BRaf and MEK Inhibitors Differentially Regulate Cell Fate and Microenvironment in Human Hepatocellular Carcinoma

Christian Breunig1,2,5, Bernadett J. Mueller2,5, Ludmila Umansky3, Kristin Wahl6, Katrin Hoffmann4, Frank Lehner7, Michael P. Manns6, Heike Bantel6, and Christine S. Falk2,5

Abstract

Purpose: Small molecule inhibitors of the mitogen-activated protein kinase (MAPK) pathway, such as sorafenib, represent novel treatment options for advanced hepatocellular carcinoma. The aim of our study was to identify downstream targets as biomarker candidates that are directly linked to the oncogenic MAPK pathway in hepatocellular carcinoma and correlate with inhibition of this pathway by multikinase inhibitors.

Experimental Design: Hepatocellular carcinoma cell lines and fresh tumor and tumor-free liver tissues from patients with hepatocellular carcinoma were incubated with different BRaf or MEK inhibitors and analyzed for kinase phosphorylation, proliferation, induction of apoptosis, and chemokine secretion.

Results: Hepatocellular carcinoma cell lines responded differentially to these inhibitors in a dose-dependent manner, even those targeting the same kinase. Sorafenib inhibited both MEK1 and ERK1/2 phosphorylation at high but increased signaling at low concentrations. Similarly, PLX4720 increased MEK/ERK signaling independently from mutations in BRaf or NRas. MEK inhibitors decreased ERK1/2 phosphorylation in a dose-dependent manner. These signaling characteristics correlated with inhibition of proliferation, induction of apoptosis, and chemokine secretion. Fresh tissues derived from patients diagnosed with primary hepatocellular carcinoma responded to these inhibitors with changes in their microenvironment following the patterns observed in hepatocellular carcinoma cells.

Conclusions: Oncogenic signaling of the MAPK pathway influences hepatocellular carcinoma sensitivity to treatment with BRaf and MEK inhibitors about cell fate independently from mutations in BRaf and NRas. MAPK inhibitors have a strong impact on chemokine secretion as a consequence of interference with oncogenic signaling. Therefore, novel biomarker candidates associated with the hepatocellular carcinoma microenvironment may be developed for prediction and monitoring of treatment response to small molecule inhibitors.

Introduction

Hepatocellular carcinoma represents a prevalent tumor disease with various etiologies and predominant resistance to systemic chemotherapy, which leads to limited treat-
MAPK Inhibition, Signaling, and Microenvironment in Hepatocellular Carcinoma

**Translational Relevance**

Treatment of patients with advanced hepatocellular carcinoma with sorafenib or other kinase inhibitors is associated with poor clinical response. Here, we demonstrate that low-dose sorafenib treatment of hepatocellular carcinoma cells increased instead of decreased signaling. Inhibition of the mitogen-activated protein kinase pathway was only achieved by high drug concentrations. This dose-dependent regulation of signaling correlated with proliferation, viability, apoptosis induction, and secretion of chemokines and growth factors. These mechanisms are likely to be involved in the variable clinical response to kinase inhibitors. Therefore, we propose that chemokines, CXCL8 in particular, and growth factors represent biomarker candidates for sorafenib treatment of patients with hepatocellular carcinoma because their plasma levels may correlate with hepatocellular carcinoma tissue response and allow an early discrimination between responders and nonresponders. Therefore, we suggest that novel candidates for biomarkers of individual treatment responses can be further developed on the basis of our investigations.

