9, 10). Cetuximab is approved by the FDA in the treatment of metastatic colorectal cancer and HNSCC (11, 12). In addition, studies show that cetuximab may have clinical benefit in NSCLC (13, 14).

Although cetuximab significantly impacts the management of HNSCC and colorectal cancer, most patients experience intrinsic resistance or develop acquired resistance to cetuximab (15–17). To understand mechanisms of resistance, our laboratory previously established models of acquired resistance to cetuximab (18). This was achieved by continually treating cetuximab-sensitive cell lines with increasing concentrations of cetuximab until single-cell–resistant clones were identified. Further evaluation of the resistant clones revealed that cetuximab-resistant lines exhibit increased activation of HER family members and downstream signaling markers such as MAPK and AKT (18, 19). We also reported that another RTK member, AXL, was the key mediator of cetuximab resistance (20). Furthermore, cetuximab-resistant cells depend heavily on HER2/HER3 signaling and therefore overexpression of HER2/HER3 significantly impacts the survival and proliferation of these cells (21). Resistance to cetuximab has also been thought to arise from copy number alterations or domain-specific mutations in EGFR or due to the key downstream effectors KRAS, BRAF, PIK3CA, and PTEN (22). In the clinical arena, RAS mutations and HER2/MET amplifications were the

Immunoblot analysis revealed the HER family and their signaling pathways were downregulated after combination treatment, most notably the proliferation (MAPK) and survival (AKT) pathways. In addition, we found a decrease in phosphorylation of DRP1 and reactive oxygen species after combination treatment in cetuximab-resistant clones, which may signify a change in mitochondrial function. Furthermore, we utilized cetuximab-resistant HNSCC patient-derived xenografts (PDX) to test the benefit of combinatorial treatment in vivo. There was significant growth delay in PDX tumors after combination treatment with a subsequent downregulation of active MAPK, AKT, and DRP1 signaling as seen in vivo. Collectively, these data suggest that honokiol is a promising natural compound in overcoming acquired resistance to cetuximab.

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most frequently detected resistance mechanism to cetuximab in a cohort of 22 metastatic colorectal cancer patients (23). Although acquired resistance to tyrosine kinase inhibitor (TKI) therapy has been attributed to mutations in the cytoplasmic tail of the EGFR, no such correlation exists for cetuximab response (24), and we did not see any EGFR mutations in these cetuximab-resistant cells (18).

Given the clinical ramifications of acquired resistance to cetuximab, overcoming acquired resistance is a critical step in successfully treating metastatic colorectal cancer, HNSSC, and potentially NSCLC. We previously tested several small-molecule inhibitors including gefitinib and erlotinib. We reported that cetuximab-resistant tumors may still rely on EGFR for growth and survival and can be successfully targeted via alternative EGFR TKIs (18, 25). Researchers began investigating natural compounds as a means to overcome resistance in part because there is lower toxicity associated with these compounds when compared with established targeted therapies (26). Honokiol is one of the natural compounds that recently emerged as a promising therapy to overcome drug resistance (27). Honokiol is a small-molecule polyphenol attained from the bark of the Chinese Magnolia tree and is used in traditional Chinese medicine (28). It has been shown to have antiangiogenic (29), anti-inflammatory (30), and antitumor properties without appreciable toxicity (31). Recent research revealed Honokiol’s antitumor effects such as apoptosis and growth inhibition in leukemia (32), melanoma (33, 34), lung (35), pancreatic (36), and colorectal cancers (37). Honokiol is also established as a potent inhibitor of EGFR, AKT, and MAPK activation, which is thought to contribute to the decrease in proliferation of HNSSCC (38). In addition, honokiol effectively suppressed growth in adriamycin- and tamoxifen-resistant breast cancer cell lines (31, 38). Thus, previous research suggests that honokiol is a powerful anticancer agent even in drug-resistant cell lines and inhibits activation of the HER family receptors in many human cancers.

Given the previous data published on honokiol, we sought to determine whether honokiol was a potential agent that could be utilized to overcome acquired resistance to cetuximab and to define the mechanism of this effect. We found that the combination treatment of cetuximab and honokiol inhibited proliferation more robustly than either honokiol or cetuximab alone in cetuximab-resistant NSCLC clones. The decrease in proliferation of these cells could potentially be explained by the decreased activation of HER3, MAPK, AKT, and Dynamin-related protein 1 (DRP1). Furthermore, the inhibition of pDRP1 led to decreased production of reactive oxygen species (ROS), which may contribute to the efficacy of honokiol treatment. In vivo, the combination treatment exhibited a robust inhibition of HNSSCC patient derived xenograft (PDX) tumor growth. Collectively, our findings suggest that honokiol may be an effective therapy in overcoming acquired resistance to cetuximab in NSCLC and HNSSCC.

