HEADCLUSION OVERCME RESISTANCE TO MEK INHIBITION IN BRAF-MUTANT COLORECTAL CANCER BY DOWNREGULATION OF c-FLIP

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Abstract

Purpose: Activating mutations in the BRAF oncogene are found in 8% to 15% of colorectal cancer patients and have been associated with poor survival. In contrast with BRAF-mutant (MT) melanoma, inhibition of the MAPK pathway is ineffective in the majority of BRAFMT colorectal cancer patients. Therefore, identification of novel therapies for BRAFMT colorectal cancer is urgently needed.

Experimental Design: BRAFMT and wild-type (WT) colorectal cancer models were assessed in vitro and in vivo. Small-molecule inhibitors of MEK1/2, MET, and HDAC were used, overexpression and siRNA approaches were applied, and cell death was assessed by flow cytometry, Western blotting, cell viability, and caspase activity assays.

Results: Increased c-MET-STAT3 signaling was identified as a novel adaptive resistance mechanism to MEK inhibitors (MEKi) in BRAFMT colorectal cancer models in vitro and in vivo. Moreover, MEKi treatment resulted in acute increases in transcription of the endogenous caspase-8 inhibitor c-FLIP, in BRAFMT cells, but not in BRAFWT cells, and inhibition of STAT3 activity abrogated MEKi-induced c-FLIP expression. In addition, treatment with c-FLIP-specific siRNA or HDAC inhibitors abrogated MEKi-induced upregulation of c-FLIP expression and resulted in significant increases in MEKi-induced cell death in BRAFMT colorectal cancer cells. Notably, combined HDAC inhibitor/MEKi treatment resulted in dramatically attenuated tumor growth in BRAFMT xenografts.

Conclusions: Our findings indicate that c-MET/STAT3-dependent upregulation of c-FLIP expression is an important escape mechanism following MEKi treatment in BRAFMT colorectal cancer. Thus, combinations of MEK with inhibitors of c-MET or c-FLIP (e.g., HDAC inhibitors) could be potential novel treatment strategies for BRAFMT colorectal cancer. Clin Cancer Res; 21(14): 3230-40. © 2015 AACR.

Introduction

BRAF is mutated in approximately 8% of all cancers, and approximately 10% of colorectal cancer tumors harbor a BRAFV600E transversion, encoding the constitutively active V600E-BRAF oncoprotein (1, 2). BRAF belongs to the RAF protein-serine/threonine kinase family, which also comprises RAF1/c-RAF and A-RAF (3). In normal signaling conditions, RAF proteins phosphorylate and activate the MAPK signaling axis in response to active GTP-bound RAS. BRAFV600E mutations lead to constitutively active BRAF, resulting in sustained MAPK signaling, independent of upstream kinase activity (4).

Recent phase III studies have shown that patients with BRAF-mutant (MT) tumors have the worst overall survival compared with patients with RAS+ MT melanoma (KRAS or NRAS) or KRAS/BRAF wild-type (WT) colorectal cancer tumors (5-7). Furthermore, some studies have suggested that the BRAFV600E mutation should be included as marker of resistance to EGFR-targeted therapies, such as cetuximab and panitumumab (8-10). Therefore, novel therapeutic strategies are urgently needed for BRAFMT colorectal cancer patients.

Tumors carrying a mutationally activated oncogene frequently show “addiction” to the oncoprotein or activated signaling pathway upon which the cancer cells became dependent (11). Recently, the FDA approved the selective BRAF inhibitor vemurafenib for the treatment of patients with unresectable or metastatic melanoma with the BRAFV600E mutation. Although the BRAF inhibitors vemurafenib or dabrafenib, MEK1/2 inhibition (trametinib), or a BRAF/MEKi combination resulted in dramatic responses between 60% and 80% in BRAFMT melanoma (12-14), disappointing responses of 5% and 12% were seen for vemurafenib alone or dabrafenib/trametinib combination, respectively, in BRAFMT colorectal cancer (15, 16). Understanding mechanisms of resistance to MAPK inhibition in BRAFMT colorectal cancer may lead to new therapeutic strategies for this poor prognostic colorectal cancer subgroup.