Alterations in the composition of cytokines, chemokines, and growth factors in hepatocellular carcinoma tissue and/or blood following treatment with small molecule inhibitors may correlate with clinical responses (8–13). In hepatocellular carcinoma, the MAPK pathway downstream of the hepatocyte growth factor (HGF) receptor c-Met is often constitutively activated even in the absence of mutations suggesting a pivotal role for this pathway in hepatocellular carcinoma development. Protooncogenes such as NRas or BRAf can either be mutated or constitutively activated because of overexpression of growth factors or their receptors (14). The constitutively active MAPK signal regulates central cellular processes, including proliferation, differentiation, angiogenesis, and survival (15). A number of small molecules, which are currently under clinical investigation inhibit these pathways at various levels (16). Among MEK inhibitors, AZD6244 (Selumetinib) and PD0325901 showed high potency of MEK1/2 inhibition and good efficacy in *in vitro* and *in vivo* models (17). However, AZD6244 was tested in a phase II study for advanced hepatocellular carcinoma but could not achieve clinical benefit (7). For treatment of other tumors like melanoma, the first BRAf*V600E* mutation-specific inhibitor, PLX4032 (Vemurafenib) has been developed (18) that showed initially promising clinical responses. The rapid development of resistance mechanisms limits the clinical benefit to a short overall and progression-free survival (19, 20). In melanoma cells not harboring the BRAf*V600E* but an upstream NRas mutation, PLX4032 treatment resulted in a strong increase in the MAPK pathway because of BRAf and CRAf dimerization by the drug instead of inhibition of the pathway (20, 21). So far, this opposing effect of increased MAPK signaling following PLX4032 treatment has not been demonstrated for hepatocellular carcinoma cells or tissues. Here, to unravel the differences between the drugs in detail, sorafenib was compared with the MEK-inhibitors U0126, AZD6244 and PD0325901, and the BRAf*V600E* mutation-specific inhibitor PLX4720 in hepatocellular carcinoma cell lines about inhibition of MAPK and PI3K signaling pathways, cell proliferation and viability, apoptosis induction, and chemokine/growth factor secretion. To demonstrate that the alterations by MAPK inhibitors with hepatocellular carcinoma cells reflect the situation in hepatocellular carcinoma tissue, the chemokine and growth factor secretion was investigated in hepatocellular carcinoma and nonmalignant liver tissue. We could demonstrate that MAPK inhibitors significantly alter the microenvironment in hepatocellular carcinoma cell lines, which could even be confirmed in *ex vivo*–treated hepatocellular carcinoma tissues. These findings might, therefore, open new strategies for monitoring of treatment response to novel kinase inhibitors in patients with hepatocellular carcinoma by following the changes in tumor microenvironment.

**Materials and Methods**

**Hepatocellular carcinoma cell lines and proliferation assays**

All hepatocellular carcinoma cell lines were obtained from the American Type Culture Collection via the laboratory of M. Müller-Schilling (Department of Gastroenterology and Hepatology, University Hospital Regensburg). HepG2 cells (hepatoblastoma, mutant NRasQ61L) were cultured in Dulbecco’s modified Eagle medium (DMEM), 10 mmol/L HEPES, 100 μg/mL gentamycin, 2 mmol/L L-glutamine, and 10% fetal calf serum (FCS; Invitrogen). Hep3B cells (hepatocellular carcinoma) were cultured in MEM medium, 10 mmol/L HEPES, 100 μg/mL gentamycin, 1× nonessential amino acids, 2 mmol/L L-glutamine, and 10% FCS. HuH-7 cells (hepatocellular carcinoma) were cultured in DMEM, 100 U/mL penicillin/streptomycin and 10% FCS. For proliferation assays, cells were stained 10 minutes with 5 μmol/L 6FUr670 (eBioScience), adjusted to 1 × 10^5 cells and seeded into 6-well plates. After 24 hours, medium was replaced by medium containing inhibitors for additional 96 hours. For cell viability assays, 1 × 10^4 cells were seeded in a 96-well plate overnight and treated with different concentrations of the inhibitors. At the end of 72 hours, incubation 10 μL of WST-1 (Roche) reagent was added to each well at the end of 72-hour incubation period for an hour and absorbance was measured at 440 nm.