**Materials and Methods**

**Cell lines**

Drs. J. Minna and A. Gazdar (University of Texas Southwestern Medical School, Dallas, TX) provided the human NSCLC cell line H226. The H226 cells were maintained in 10% FBS in RPMI1640 (Mediatech, Inc) with 1% penicillin and streptomycin. The development of cetuximab-resistant cells has been described previously (18). The validity of these cell lines was consistently verified on the basis of cell morphology and genomic short tandem repeat (STR) profile of each cell line. Four to 6 months prior to the initiation of our investigation, H226 cells were tested for authenticity in agreement with ATCC guidelines.

**Materials**

Cetuximab (IMC-225, Erbitux) was purchased from the University of Wisconsin Pharmacy. Honokiol was generously provided from Dr. Jack Arbiser at Emory University (Atlanta, GA). Intralipid was purchased from Santa Cruz Biotechnology, Inc.

**Antibodies**

All antibodies were purchased from commercial sources as indicated below: EGFR, HER2, HER3, and HRP-conjugated goat anti-rabbit IgG and goat anti-mouse IgG were obtained from Santa Cruz Biotechnology, Inc. pMAPK (T202/Y204), pAKT (S473), AKT, rpS6 (S235/236), pS6, pDRP1 (S616), DRP1, p-C-RAF (S338), C-RAF, were obtained from Cell Signaling Technology. α-tubulin was purchased from Calbiochem.

**Cell proliferation (CCK8) assay**

This assay was performed as described previously (39).

**Evaluation of therapeutic interactions**

The fractional product method was used for analysis of interactions in cetuximab-resistant cells (40, 41). Briefly, this method utilizes the relative cell density following treatment with each individual agent and calculates the expected (E) effect of combination therapy as a product of the individual responses. The observed (O) effect is the relative cell density following dual treatment. A ratio of the observed to expected (O:E) values was calculated and used to estimate the synergy, additivity, or antagonism. A value less than 1 indicated synergism, greater than 1 demonstrated antagonism, and 1 represented additivity.

**Immunoblot analysis**

Whole-cell protein lysate was attained using Tween-20 lysis buffer [50 mmol/L HEPES, pH 7.4, 150 mmol/L NaCl, 0.1% Tween-20, 10% glycerol, 2.5 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L DTT, 1 mmol/L Na3VO4, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L L-b-glycerophosphate (BGP), and 10 mg/mL leupeptin and aprotinin]. Immunoblot analysis was performed as described previously (42).

**Phospho-receptor tyrosine kinase array**

Using an antibody-based array from R&D Systems, the activity of several RTKs were screened. This phospho-RTK array selectively screens for proteins/receptors associated with the membrane. Antibodies against 42 RTKs were presotted in duplicate on nitrocellulose membranes. Cetuximab-resistant HCO4 cells were treated with vehicle, cetuximab (100 nmol/L), and/or honokiol (20 μmol/L) for 24 hours, and cell lysates were collected and incubated with the prepared membrane. Phosphorylated tyrosines on activated RTKs were detected using a pan-anti-phosphotyrosine antibody. Scanned images were quantified by Image Quant software and relative expression of specific phosphorylated RTKs was determined.
Bromodeoxyuridine cell-cycle distribution analysis

Cells were plated at a density of $8 \times 10^5$ cells per 100-mm² plate and allowed 24 hours to adhere. The cells were treated with vehicle, 100 nmol cetuximab, 20 μmol/L honokiol, or 100 nmol cetuximab and 20 μmol/L honokiol in combination for 24 hours. Bromodeoxyuridine (BrdUrd) cell distribution analysis was executed as described previously (43). Twenty-four hours after the last cetuximab and/or honokiol treatment, tumors were harvested. Details of tumor collection and protein isolation were described previously (43). Protein quantitation and immunoblot analysis were completed following procedure as stated above.

Statistical analysis

Statistical analyses were performed using Prism 7 software (GraphPad Software, Inc.). Paired t-test analysis was performed for in vivo study and $P \leq 0.01$ was considered significant at the 99% confidence level as shown by the asterisks (**).