Herein, we report a novel resistance mechanism to MEK1/2 inhibition in BRAFMT colorectal cancer, which is mediated via STAT3 and the antiapoptotic protein c-FLIP. This survival
Regulation of STAT3 and c-FLIP<sub>L</sub> in BRAF-Mutant Colon Cancer

Translational Relevance

Oncogenic V600E BRAF mutations are associated with poor clinical prognosis in colorectal cancer. Inhibitors of the BRAF-MAPK pathway are effective therapies for BRAF-mutant (MT) melanoma, but are ineffective in the majority of BRAFMT colorectal cancers. Understanding MAPK inhibitor resistance is therefore an important unmet clinical need in BRAFMT colorectal cancer. In this study, we have identified a novel resistance mechanism to MEK1/2 inhibition that is mediated via STAT3 and the antiapoptotic protein c-FLIP<sub>L</sub>, and which is dependent on c-MET activity. Furthermore, inhibiting c-MET or downregulating c-FLIP using RNAi or HDAC inhibitors significantly increased MEK inhibitor-induced apoptosis in BRAFMT colorectal cancer in vitro and in xenograft models. Taken together, our results indicate that combining MEK1/2 inhibitors with c-MET or HDAC inhibitors may be promising therapeutic strategies for this poor prognostic colorectal cancer subgroup and should be explored in future clinical trials.

Materials and Methods

Materials
AZD6244, AZD1480, and AZD9150 were obtained from AstraZeneca, crizotinib from Pfizer, TG101348 from Axon Medchem, trametinib, AZD8931, vorinostat and entinostat (MS-275) from Selleck Chemicals LLC, statin from Sigma-Aldrich, UO126 from Promega, and PD98059 from Cell Signaling Technology. siRNAs were purchased from Qiagen; the c-FLIP targeting STAT3 (STAT3_7), JAK1 (JAK1_1), JAK2 (JAK2_7), and c-MET and mucin-related antigens/mutational analysis (21, 22).

Authentication and culture of LIM2405, LIM1215, HT-29, VACO432/VT1, RKO F6-8, and CACO-2 colorectal cancer cells have previously been described (18–20). All cells were passaged for a maximum of 2 months, after which new seed stocks were thawed for experimental use. LIM2405 and LIM1215 cell lines, established in 1992, were a gift from Dr. Whitehead (Ludwig Institute of Melbourne and Vanderbilt University, Nashville, TN) in 2010. These cell lines were tested for morphology/growth rate/response to mitogens/xenograft growth/expression of brush-border and mucin-related antigens/mutational analysis (21, 22). VACO432, VT1, and RKO F6-8 were kindly provided by Prof B. Vogelstein (Johns Hopkins University School of Medicine, Baltimore, MD) in 2012. HT-29 (2001), CACO2 (2005), COLO205 (2012), and COLO320 (2012) cells were obtained from the ATCC (authentication by short tandem repeat profiling/karyotyping/isoenzyme analysis).

Generation of stable c-FLIP<sub>L</sub>-overexpressing cells

c-FLIP<sub>L</sub>, containing pBabe puro vector was transfected into Phoenix 293T cells along with a VSVG (vesicular stomatitis virus glycoprotein) coat protein plasmid. Following 24-hour transfection, filtered medium with polybrene (1:1000) was added to HT-29 cells for 48 hours, and thereafter 1 µg/mL puromycin (Life Technologies, Inc.) added for selection of c-FLIP<sub>L</sub>-overexpressing HT-29 cells. Overexpression of c-FLIP<sub>L</sub>, was confirmed by Western blotting.

Cell viability assays
Cell viability assays were done as previously described (20). Representative results of at least three independent experiments are shown.

Flow-cytometric analysis and cell death measurement
Apoptosis was determined using propidium iodide (PI) staining to evaluate the percentage of cells with DNA content <2 N as previously described (18). For annexin/PI analysis, cells were harvested and analyzed according to the manufacturer’s instructions (BD Biosciences). Representative results of at least three independent experiments are shown.

Western blotting
Western blot analysis was carried out as previously described (20). Anti-FLIP (NF6; Alexis), anti-caspase-8 (12F5; Alexis), and anti-PARP (eBioscience) mouse monoclonal antibodies were used in conjunction with a horseradish peroxidase (HRP)-conjugated anti-mouse secondary antibody (Amersham). Anti-cleaved caspase-3 (Cell Signaling Technology), anti-hyperacetylated Hystone H4 (Millipore) were used in conjunction with a HRP-conjugated anti-rabbit secondary antibody (Amersham). Equal loading was assessed using β-actin (Sigma) mouse monoclonal primary antibody.

Caspase-activity assays
Caspase-8 or caspase-3/7-GLO reagents (25 µL; Promega) were incubated with 5 µg of protein lysate diluted in cell culture medium in a total volume of 50 µL for 45 minutes at room temperature. Luciferase activity was measured using a luminometer.

STAT3 reporter assay
The STAT3 reporter, negative and positive control constructs were obtained from Qiagen. Luminescence was measured following addition of 50 µL of luciferase assay reagent into each well.

siRNA transfections
siRNA transfections were carried out using Hiperfect (Qiagen) as previously described (20).

ELISA
Soluble c-MET and HGF ELISA assays were carried out as previously described (20).