**Quantification of apoptosis**

Quantification of apoptosis was performed by measuring DNA fragmentation of propidium iodide–stained nuclei (sub-G, peak). After treatment with inhibitors, hepatocellular carcinoma cells were incubated with 300 μL Nicotelli solution (0.1% sodium citrate, 0.1% Triton X-100, pH 7.4) containing 20 μg/mL propidium iodide (Sigma) and measured by flow cytometry.
Quantification of phosphorylated signaling proteins

Hepatocellular carcinoma cells treated with inhibitor were lysed using cell lysis solution (Bio-Rad). Protein concentrations were determined with the BCA protein assay (Thermo Scientific) according to the manufacturer’s manual and adjusted to 100 μg/mL with pervanadate-containing assay buffer (Bio-Rad). To quantify phospho- and total proteins, 5 μg total protein of each sample were incubated overnight with microbeads coated with capture antibodies. Phospho- or total proteins were quantified by biotinylated secondary antibodies and staining with SA-PE. For each protein, >50 beads were analyzed for PE-fluorescence and calculated as mean fluorescence intensity (MFI) by the Bio-Rad Manager 6.0 program.

Treatment with inhibitors

Hepatocellular carcinoma cells (1 × 10^5 per 6-well plate or 1 × 10^6 per 96-well plate) were treated with 0.2, 1, 5, 6.5, and 10 μmol/L or 7.5 μg/mL sorafenib ( Nexavar Bayer AG), PLX4720, PD0325901, AZD6244, sunitinib, GW5074 (all Selleck Chemicals), U0126 (Promega), or with control solvent dimethyl sulfoxide (DMSO; Sigma-Aldrich).

Ex vivo treatment of liver tissue samples

Hepatocellular carcinoma explants (n = 6) were obtained from patients (45–77 years; mean age 63.2 ± 4.3; 83.3% male) with chronic hepatitis C virus (HCV) infection (n = 2), chronic hepatitis B virus (HBV) infection (n = 1), alcoholic liver disease (n = 1), hemochromatosis (n = 1), or cryptogenic liver cirrhosis (n = 1). Explants of healthy liver tissue (n = 8) of patients were analyzed who underwent partial hepatectomy because of single metastasis of nonhepatic origin (years 38–75; mean age 58.6 ± 5.1 years; 50% male). The study was performed according the Ethics committees of Hannover Medical School and the University of Heidelberg, Germany. Culturing of ex vivo liver of patients with hepatocellular carcinoma was described (22). Hepatocellular carcinoma or healthy liver tissue was cut into 125 mm² pieces under sterile conditions and incubated in 24-well plates with medium control or 7.5 μg/mL sorafenib. Paraffin of tumor and tumor-free liver tissues from patients with hepatocellular carcinoma (n = 2) were treated with or without 6.5 μmol/L sorafenib, 5 μmol/L PLX4720, AZD6244, U0126, or PD0325901 for 8 hours at 37°C. Supernatants were collected and tissue lysates were generated as described. All hepatocellular carcinoma tissues carried wild-type sequences of Braf codon 600, NRAs codon 12 and 13 (data not shown).

Chemokine and growth factor detection in supernatant and lysates

Chemokine and growth factor concentrations in supernatants of hepatocellular carcinoma cells (48-hour treatment) or tissues (8-hour treatment) were quantified by the Luminox-based multiplex technique according to manufacturer’s instructions (Bio-Rad). Standard curves and concentrations were calculated with Bio-Plex Manager 6.0. The detection range of all proteins was between 2 pg/mL and 40 μg/mL.

Statistics

All statistical calculations were performed with the GraphPad Prism 5.01 program. The sample values and replicates, respectively, were applied first to Komolgorow–Smirnov normality test followed by either t test for parametric or Mann–Whitney U tests for nonparametric datasets. Significances of multiplex assays were calculated using two-way ANOVA statistics on the basis of mean and median values, SDs and numbers of replicates between 25 and 100 beads. Indicated P values are defined as *P < 0.05, **P < 0.01, and ***P ≤ 0.001.