Results

Honokiol inhibits proliferation of cetuximab-resistant NSCLC clones

We previously established a model of cetuximab resistance using the NSCLC H226 cell line. Cetuximab-sensitive H226 clones were continually treated with increasing doses of cetuximab and we selected clones based on continued proliferation despite treatment (18). Cetuximab-resistant clones (HC1, HC4, HC8) were found to have increased activation of EGFR/HER2/HER3 and subsequent increased activation of the MAPK/AKT pathways. Given these results, we hypothesized that honokiol could have potent antiproliferative affects in cetuximab-resistant NSCLC lines. To test this hypothesis, we performed a comparative cell proliferation assay looking at cetuximab-resistant clones (HC1, HC4, HC8) versus the parental, cetuximab-sensitive, control (HP) following 72 hours of treatment with increasing concentrations of honokiol (Fig. 1A). This revealed a statistically significant, dose-dependent growth inhibition of all resistant clones. Treatment with 25 μmol/L of honokiol resulted in approximately 30%–50% decrease in proliferation, whereas proliferation of the parental line remained relatively stagnant despite increased concentrations of honokiol. The IC_{50} value of honokiol is 32, 24, 24, and 23 μmol/L in HP, HC1, HC4, and HC8 cells, respectively. To determine honokiol’s effect on the HER family members and downstream signaling, we analyzed the expression and activity of the HER family networks in cetuximab-resistant clones (HC1, HC4, HC8) versus the cetuximab-sensitive parental line (HP) after treatment with increasing concentrations of honokiol (Fig. 1B). Phosphorylation of all three receptors decreased after 25 μmol/L of honokiol. In addition, phosphorylation of downstream cascades in resistant clones (HC1, HC4, HC8) specifically C-RAF, MAPK, AKT, and ribosomal protein S6 (rpS6) was inhibited after treatment with honokiol. HER3 total expression also decreased following 25 μmol/L of honokiol; however, total expression of HER2 and EGFR remained steady following honokiol treatment. Collectively, these findings suggest that honokiol effectively decreases proliferation of cetuximab-resistant clones which may be explained by the decreased activation of HER family receptors and downstream signaling networks.

Combination treatment of honokiol plus cetuximab inhibits cell proliferation and induces G_1-phase cell-cycle arrest in cetuximab-resistant clones

To determine whether honokiol could resensitize cetuximab-resistant clones to cetuximab treatment, we treated cetuximab-resistant clones (HC1, HC4, HC8) with vehicle, cetuximab (100 nmol/L), honokiol (20 μmol/L) or the combination (Fig. 2A). In all cetuximab-resistant clones, cell proliferation...
decreased by approximately 40% after dual treatment as compared with the vehicle control. Furthermore, synergistic interactions between cetuximab and honokiol were found in all cetuximab-resistant clones we tested as analyzed by the fractional product method (Fig 2A). The honokiol treatment of the cetuximab-sensitive parental control (HP) did not elicit any additional effects to the cetuximab treatment. To determine the effect of combination treatment on the activation of RTKs, an RTK array of a cetuximab-resistant clone, HC4, was preformed following vehicle, cetuximab, honokiol, or combination treatment (Fig. 2B). The combinatorial treatment showed an approximately 20% decrease in activation of HER2 and HER3. This was greater inhibition than either treatment alone. Interestingly, activation levels of insulin receptor (IR), insulin growth factor 1 receptor (IGF1R), and c-Ret were increased after honokiol treatment (Fig. 2B). This activation, however, appeared to be downregulated in the combination arm. We performed an immunoblot analysis after the same treatment.
regime as stated above in HP, HC1, HC4, and HC8 to examine the expression and activity of downstream signaling (Fig. 2C). Activation of HER2, HER3, and downstream signaling markers robustly decreased after combination treatment, more so than either treatment strategy alone. The most significant inhibition of phosphorylation was found in MAPK, AKT, rpS6, and C-RAF. Across all cetuximab-resistant lines (HC1, HC4, HC8), total expression of the HER family and downstream markers remained constant across treatments. Furthermore, combination treatment induced a G1-phase cell-cycle arrest in cetuximab-sensitive and -resistant lines (Fig. 3). Dual treatment led to the greatest inhibition of the S-phase followed by honokiol and then cetuximab in all cetuximab-resistant lines. Overall, combination treatment of honokiol and cetuximab in
resistant clones led to robust inhibition of proliferation and activation of the HER family network and induced a G1-phase cell-cycle arrest.