Real-time reverse transcription PCR analysis
RNA was isolated using the GeneJet RNA purification kit (Thermo Scientific) and reverse transcribed using the Moloney

Response is dependent on c-MET and JAK1/2 activity and is abrogated using c-MET and JAK1/2-STAT3 inhibitors. We also show that concomitant treatment with inhibitors of c-MET or c-FLIP (using HDAC inhibitors) and MEK1/2 inhibition leads to marked increases in therapeutic efficacy in BRAFMT in vitro and xenograft models. Taken together, our results indicate that combining MEK1/2 inhibitors with c-MET or HDAC inhibitors could be potential novel treatment strategies for BRAFMT colorectal cancer.

Cell culture
Authentication and culture of LIM2405, LIM1215, HT-29, VACO432/VT1, RKO F6-8, and CACO-2 colorectal cancer cells have previously been described (18–20). All cells were passaged for a maximum of 2 months, after which new seed stocks were thawed for experimental use. LIM2405 and LIM1215 cell lines, established in 1992, were a gift from Dr. Whitehead (Ludwig Institute of Melbourne and Vanderbilt University, Nashville, TN) in 2010. These cell lines were tested for morphology/growth rate/response to mitogens/xenograft growth/expression of brush-border and mucin-related antigens/mutational analysis (21, 22). VACO432, VT1, and RKO F6-8 were kindly provided by Prof B. Vogelstein (Johns Hopkins University School of Medicine, Baltimore, MD) in 2012. HT-29 (2001), CACO2 (2005), COLO205 (2012), and COLO320 (2012) cells were obtained from the ATCC.
Carson et al.

murine leukemia virus-based reverse transcriptase kit (Invitrogen). qPCR analysis was performed using the LightCycler 480 probes master mix or the Roche SYBR Green method (LightCycler 480II, Roche).

**Results**

In vivo studies were conducted as previously described using BALB/c nude mice (18). AZD6244 10 mg/kg/twice-a-day (bid) p. o., entinostat (MS-275) 10 mg/kg/every day (qd) p. o., and crizotinib 25 mg/kg/qd were administered by oral gavage. The control groups received vehicle (methocel/polysorbate buffer). In the combination groups, AZD6244 was administered together with MS-275 or crizotinib. Each treatment group contained 8 animals. Two hours following final dosing, mice were sacrificed and tumor tissue was excised and snap frozen in liquid nitrogen for immunoblotting (PPL2704). All animal experiments were carried out according to U.K.C.C.C.R. guidelines.

**Statistical analysis**

Student t tests and two-way ANOVA were calculated using the GraphPad software (Prism4). Two-way ANOVA test was used to determine the significance of change in levels of apoptosis between different treatment groups. All changes in levels of apoptosis that are described as significant had P values that were <0.05 (*, P < 0.05; **, P < 0.01; ***, P < 0.001; ns, not significant compared with control).

**Results**

MEK1/2 inhibition decreases cell viability but does not induce apoptosis in BRAFV600E colorectal cancer

To explore the sensitivity of BRAFMT colorectal cancer cells to MEK1/2 inhibition, we evaluated the effects of the clinically used MEK1/2 inhibitors AZD6244 and trametinib (GSK1120212) on viability of the parental VACO432 colorectal cancer cell line, which carries the V600E mutation in BRAF (BRAFV600E/+). Its isogenic VTI clone with a disrupted BRAFV600E allele (23), and a panel of BRAFMT (LIM2405, HT-29, COLO205, RKO F6-8) and WT (Caco-2, LIM1215 and COLO320) colorectal cancer cells (Supplementary Fig. S1A, top). BRAFMT colorectal cancer cells showed a higher sensitivity to MEK1/2 inhibition with IC50 values between 20 nmol/L to 3.7 µmol/L and 0.1 nmol/L to 9.4 nmol/L for AZD6244 and trametinib, respectively, compared with IC50 values of >6.8 µmol/L and >13 nmol/L for BRAFWT cells (P < 0.001 for both AZD6244 and trametinib). Although BRAFMT colorectal cancer cells were more sensitive to MEK1 than BRAFWT cells, apoptosis induction 24 hours after treatment with MEK1/2 inhibitor was relatively inefficient as assessed by activation of caspase-3 (Supplementary Fig. S1A, bottom) and flow cytometry (Supplementary Fig. S1B).