Results

MAPK inhibitors have differential effects on MEK1 and ERK1/2 phosphorylation

Efficiency and kinetics of kinase inhibitors were determined by measuring the phosphorylation of kinases, such as MEK1 at Ser217/221 and ERK1/2 at Thr185/202 Tyr187/204 at different drug concentrations. The influence of BRAf inhibitors sorafenib and PLX4720, an analogue of PLX4032 (Vemurafenib), and MEK inhibitors U0126, AZD6244, and PD0325901 on signaling was analyzed in 3 hepatocellular carcinoma lines, HepG2, Hep3B, and Huh-7 (Fig. 1). Substantial differences were detected in p-MEK1 and p-ERK1/2 phosphorylation in the 3 cell lines at 5 μmol/L and higher concentrations (Fig. 1, Supplementary Fig. 1A). However, increased p-MEK and p-ERK1/2 levels were observed at low concentrations of 0.2 and 1 μmol/L in HepG2 and Hep3B but not in Huh-7 cells (Fig. 1A–C). Thus, this sorafenib effect at low doses may be cell specific but independent of mutated Braf or NRas because only HepG2 carries an NRasQ61L mutation. As expected from the absence of Braf mutations in HepG2 and Hep3B cells, PLX4720 treatment significantly increased both MEK1 and ERK1/2 phosphorylation with rising drug concentrations. As reported for melanoma cells (19, 20), binding of PLX4720 to wild-type Braf seems to mediate dimerization with CRaf, which leads to phosphorylation of MEK1 and ERK1/2. Because low-dose treatment with sorafenib also resulted in elevated p-MEK1 and p-ERK1/2 levels in HepG2 and Hep3B cells, this drug-induced induction of MAPK signaling at low doses seems not to be a unique feature of PLX4720. Neither the pan-Raf inhibitor GW5074 nor the receptor tyrosine kinase inhibitor sunitinib were able to suppress MEK/ERK phosphorylation in this low concentration range (Supplementary Fig. S1C). In contrast to Braf inhibitors, MEK inhibitors had dose-dependent suppressive effects on ERK1/2 phosphorylation in all 3 cell lines. Because of interference of ADZ6244 and PD0325901 with the ATP binding site, AZD6244 phosphorylation is a unique feature of PLX4720. Neither the pan-Raf inhibitor GW5074 nor the receptor tyrosine kinase inhibitor sunitinib were able to suppress MEK/ERK phosphorylation in this low concentration range (Supplementary Fig. S1C). In contrast to Braf inhibitors, MEK inhibitors had dose-dependent suppressive effects on ERK1/2 phosphorylation in all 3 cell lines. Because of interference of ADZ6244 and PD0325901 with the ATP binding site, AZD6244 phosphorylation is simultaneously increased significantly while in U0126-treated cells, p-MEK1 levels were only weakly elevated whereby ERK1/2 phosphorylation was dose-dependently inhibited. Although similar MEK1 patterns were observed upon treatment with sunitinib (Supplementary Fig. S1C), this drug was...
unable to suppress p-ERK1/2 levels at this concentration range.

In addition to the phosphorylated proteins, the total amount of MEK1 and ERK1/2 kinases was also quantified in the same cell lysates. Treatment with low concentrations of 0.2 and 1 μmol/L had no substantial effect on the total amount of these proteins (Supplementary Fig. S3A–S3C). However, at higher concentrations of 5 or 6.5 μmol/L, some inhibitors had a strong impact at later time points (Supplementary Fig. S3). Because protein degradation can be a sign of drug toxicity, cell viability, proliferation, and induction of apoptosis were also analyzed at different inhibitor concentrations.

Figure 1. Modulation of MEK1 and ERK1/2 phosphorylation by MAPK inhibitors. Phospho-MEK1 and p-ERK1/2 at 0, 6, 24, 48, and 72 hours were detected in lysates of hepatocellular carcinoma cell lines HepG2 (A), Hep3B (B), and Huh-7 (C) following treatment with DMSO (gray circle) as control solvent, 0.2 (black cube), 1 (black triangle), or 5 μmol/L (black circle) sorafenib, PLX4720, U0126, AZD6244, or PD0325901. Values represent phosphoplex data of mean fluorescence intensities (MFI) of 30 to 60 beads for each kinase at each time point and significance levels using two-way ANOVA statistics are listed in Supplementary Table S1.
concentrations (Fig. 3). To exclude a direct interference of the drugs with caspase activation, cells were treated in the presence of the pan-caspase inhibitor ZVAD-fmk. Addition of ZVAD-fmk did not block MEK1 or ERK1/2 degradation, indicating that this process is independent from caspase activation (data not shown).