**Combinatorial treatment suppresses activation of DRP1 in cetuximab-resistant clones**

Activation of the RAS/RAF/MAPK pathway alters mitochondrial function through the activation of DRP1 (45). Activated DRP1 promotes mitochondrial fission which increases mitochondrial function as the mitochondria have increased surface area after fission. In addition, increased mitochondrial function is shown to contribute to the tumor growth of oncogenic MAPK-dependent cancers (45). Inhibition of DRP1 could affect the rate of mitochondrial fission which could decrease mitochondrial output, limiting the energy available for proliferation. Recent data suggests that honokiol downregulates mitochondrial function in melanoma (34). Given the decrease in proliferation and activation of C-RAF and MAPK after honokiol treatment, we hypothesized that honokiol may inhibit phosphorylation of DRP1 in NSCLC cetuximab-resistant clones. We compared the phosphorylation of DRP1 after cetuximab, honokiol, and combination treatment in cetuximab-sensitive and -resistant NSCLC clones (Fig. 4A). Interestingly, we found that the parental cetuximab-sensitive line had low endogenous levels of pDRP1 while all cetuximab-resistant lines had higher endogenous levels of pDRP1 (Fig. 4A). Results from this study indicated that the combination of honokiol and cetuximab treatment led to greater inhibition of pDRP1 when compared with either treatment alone. These results were corroborated by immunofluorescence analysis of pDRP1 (Fig. 4B). Quantification of average cell fluorescence revealed that HC4 clones had almost 2 times the amount of pDRP1 fluorescence after vehicle treatment. In addition, it was shown that pDRP1 fluorescence had approximately 65% decrease after combination treatment. These results suggest that cetuximab-resistant clones have increased activation of DRP1 endogenously. To examine the changes in mitochondrial function after combination treatment, we used Mitosox immunofluorescence (red) to stain ROS in cetuximab-resistant (HC4) and sensitive clone (HP). Interestingly, the cetuximab-resistant clone (HC4) had increased endogenous levels of ROS compared with the cetuximab-sensitive clone (HP). Quantification of average cell fluorescence showed that HC4 clones contained nearly 2.5× the amount of ROS staining after vehicle treatment. The combination treatment led to the most robust decrease (~70%) in ROS staining in our cetuximab-resistant clone (Fig. 4C). Remarkably, both pDRP1 and ROS staining increased after cetuximab treatment in our cetuximab-resistant clone. Combination treatment of honokiol plus cetuximab effectively inhibited the phosphorylation of DRP1 and thus suppressed the production of ROS.

**Combination treatment delayed growth of HNSCC patient-derived xenograft (PDX) tumors**

Given the promising in vitro data and the established effects of pDRP1 on tumor growth (46), we tested the efficacy of a combination treatment of cetuximab and honokiol in vivo (Fig. 5A). Cetuximab-resistant UW-SCC1 PDX tumors (44) were inoculated in each dorsal flank of the mice on day 0 and were allowed to reach 200 mm3 before animals were randomly assigned to treatment groups (vehicle, cetuximab, honokiol, or combination). Thirty-two mice were analyzed: 8 per treatment group. Each group was treated with cetuximab (0.2 mg/mouse twice weekly), honokiol (3 mg/mouse three times/week), or combination that was administered through intraperitoneal injection for 30 days. This experiment demonstrated that tumor growth was significantly inhibited when compared to vehicle or either treatment alone. Average volume of tumors after 30 days of vehicle treatment was approximately 1,000 mm3, after cetuximab treatment approximately 900 mm3, after honokiol treatment approximately 600 mm3, and after combination treatment approximately 250 mm3. Importantly, the size of the tumors after 30 days of combination treatment was approximately the same as the volume when treatment began. Further evaluation of tumor samples revealed a similar downregulation of HER family phosphorylation and activation of downstream signaling markers as seen in vitro (Fig. 5B). Specifically, activation of HER3, C-RAF, MAPK, AKT, and rpS6 was effectively inhibited following the combinatorial treatment. pDRP1 expression was also inhibited in tumor lysates after combination treatment. Collectively, these data suggest that combination treatment of cetuximab and honokiol inhibits phosphorylation of the HER family, downstream signaling cascades, and DRP1. This may offer an explanation for the effective growth delay observed in the HNSCC PDX tumors.