STAT3 pathway activation and JAK1/JAK2 are key mediators of resistance to MEK inhibitors in BRAFMT colorectal cancer cells

We have recently shown that KRAS-mutant cells respond to MEK1/2 inhibitors with increased STAT3 phosphorylation (20). Hence, we investigated the effect of AZD6244, PD98059, UO126, and trametinib on STAT3 activation in our panel of BRAFMT and WT colorectal cancer cell lines and found that STAT3 phosphorylation (Y705) was acutely increased in the BRAFMT cell lines in response to each agent, but not in the BRAFWT/KRASWT cells (Fig. 1A). In addition, we also found acute increases in STAT3 transcriptional activity and expression of the STAT3 target genes IRF-7, RPS18, and SOCS3 following AZD6244 treatment in the BRAFMT colorectal cancer cells (Supplementary Fig. S1C). Constitutive pSTAT3 levels were higher in BRAFMT VACO432 cells compared with its isogenic WT clone; however, there was no clear pattern of pSTAT3 expression across a panel of non-matched BRAFMT and WT colorectal cancer cells (Supplementary Fig. S1D). STAT3 is activated in the context of MEKi in BRAFMT but not BRAFWT cells, a phenomenon known as an adaptive, acutely induced resistance mechanism (24). We also assessed the effect of STAT3 silencing on apoptosis induction by flow cytometry and Western blotting for PARP cleavage and activation of caspase-3. A significant increase in apoptosis was observed when BRAFMT cells were cotreated with STAT3 siRNA and AZD6244 (Supplementary Fig. S1B and S1E). Collectively, these data indicate that STAT3 is an important survival pathway following MEK1/2 inhibition in BRAFMT colorectal cancer.

Next, we investigated the involvement of the upstream kinases JAK1 and JAK2 in regulating STAT3 activity and survival of BRAFMT and WT colorectal cancer cells. MEK inhibitor-induced STAT3 phosphorylation was associated with increased JAK1 and JAK2 phosphorylation (Supplementary Fig. S2A). In addition, RNAi against JAK1 decreased basal and MEKi-induced pJAK2 levels and resulted in more potent inhibition of both basal and MEKi-induced STAT3 activity compared with the effects of JAK2 silencing (Fig. 1B). Moreover, treatment with the JAK1/2 inhibitor AZD1480 (25) resulted in potent inhibition of MEKi-induced STAT3 transcriptional activity, as indicated by decreased STAT3 reporter activity and mRNA expression of IRF-7 (Fig. 1C, top and bottom). These results indicate that JAK1 and JAK2 cooperate to regulate basal and MEKi-induced STAT3 survival response in BRAFMT colorectal cancer, which is consistent with our previous data in KRASMT colorectal cancer cells (20).

We next investigated the involvement of JAK1/2 in regulating survival of BRAFMT and WT colorectal cancer cells. Combined treatment of AZD1480 or TG101348 (20, 26) with AZD6244 resulted in potent increases in apoptosis as indicated by flow cytometry and increased PARP cleavage and caspase-3 processing in the BRAFMT VACO432 cell line but not the WT VTI clone (Fig. 1D, top and bottom). Calculation of combination index (CI) values confirmed strong synergy between AZD1480 or TG101348 and AZD6244 in the BRAFMT VACO432 cell line (Supplementary Fig. S2B). Importantly, similar results were obtained in a wider panel of BRAFMT (HT-29, LIM2405, RKO F6-8, COLO205), but not BRAFWT colorectal cancer cell line models (Fig. 1D and Supplementary Fig. S2B–S2D).

c-MET regulates MEK1-induced STAT3 activation and survival in BRAFMT colorectal cancer cells

Previous studies have shown the role of the EGFR family in resistance to BRAF inhibitors in BRAFMT cancers, including colorectal cancer (27, 28). Secretion of the hepatocyte growth factor (HGF), the ligand for c-MET, has also been shown to confer resistance to BRAF inhibition in BRAFMT melanoma (29). On the basis of these studies and our previous data in KRASMT colorectal cancer (20), we assessed the effect of MEK inhibition on EGFRY1066, HER2Y1248, and c-METY1234/1235 phosphorylation and found acute increases in phosphorylation of these receptors, as early as 3 hours following treatment with AZD6244 in BRAFMT cells (Supplementary Fig. S3A). Basal and AZD6244-induced STAT3 phosphorylation was inhibited following treatment with...
the c-MET/ALK inhibitor crizotinib, but unaffected by treatment with the dual EGFR/HER2 inhibitor AZD8931 in a panel of BRAFMT colorectal cancer cells, indicating that activation of STAT3 following MEK inhibition is not driven by EGFR or HER2 in BRAFMT colorectal cancer (Fig. 2A and Supplementary Fig. S3B).