**Influence of MAPK inhibitors on c-Jun and Akt pathways**

Because other signaling pathways are linked to the MAPK cascade, the drug effect was tested about phosphorylation of the kinases c-Jun, Akt, and the transcription factor ATF2 (Fig. 2 and Supplementary Fig. S2). In HepG2 cells treated at high concentrations of 5 and 6.5 μmol/L sorafenib for more than 6 hours, phosphorylation of c-Jun\(^{\text{Ser63}}\) and ATF2\(^{\text{Thr71}}\) was strongly increased and weak stabilization was detected at later time points by PLX4720, AZD6244, and PD0325901 (Fig. 2 and Supplementary Fig. S2). Increased p-c-Jun levels were also detected in both Hep3B and Huh-7 cells treated with AZD6244 and PD0325901 and, to a lesser extent, by sorafenib whereas PLX4720 treatment had minor effects on p-c-Jun. In Huh-7 cells, treatment with sorafenib and

![Figure 2. Effects of MAPK inhibitors on c-Jun, Akt, and ATF2 phosphorylation. Kinetics of p-c-Jun (top), p-Akt (middle), and p-ATF2 (bottom) phosphorylation in HepG2 (A) and Hep3B (B) or Huh-7 (C) cell lines. (Continued on the following page.)](image-url)
AZD6244 resulted in dose-dependent enhancement of p-ATF2. Phosphorylation of AktSer473, an important regulatory site for apoptosis and cell proliferation, was also affected by BRAf and MEK inhibitors. AktSer473 phosphorylation was significantly increased by 5 μmol/L sorafenib treatment after 6 hours in all 3 hepatocellular carcinoma cell lines and returned to baseline levels at 24 hours (Fig. 2). Decreased p-Akt levels were detected in PLX4720 treated HepG2 and Hep3B cells whereas only minor changes were observed in Huh-7 cells. In contrast, low-dose treatment (0.2 μmol/L) with AZD6244 or PD0325901 resulted in weakly increased p-Akt levels at different time points. Treatment with GW5074 or sunitinib had minor effects on c-Jun, ATF2, or Akt phosphorylation as well as on the total amount of these proteins (Supplementary Figs. S2 and S3). Because these signaling pathways are important for cell survival, proliferation, and apoptosis, alterations in the phosphorylation status are likely to impinge on cell fate as well as secretion of chemokines and growth factors.

**BRaf and MEK inhibitors display dose-dependent antiproliferative and proapoptotic activities**

The MAPK cascade is one major signaling pathway implicated in hepatocarcinogenesis and a driving force in tumor cell proliferation. Therefore, we investigated whether the differences in signaling had an impact on proliferation, adhesion, and apoptosis. For sorafenib treatment, significantly increased apoptosis was detected at concentrations higher than 5 μmol/L in all 3 hepatocellular carcinoma cell lines (Fig. 3A, Supplementary Fig. S4A and S4B). HepG2 and Hep3B cells were also sensitive to treatment with AZD6244 and PD0325901 in a dose-dependent manner, followed by weak effects of U0126. Huh-7 cells were resistant to apoptosis induction by these drugs, except high concentrations of sorafenib. In contrast, PLX4720 treatment that increased ERK1/2 phosphorylation, did not induce apoptosis. These findings indicate that induction of apoptosis is highly dependent on the concentration of the drugs as well as the individual susceptibility of the hepatocellular carcinoma cells.