**Discussion**

Cetuximab, an mAb against the EGFR, is approved by the FDA for treatment of metastatic colorectal cancer and HNSCC (12, 47).
Figure 4. Cetuximab and honokiol in combination suppresses DRP1 phosphorylation. 

A, Combination treatment downregulates phosphorylation of C-RAF, MAPK, and DRP1. The parental cetuximab-sensitive line (HP) had relatively low expression of pDRP1 and DRP1 after vehicle treatment when compared with cetuximab-resistant clones. Cetuximab-sensitive and cetuximab-resistant lines were treated with vehicle, cetuximab (100 nmol/L), honokiol (20 μmol/L), or combination of cetuximab and honokiol for 24 hours. Cells were lysed and fractionated using SDS-PAGE prior to immunoblotting for proteins of interest. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used to validate loading concentrations. 

B, Immunofluorescent microscopy and imagery exposed decreased expression of pDRP1 after combination treatment. The parental cetuximab-sensitive clone (HP) had low baseline expression of pDRP1 which remained relatively constant after treatments. To visualize pDRP1 expression in HP and HC4, we used a pDRP1 antibody and Alexa Fluor-488 secondary antibody (green). To visualize the nucleus, DAPI was utilized (blue). Average cell fluorescence was then quantified using approximately 50 randomly chosen cells per treatment. Magnification: 400×. 

C, Immunofluorescent staining with MitoSox (red) revealed decreased ROS presence in our cetuximab-sensitive line but relatively high amounts of ROS in our cetuximab-resistant line. The abundance of ROS in our cetuximab-resistant line (HC4) was effectively downregulated after combination treatment. The inhibition of ROS after combination treatment in HC4 was more robust that either honokiol or cetuximab alone. Average cell fluorescence was then quantified by measuring the fluorescence of approximately 20 cells randomly chosen per treatment.
In addition, recent studies demonstrate that it is also a promising therapy for NSCLC (13, 14). Although some patients benefit from cetuximab treatment, many patients are intrinsically resistant or develop acquired resistance to the drug. To study potential mechanisms of acquired resistance, our laboratory previously developed a model of acquired resistance in NSCLC and HNSCC. Further evaluation revealed that cetuximab-resistant clones over-expressed HER family members as well as MAPK and AKT (18, 21, 43). A recent study conducted by Leeman-Neill and colleagues concluded that honokiol, a small-molecule polyphenol, decreased phosphorylation of MAPK and AKT in HNSCC. In addition, honokiol is effective in overcoming drug resistance in myeloma, melanoma, and breast cancer (31, 33, 34). It also elicited robust anticancer activity in leukemia (32), melanoma.

Figure 5.
Combinatorial treatment delays the growth of cetuximab-resistant HNSCC patient-derived xenograft (PDX) tumors. A, Combination treatment significantly delayed growth in cetuximab-resistant HNSCC PDX tumors when compared with either cetuximab or honokiol alone. UW-SCC1 HNSCC PDX tumor was inoculated into mice and tumors grew to 200 mm³. Mice were randomized into control or treatment groups. Treatment groups received either 0.2 mg/mouse of cetuximab per day twice a week, 3 mg/mouse of honokiol per day three times a week, or a combination of both treatments by intraperitoneal injection. B, Tumors were harvested by the protocol listed previously in Materials and Methods. Tumor lysate was further analyzed through immunoblotting of targeted proteins. Alpha-tubulin was used as a loading control.
downregulated proteins that are abundant in our acquired resistance to cetuximab in NSCLC.

In this study, we found that combination treatment of cetuximab and honokiol had a robust antiproliferative effect in cetuximab-resistant clones in vitro and in vivo (Figs. 2A and 5A). This can largely be attributed to the down regulation of cell proliferation and survival pathways that are hyperactive in cetuximab-resistant clones. Dual treatment with honokiol and cetuximab showed significant decreases in the levels of pHER2, pHER3, pMAPK, pAKT, and p-C-RAF (Fig. 2C). This inhibition was greater than either treatment alone in the cetuximab-resistant setting. Consequently, it has been shown that although targeting AKT using MK-2206 resulted in growth inhibition, the greatest growth inhibition was achieved after dual treatment of MK-2206 and cetuximab in NSCLC cetuximab–resistant lines (19). Recent studies observed an inhibition of pMAPK in HNSCC, melanoma, and myeloma after honokiol treatment (33, 34, 38). In addition, inhibition of pAKT was demonstrated in HNSCC, myeloma, and breast cancers after honokiol treatment (31, 33, 38). These results corroborate our findings. pMAPK and pAKT inhibition might offer a further explanation for the G_{1} cell-cycle arrest after dual treatment found in our study (Fig. 3). Decreased phosphorylation of MAPK and AKT were also found in the in vitro setting (Fig. 5B). Inhibition of pAKT and pMAPK and G_{1} phase cell-cycle arrest after combination treatment helps explain the delayed PDX tumor growth found in our experiment. Furthermore, honokiol has also been found to have antitumor properties in skin, lung, breast, ovarian, prostate, colorectal, and even malignant gliomas (27). These data suggest that across a wide variety of cancers, honokiol successfully inhibits both pMAPK and pAKT. This inhibition likely contributes to the decrease in cell proliferation and delayed tumor growth found in cetuximab-resistant cells and PDX tumors after combination treatment of cetuximab and honokiol.