We next assessed the effect of c-MET inhibition on MEK-induced apoptosis in BRAFMT colorectal cancer cells. Cotreatment with crizotinib and AZD6244 resulted in significant increases in apoptosis in the BRAFMT VACO432 cell line, but not in the BRAFVT daughter cell line (Fig. 2B). Similar results were obtained when apoptosis was assessed by Western blotting for PARP and caspase-3 cleavage, flow cytometry, and caspase-8 and -3/7 activity assays following combined AZD6244/crizotinib treatment in BRAFMT LIM2405, COLO205, HT-29, and RKO cells, but not in BRAFVT cells (Fig. 2B and C and Supplementary Fig. S3C–S3E). Importantly, although both AZD8931/AZD6244 and crizotinib/AZD6244 combinations resulted in significant decreases in cell viability in BRAFMT cells, only crizotinib enhanced apoptotic cell death when combined with AZD6244 in the panel of BRAFMT cells (Fig. 2B and C and Supplementary Fig. S3C). In order to define the relative importance of the extrinsic and intrinsic apoptotic pathways in mediating AZD6244/crizotinib-induced apoptosis, we used siRNA specifically directed against caspase-8 (extrinsic pathway) or caspase-9 (intrinsic pathway; Supplementary Fig. S3G). Notably, silencing of either caspase significantly decreased apoptosis following AZD6244/crizotinib treatment in BRAFMT LIM2405 cells. This suggests that the cell death induced by this combination proceeds via a caspase-8–mediated activation of the caspase-9–dependent intrinsic apoptosis pathway. Taken together, these data would indicate that the c-MET/STAT3 signaling is an important survival response induced by MEK1/2 inhibition in BRAFMT colorectal cancer.

On the basis of the compelling in vitro evidence, we next assessed the therapeutic efficacy of combined c-MET and MEK inhibition in a BRAFMT HT-29 xenograft model using AZD6244 and crizotinib (Fig. 2D). We detected significantly increased
pSTAT3 levels in xenografts treated with AZD6244; moreover, crizotinib resulted in decreased basal and AZD6244-induced pSTAT3 levels, consistent with our in vitro observation (Supplementary Fig. S3H). Importantly, the METi/MEKi combination led to a marked reduction in growth of BRAF MT HT-29 xenografts compared with treatment with each agent individually (Fig. 2D, left). Furthermore, caspase-3 cleavage was observed in the HT-29 tumors following treatment with AZD6244 and combination with crizotinib, indicative of apoptosis induction (Fig. 2D, right).

These results indicate that agents directed against c-MET may be highly effective combination partners for MEK1/2-targeted therapies in BRAF MT colorectal cancer.

Inhibitors of MEK1/2 upregulate c-FLIP L expression in BRAFMT colorectal cancer

To investigate the mechanism of apoptosis further, we assessed expression levels of pro- and antiapoptotic proteins following AZD6244/crizotinib treatment in BRAFMT HT-29, COLO205, and LIM2405 colorectal cancer cells (Fig. 3A). Notably, expression of c-FLIP L, but not c-FLIP S, was acutely increased following AZD6244 treatment in all three BRAFMT cell lines (Fig. 3A). In contrast, AZD6244 treatment had little impact on c-FLIP L levels in BRAFWT colorectal cancer models (Supplementary Fig. S4A). We also found that the increase in c-FLIP L levels in BRAFMT colorectal cancer cells was a transcriptional event, as AZD6244 increased c-FLIPL mRNA levels as early as 6 hours after treatment (Fig. 3B and Supplementary Fig. S4B). Importantly, cotreatment with AZD6244 and crizotinib, but not AZD8931, abrogated AZD6244-induced c-FLIPL expression, which was associated with processing of pro-caspase-8 and potent caspase-3, -9, and PARP cleavage (Fig. 3A).

c-FLIP L is important in regulating sensitivity to MEKi in BRAFMT colorectal cancer cells