The differences in sensitivity to apoptosis were also reflected by cell viability and proliferation using CFSE (Fig. 3B) or WST-1 assays (Fig. 3C). Cell proliferation was suppressed in all 3 hepatocellular carcinoma cell lines by high-dose sorafenib treatment. In addition, HepG2 cell proliferation was significantly inhibited by U0126 and PD0325901 in a dose-dependent manner whereas AZD6244 treatment was less suppressive. In addition to sorafenib at high concentrations, Hep3B cell proliferation was suppressed by PD0325901 and by high-dose U0126 treatment. Similar to their apoptosis resistance, proliferation and viability of Huh-7 cells was not inhibited by AZD6244 and U0126 whereas PD0325901 had some suppressive effect. None of the cells responded with enhanced proliferation to PLX4720 treatment despite ERK1/2 hyper-phosphorylation. Quantification of cell viability revealed a mirror image of the apoptosis and proliferation data because the influence of the drugs was either dose dependent, that is treatment of all cells with sorafenib, or had little effect on cell survival like treatment of Huh-7 cells. These findings were
confirmed under real-time conditions by measuring the impedance of proliferation/adhesion (Supplementary Fig. S4C) (23). Immediate alterations in impedance upon sorafenib treatment could be detected in a dose-dependent manner. In contrast, treatment with AZD6244 and PD0325901 resulted in stable or even increased impedance for 24 hours in HepG2 and Hep3B cells followed by a constant decrease. In Huh-7 cells, even enhanced impedance was measured upon AZD6244 and PD0325901 treatment. Enhanced MEK1 and ERK1/2 phosphorylation in PLX4720-treated cells did not lead to increased cell division, proliferation, or impedance, which supports the interpretation that hepatocellular carcinoma lines have already reached their proliferative maximum. Thus, the inhibitory capacity of the drugs correlated with their negative impact on MAPK signaling suppression.

MAPK inhibitors modulate chemokine and growth factor secretion by hepatocellular carcinoma cells

The release of chemokines and growth factors such as CXCL8 [interleukin-8 (IL-8)], VEGF, and others can lead to “remodeling” of the tumor microenvironment, which then favors a pro-angiogenic “tumor escape” situation (9, 24). Because many of these factors are downstream targets of the MAPK pathway, we hypothesized that inhibition of this pathway may exert an influence on tumor microenvironment. The hepatocellular carcinoma lines showed baseline secretion of chemokines, IL-1 receptor antagonist (IL-1RA) and growth factors with individual variation (Fig. 4A and B). Sorafenib treatment of Hep3B, HepG2, and Huh-7 cells reduced secretion of CXCL8, CXCL10, and VEGF dependent on drug concentration and, thus followed the pattern observed for signaling, proliferation, and apoptosis. Enhanced secretion of CXCL8 and IL-1RA was observed in PLX4720-treated HepG2 cells, which also coincided with activation of the MEK/ERK signaling shown in Fig. 1A. In Hep3B cells, this ERK1/2 phosphorylation was insufficient for induction of chemokines and growth factors by PLX4720 that, instead, decreased CXCL8 secretion. Among the MEK inhibitors, AZD6244 and PD0325901 had the strongest suppressive effect on CXCL8, IL-1RA, and VEGF secretion in HepG2 cells, followed by U0126 treatment. In Hep3B and Huh-7 cells, suppression of CXCL8 and VEGF secretion was achieved at high concentrations of MEK.
inhibitors, whereas a minor influence of the drugs was observed on IL-1RA and SCGF secretion (Fig. 4B). These differences in response to MEK inhibitors were even more pronounced about the chemokines CXCL12 and CCL5 (data not shown) and the macrophage migration inhibitory factor (MIF) that was strongly enhanced in MEK inhibitor-treated hepatocellular carcinoma cells, HepG2 and Hep3B (Fig. 4B). The hepatocyte growth factor (HGF) was not expressed by hepatocellular carcinoma lines (data not shown), which excludes an autocrine growth factor loop by HGF binding to its receptor c-Met. These observations indicate that interference with the MAPK signaling selectively targets specific chemokines and growth factors that are addicted to this pathway.

MAPK inhibitors modulate chemokine and growth factor secretion in hepatocellular carcinoma but not in healthy liver tissue