In addition to pMAPK and pAKT downregulation, combination treatment also inhibited p-C-RAF and pDRP1 expression (Fig. 4A). Kishatsus and colleagues showed that phosphorylation of DRP1 by pMAPK can lead to mitochondrial fission and increased mitochondrial function. A similar mechanism proposed by Tailor and colleagues also links pDRP1 to mitochondrial fission (48). Furthermore, honokiol can alter mitochondrial function in lung cancer (49) and can inhibit pDRP1 expression in melanoma (34). Our results supported these mechanisms as we observed decreased activation of C-RAF, MAPK, and DRP1 following honokiol and combination treatments. We speculate that mitochondrial fission and subsequent increased mitochondrial function contributes to the survival and proliferation of cetuximab-resistant clones and PDX tumors as the mitochondria are providing energy for these activities. The balance between mitochondrial fission and fusion has significant implications on mitochondrial function as changes to morphology affect the surface area and the electron transport chain which could alter the production of reactive oxygen species. Important mitochondrial function including proliferation, metabolic efficiency, apoptosis, and autophagy are therefore impacted by the balance between mitochondrial fission and fusion. Previous research also revealed that downregulation of DRP1 led to decreased cell proliferation in lung cancer and colorectal cancer (46, 48, 50). This could further provide an explanation for the decrease in proliferation found in cetuximab-resistant clones and PDX tumors after combination treatment.

Our study also found that decreased pDRP1 expression after honokiol and cetuximab treatment correlated with decreased ROS production (Fig. 4). The antioxidant properties of honokiol were previously established, although the biological ramifications of ROS production are still debated (27, 30). It is speculated that increased ROS production in cells could lead to apoptosis due to increased oxidative stress (49). Alternatively, ROS-driven tumors with increased MAPK activation have been identified in tobacco-induced oral and lung carcinoma and other cancers caused by common viruses such as Epstein–Barr virus and hepatitis C virus. These cancers have been found to use reactive oxygen in molecular signaling pathways that can contribute to the increased survival of these tumors (51). Results from our current study demonstrated ROS inhibition following combination treatment of honokiol and cetuximab (Fig. 4C). This inhibition was also correlated with decreased proliferation, survival, G_{1} phase cell-cycle arrest, and delayed PDX tumor growth.

Collectively, the results reported indicate that combination treatment of cetuximab and honokiol successfully leads to decreased proliferation and survival of cetuximab-resistant clones and decreased tumor growth of intrinsically resistant HNSCC PDX tumors. This was likely achieved through the decreased phosphorylation of AKT, MAPK, S6rp, and DRP1. Our previous findings targeting other members of the HER family such as HER3 (42) or using a pan-HER antibody targeting all family members (21) as well as other key mediators such as AKT (19) can overcome resistance to cetuximab due to the compensatory mechanisms that allow for sustained proliferation and survival upon inhibition of both AKT or MAPK signaling pathways. Although work has shown that honokiol can enhance cetuximab, this is the first study to identify honokiol as a potential therapy to overcome acquired cetuximab resistance and is also the first study to examine the link between honokiol, mitochondrial function, and ROS in this setting. Given honokiol’s wide-ranging affects in multiple cancers, low appreciable toxicity, and the ability to overcome drug resistance, it is a promising agent for the future of cancer therapy.

Disclosure of Potential Conflicts of Interest

R.J. Kimple reports receiving commercial research grants from Threshold Pharmaceutical and Peloton Therapeutics. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Conception and design: H.E. Pearson, M. Iida, J. Arbiser, D.L. Wheeler
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References
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