To assess further the importance of c-FLIP L in regulating response to MEK1/2 inhibition, we developed a BRAFMT HT-29 cell line model that stably overexpresses c-FLIP L (Fig. 3C). c-FLIP L expression in HT-29-F3 cell line was approximately 2- to 2.5-fold higher compared with EV (empty vector; Fig. 3C, top). Overexpression of c-FLIP L in this cell line correlated with reduced levels of AZD6244-induced apoptosis compared with the EV cell line (Fig. 3C, bottom). These results suggested a role for c-FLIP L in mediating resistance to MEK-targeted agents in BRAFMT colorectal cancer cells. To investigate this, we assessed the effect of c-FLIP silencing on apoptosis induction using Western blotting for PARP cleavage and activation of caspase-3. Cotreatment with c-FLIP
siRNA and AZD6244 resulted in significant increases in apoptosis in the BRAF MT colorectal cancer cells to MEK inhibition. A, HT-29, COLO205, and LM2405 cells were pretreated with crizotinib or AZD6244 for 24 hours and PARP, pro–caspase-8, caspase-8 p41/43, caspase-8 p18, cleaved caspase-3, cleaved caspase-9, FLIP, FLIP, XIAP, BCL-2, and BAX levels determined by Western blotting. B, HT-29 and COLO205 cells were treated with AZD6244 for the indicated time. Top, FLIP, FLIP, pSTAT3Y705, STAT3, pERK1/2, and ERK1/2 levels were determined by Western blotting. Bottom, c-FLIP mRNA levels were determined by real-time PCR. Relative mRNA expression was calculated using the DD^Ct method with normalization to β-actin and GAPDH. C, top: expression levels of FLIP, FLIP, FLIP in HT-29 empty vector (EV) and FLIP-overexpressing cells, determined by Western blotting and real-time PCR. Bottom, HT-29 empty vector (EV) and FLIP-overexpressing cells were treated with AZD6244 for 24 hours and apoptosis assessed by flow-cytometric analysis. D, BRAF MT LIM2405, RKO F6-8, HT-29, and VACO432 cells and BRAF WT CACO-2, LIM-1215, and COLO320 cells were transfected with c-FLIP siRNA (30 nmol/L) for 24 hours and thereafter treated with AZD6244 for 24 hours. PARP, cleaved caspase-3, FLIP, FLIP, pERK1/2, and ERK1/2 levels were determined by Western blotting. *P < 0.01, **P < 0.001.

Figure 3. c-FLIP, regulates sensitivity of BRAF MT colorectal cancer cells to MEK inhibition. A, HT-29, COLO205, and LM2405 cells were pretreated with crizotinib or AZD6244 for 3 hours, and thereafter treated with 1 μmol/L AZD6244 for 24 hours and PARP, pro–caspase-8, caspase-8 p41/43, caspase-8 p18, cleaved caspase-3, cleaved caspase-9, FLIP, FLIP, XIAP, BCL-2, and BAX levels determined by Western blotting. B, HT-29 and COLO205 cells were treated with AZD6244 for the indicated time. Top, FLIP, FLIP, pSTAT3Y705, STAT3, pERK1/2, and ERK1/2 levels were determined by Western blotting. Bottom, c-FLIP mRNA levels were determined by real-time PCR. Relative mRNA expression was calculated using the DD^Ct method with normalization to β-actin and GAPDH. C, top: expression levels of FLIP, FLIP, FLIP in HT-29 empty vector (EV) and FLIP-overexpressing cells, determined by Western blotting and real-time PCR. Bottom, HT-29 empty vector (EV) and FLIP-overexpressing cells were treated with AZD6244 for 48 hours and apoptosis assessed by flow-cytometric analysis. D, BRAF MT LIM2405, RKO F6-8, HT-29, and VACO432 cells and BRAF WT CACO-2, LIM-1215, and COLO320 cells were transfected with c-FLIP siRNA (30 nmol/L) for 24 hours and thereafter treated with AZD6244 for 24 hours. PARP, cleaved caspase-3, FLIP, FLIP, pERK1/2, and ERK1/2 levels were determined by Western blotting. *P < 0.01, **P < 0.001.

Coinhibition of HDAC 1-3 and MEK1/2 results in cell death and inhibits growth of BRAF MT xenograft models

Our previous studies and those of others have shown that HDAC inhibitors with anti-HDAC1-3 activity are efficient post-transcriptional suppressors of FLIP expression (30, 31). Treatment with the pan-HDAC inhibitor vorinostat or the HDAC 1-3 inhibitor entinostat inhibited both basal and AZD6244-induced c-FLIP, expression, and this was associated with marked increases in apoptosis in the BRAF MT cell line panel (Fig. 4A and Supplementary Fig. S5A and S5B). We also determined that the apoptosis induced by entinostat/AZD6244 was highly dependent on caspase-8 (Fig. 4B), consistent with a FLIP-dependent mechanism of cell death.

To extend these in vitro findings, we next assessed the therapeutic efficacy of combined Entinostat and MEK inhibition in the BRAF MT xenograft model. Although both entinostat and AZD6244 slowed tumor growth, the entinostat/MEKi combination led to a supra-additive reduction in growth in HT-29 BRAF MT xenograft model (Fig. 5). Treatment with entinostat resulted in increased acetylation of H4, a marker of HDAC inhibition, and decreased basal and AZD6244-induced STAT3 activation (Fig. 5 and Supplementary Fig. S5C). In addition, combined entinostat/AZD6244 treatment resulted in high levels of caspase-3 activation in HT-29 tumors (Fig. 5). These findings indicate that HDAC1-3–targeted agents may be highly effective when used in conjunction with MEK1/2-targeted therapies to treat BRAF MT colorectal cancer.
STAT3 regulates MEK1/2 inhibitor-induced increases in c-FLIP expression