In liver tissue, cytokines, chemokines, and growth factors are secreted by different cell types and the composition varies between healthy, cirrhotic, and tumor tissue (24–26). Because chemokine and growth factor secretion by hepatocellular carcinoma cells was modulated by MAPK inhibitors in vitro, we analyzed the effects of sorafenib treatment on the microenvironment on ex vivo liver tissue in a standardized fashion (22, 27). Fresh hepatocellular carcinoma liver tissue (n = 6) was obtained from patients with hepatocellular carcinoma and healthy liver tissue (n = 38) from patients with liver metastases of nonhepatic origin. Molecular analyses of mutations in codon 600 of B-raf, codons 12 and 13 of NRas revealed that all hepatocellular carcinoma samples carried wild-type sequences of these kinases (data not shown). Defined tissue pieces were treated with inhibitors as described (22, 27). Treatment with sorafenib reduced secretion of CXCL8, CXCL10, CCL5, and IL-1RA in both supernatant and tissue of most patients (Figs. 5 and 6). The strongest reduction was observed with CXCL8 in 5 patients, indicating that in these patients, CXCL8 expression was addicted to the MAPK pathway. Because secretion of CXCL8 was not modulated, a general suppressive effect on protein expression can be excluded (Fig. 5A). A more variable response to sorafenib was observed for VEGF, HGF, and SCGF secretion ranging from reduction to an increase in hepatocellular carcinoma tissue of one patient. No influence of sorafenib was seen for M-CSF or MIF expression. In comparison to hepatocellular carcinoma tissue, the microenvironment of healthy liver tissue was not altered by sorafenib because concentrations of chemokines and growth factors remained unchanged in supernatants of healthy liver (Fig. 5B). This striking difference between hepatocellular carcinoma and tumor-free liver tissue strongly supports our hypothesis that chemokine and growth factor expression in hepatocellular carcinoma tissue is driven by an oncogenic MAPK pathway, independently from B-raf or NRas mutations. From 2 additional patients with hepatocellular carcinoma, sufficient tumor and corresponding nonmalignant liver tissue pairs were available for treatment with all 5 drugs (Fig. 6). With the low baseline secretion of chemokines and growth factors, hepatocellular carcinoma and nonmalignant tissue of patient 7 resembled rather the HepG2 pattern whereas the high concentrations in hepatocellular carcinoma and tumor-free tissue of patient 8 rather followed the Hep3B pattern. Hepatocellular carcinoma tissue of patient 7 showed lower baseline expression of HGF, M-CSF, MIF, and CCL5 compared with tumor-free tissue suggesting that hepatocellular carcinoma tissue may not be characterized in general by higher chemokine and growth factor levels. Treatment with sorafenib, U0126, AZD6244, or PD0325901 did not suppress secretion of these factors. In this hepatocellular carcinoma tissue, only sorafenib treatment showed at least some suppressive effect. A different treatment response was observed with tissues of patient 8 where sorafenib treatment had the strongest suppressive effect on both hepatocellular carcinoma and tumor-free tissue about CXCL8 secretion followed by CXCL10, CXCL12, IL-1RA, VEGF, and M-CSF. The effect of the MEK inhibitors varied between suppression and enhancement of several factors. More importantly, PLX4720 did not induce an enhanced production of CXCL8, CCL5, or IL-1RA as seen in hepatocellular carcinoma cell lines (Fig. 4).