To investigate the relationship between MEK1/2 inhibitor-induced increases in STAT3 activity and c-FLIP expression levels, we used the STAT3 small-molecule inhibitor Stattic, which targets the STAT3 SH2 domain and prevents dimerization (32), RNAi and antisense against STAT3. Treatment with Stattic resulted in decreases in basal and AZD6244-induced STAT3Y705 phosphorylation levels, and this was associated with decreased AZD6244-induced c-FLIP expression levels (Fig. 6A and Supplementary Fig. S6A). To exclude any off-target effects from Stattic that may affect c-FLIP expression, we analyzed the effect of STAT3 siRNA and AZD9150, a 16-mer oligonucleotide antisense molecule targeting the 3' untranslated region of the STAT3 mRNA, which is in clinical development (33). Both AZD9150 and STAT3 silencing decreased basal and AZD6244-induced STAT3Y705 phosphorylation levels and c-FLIP expression levels (Fig. 6B and Supplementary Fig. S6B). In addition, cotreatment with AZD6244 and Stattic or AZD9150 resulted in significant increases in apoptosis (Fig. 6), similar to those observed with HDACi/MEKi combination (Fig. 4). Taken together, these results would suggest that STAT3 directly regulates c-FLIP survival response in response to MEK inhibition in BRAFMT colorectal cancer.

Discussion

Activating mutations in the BRAF oncogene occur in 8% to 15% of colorectal cancer and are associated with the poor clinical outcome (34). The studies showing a lack of clinical benefit from EGFR-targeted agents in BRAFMT colorectal cancer remain controversial (9, 35). Although selective BRAF and MEK1/2 inhibitors such as vemurafenib and trametinib have resulted in dramatic responses in BRAFVT600E melanoma, BRAFMT colorectal cancer with the identical missense mutation has shown very poor responses to these treatments (15, 16). In both our in vitro and in vivo data, we corroborate the findings seen in human patients. Understanding of the intrinsic resistance mechanisms to MAPK inhibition in BRAFMT colorectal cancer has the potential to
identify novel therapeutic strategies for this poor prognostic genetic subgroup. Recently, a number of studies have investigated resistance mechanisms to the BRAF inhibitor vemurafenib in BRAFMT colorectal cancer. Indeed, two studies have observed increased activity of EGFR following treatment with vemurafenib in BRAFMT colorectal cancer cells (27, 36). Moreover, BRAFMT melanoma cells were found to have low levels of EGFR, which might explain the favorable response of BRAFMT melanoma to BRAFI. Other studies reported the role of the PI3K/AKT and mTOR pathways in mediating resistance to vemurafenib in BRAFMT colorectal cancer cells (37–39). Our study provides evidence that STAT3-dependent c-FLIP upregulation is a major resistance mechanism to MEK inhibitors in BRAFMT colorectal cancer models.

Constitutive activation of STAT3 has been reported in a broad range of tumors and tumor-derived cell lines, including melanoma and colon cancer (40). A number of studies have shown that deregulated STAT3 not only enhances tumor cell proliferation and survival, but also can promote oncogenesis by bridging chronic inflammation to tumor formation (41–43). Furthermore, recent data from our laboratory and other studies have shown a role for STAT3 in regulating resistance to EGFR and MEK1/2 targeted therapies in lung and colon cancer, driven by activated EGFR, HER2, ALK, MET, and mutant KRAS (20, 44). In this study, we have found that treatment with different MEK inhibitors resulted in acute increases in STAT3 phosphorylation in BRAFMT colorectal cancer cells. Although recent studies have identified mutations within the SH2 dimerization and activation domain of STAT3 in large granular lymphocytic leukemia (45) and inflammatory hepato-cellular adenomas (46), STAT3 activation is usually dependent on growth factor receptors signaling and their associated janus kinases. Here, we found that basal and MEKi-induced STAT3 activation in BRAFMT colorectal cancer cells was mediated through JAK1 and JAK2. Using selective inhibitors of JAK1/2, we further demonstrated the differential dependency of BRAFMT and BRAFMT cells on STAT3 for survival, particularly in the context of cotreatment with MEK inhibitors. Collectively, these results indicate that JAK activation in response to inhibition of MEK1/2 in BRAFMT colorectal cancer cells contributes to resistance to MEK inhibitors in these cancer cells.

In addition, we found that STAT3 was activated by upstream activation of c-MET. Soluble HGF, the ligand for c-MET, was not detected in the culture medium of BRAFMT cell line models; however, consistent with our data in KRASMT cell lines (20), we found that MEK regulated the levels of soluble decoy MET, the natural antagonist of c-MET, in BRAFMT colorectal cancer models in vitro and in vivo (Supplementary Fig. S7). On the basis of these findings of a vemurafenib-induced feedback activation of EGFR and their in vivo data, previous studies have suggested that the addition of an EGFR inhibitor to vemurafenib could be a novel treatment approach for BRAFMT colorectal cancer (27). Interestingly, our studies showed that the c-MET-STAT3 signaling axis appears to have a more significant role in survival and MEKI-induced resistance in BRAFMT colorectal cancer cells, as the crizotinib/AZD6244 combination was a more effective inducer of apoptosis compared with AZD6244 combined with gefitinib (EGFR inhibitor) or AZD8931 (EGFR/HER2 inhibitor). The importance of c-MET as mediator of acute resistance to MEK1/2 inhibitors was demonstrated in vivo, where cotreatment of BRAFMT colorectal cancer xenografts with crizotinib blocked AZD6244-induced STAT3 activation and resulted in supra-additive reductions in tumor growth and marked induction of apoptosis (24). Collectively, these results indicate that inhibitors of the c-MET pathway in conjunction with MEK inhibitors could be a novel treatment strategy for BRAFMT colorectal cancer tumors.

Mechanistically, we found that STAT3 activation protects BRAFMT cancer cells from apoptosis following MEK inhibition, by acutely upregulating expression of the antiapoptotic protein c-FLIP_

L. Notably, treatment with AZD6244 resulted in acute increases in c-FLIP_

L mRNA and protein levels only in BRAFMT cells, and inhibition of STAT3 using statin or AZD9150 attenuated MEKi-induced upregulation of c-FLIP_

L expression and resulted in significant increases in MEKi-induced apoptosis. Moreover, we also demonstrate for the first time that FLIP_

L overexpression abrogates the effect of MEKi in BRAFMT colorectal cancer cells. We previously have shown that c-FLIP is an important regulator of apoptosis and drug resistance and a poor prognostic marker in colorectal cancer (17, 47, 48). Using siRNA against c-FLIP and multiple cell line models, we now show that c-FLIP is also a critical mediator of resistance to MEKi in BRAFMT colorectal cancer. Our previous studies and those of others have shown that...
pan-HDAC inhibitors and more specific HDAC1-3 inhibitors act as efficient posttranscriptional suppressors of FLIP expression (30, 49). Combined treatment of BRAFMT colorectal cancer models with the HDAC1-3 inhibitor entinostat and AZD6244 blocked AZD6244-induced c-FLIP expression and resulted in enhanced levels of apoptosis induction. In addition, we also demonstrated that MEK inhibition in conjunction with entinostat was highly effective at blocking the growth of BRAFMT colorectal cancer xenografts. This is the first study showing that combined HDAC/MEK inhibition could be a promising treatment strategy for BRAFMT colorectal cancer patients. Before carrying forward into a clinical trial, this combination study needs to be confirmed in additional BRAFMT models.

In conclusion, we have identified STAT3-mediated c-FLIP upregulation as an important resistance mechanism to MEKi in BRAFMT colorectal cancer patients. Before carrying forward into a clinical trial, this combination study needs to be confirmed in additional BRAFMT models.

Disclosure of Potential Conflicts of Interest
P.G. Johnston reports receiving speakers bureau honoraria from Chugai Pharmaceuticals and Pfizer, has ownership interest (including patents) in CV6 Therapeutics and Fusion Antibodies, and is a consultant/advisory board member for Chugai Pharmaceuticals. No potential conflicts of interest were disclosed by the other authors.

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Grant Support
This work was financially supported by Cancer Research UK (C212/A7402) and Cancer Research UK fellowship (C13749/A7261).

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Received October 20, 2014; revised February 14, 2015; accepted March 6, 2015; published OnlineFirst March 26, 2015.

Figure 6. STAT3 regulates MEKi-induced FLIP expression levels. A, top: HT-29 cells were cotreated with Stattic and AZD6244 for 24 hours and pSTAT3Y705, STAT3, FLIP1, FLIP3, pERK1/2, and ERK1/2 levels determined by Western blotting. Bottom, flow-cytometric analysis of HT-29 following treatment with 5 μmol/L Stattic and 1 μmol/L AZD6244 for 48 hours. B, top, pSTAT3Y705, STAT3, FLIP1, FLIP3, cleaved C3, pERK1/2, and ERK1/2 levels in LIM2405 cells following transfection with 10 nmol/L AZD9150 for 48 hours and treatment with AZD6244 for 24 hours. Bottom, flow-cytometric analysis of LIM2405 following transfection with 10 nmol/L AZD9150 for 48 hours and treatment with 1 μmol/L AZD6244 for 24 hours. C, schematic overview of mechanism of STAT3-mediated c-FLIPs expression following MEKi treatment in BRAFMT colorectal cancer. **, P < 0.01, ***, P < 0.001.
References


Carson et al.

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