Discussion

In hepatocellular carcinoma, the MAPK pathway is often activated and has been shown to drive tumor progression, although mutations directly affecting BRaf or NRas are rare in hepatocellular carcinoma (3, 15, 28, 29). In contrast to other cancers, no oncogene addition loops have been identified so far for hepatocellular carcinoma (24). Nevertheless, intensive research has been performed on components of the MAPK pathway as therapeutic target (30). The MEK/ERK signaling cascade can be inhibited by the multikinase inhibitor sorafenib, as well as MEK inhibitors such as U0126, AZD6244, and PD0325901. Inhibition is usually demonstrated by a decrease in ERK1/2 phosphorylation also in tissues which can be used as prognostic factor of patients treated with sorafenib (8). In these patients with advanced hepatocellular carcinoma treated with sorafenib, higher staining intensity of p-ERK and p-VEGFR2 was identified as independent prognostic factor for predicting time to progression (TTP) together with Child-Pugh A staging. In another phase II study with sorafenib-treated patients with advanced hepatocellular carcinoma, high p-ERK staining in hepatocellular carcinoma tissue was also associated with increased TTP (31), suggesting that p-ERK levels in tumor tissue may be useful as biomarker for response to sorafenib treatment. Here, we demonstrate that low-dose treatment of hepatocellular carcinoma cells with sorafenib can increase MEK1 and ERK1/2 phosphorylation most likely by a similar mechanism to PLX4720, that is dimerization of B- and CRaf without simultaneous inhibition of kinase activity. This observation implies that in hepatocellular carcinoma patients treated with sorafenib, locally low drug concentrations in the tumor tissue may have opposite effects by sustained or even increased MAPK signaling via B- and CRaf dimerization.
At the level of hepatocellular carcinoma cell lines, MEK inhibitors displayed a high, dose-dependent efficacy in inhibition of pERK1/2 phosphorylation compared with sorafenib, which is in concordance to other reports (17, 32). MEK inhibitors did not block the phosphorylation site of MEK, which resulted in an accumulation of p-MEK1 as demonstrated in previous studies (17, 32). Most importantly, treatment of HepG2 and Hep3B cells with low-dose sorafenib resulted in increased MEK1 and ERK1/2 phosphorylation, whereas inhibition of the MAPK pathway was only achieved at concentrations higher than 5 μmol/L. Hyperphosphorylation was also observed following treatment with PLX4720 in NRas-mutant HepG2 as well as NRas wild-type Hep3B cells, indicating that this mutation is not necessary for this opposing regulation of p-ERK1/2. Biochemical analyses of melanoma cells demonstrated that in a first step, PLX4720 induces B- and CRAF dimerization and, in a second step, inhibits phosphorylation of mutated BRAFV600E. However, in BRAF wild-type, NRas-mutant cells, dimerization results in increased MEK1 and ERK1/2 phosphorylation and, thus, promotes the MAPK pathway, proliferation and metastasis formation (19, 33, 34). Moreover, in vemurafenib-resistant melanoma cells, aberrantly spliced BRAFV600E variants were shown to promote dimerization independently from activated NRAs, indicating that BRAF/CRAF dimerization is the key event in activation of the MAPK pathway (21). In hepatocellular carcinoma cells, the first step of BRAF/CRAF dimerization is obviously mediated by low-dose sorafenib treatment that is insufficient to mediate the second step, that is inhibition of the BRAF/CRAF complex, which finally leads to enhanced downstream signaling by MEK1 and ERK1/2 phosphorylation. Remarkably, low-dose treatment of these 2 hepatocellular carcinoma cells with a pan-Raf inhibitor, GW5074, also resulted with increased p-MEK1 and p-ERK1/2 levels suggesting that this hierarchy of dimerization without inhibition of phosphorylation is not unique to PLX420 and sorafenib. Although PLX4720 treatment of HepG2 and Hep3B cells enhanced MEK1 and ERK1/2 phosphorylation, cell division and viability was not increased. This phenomenon reflects observations in melanoma cells in which PLX4720 treatment did not enhance cell-cycle progression and conferred resistance to apoptosis (34). As expected, neither induction of apoptosis nor decreased cell viability or proliferation was observed in PLX4720-treated hepatocellular carcinoma cells. In contrast to HepG2 and Hep3B cells, Huh-7 cells were generally less sensitive to drug-treatment about signaling, proliferation, and apoptosis induction, which might be because of point mutation of p53 at codon 220 (35). This relative insensitivity to drug treatment indicates that Huh-7 cells and eventually other liver cancer cells are relative independent of the ERK pathway about proliferation and viability.

The comparison of MEK inhibitors revealed different downstream effects on other signaling pathways, for example the INK pathway that regulates transcription factors like c-Jun and ATF2 involved in differentiation, cell migration, and apoptosis (36). High p-c-Jun levels in HepG2 cells were further activated by high-dose sorafenib treatment, which was not observed with PLX4720 and MEK inhibitors. This difference may be important for hepatocarcinogenesis because in a murine model, activated c-Jun has been shown to protect from liver damage and to promote tumorigenesis (37). In several tumor cells, the PI3K/Akt pathway is connected to the MAPK pathway by the various Ras isoforms that are involved in the regulation of cell growth and tumorigenesis (38) and high p-Akt was associated with poor prognosis of patients with hepatocellular carcinoma (39). In addition, several studies have shown that increased p-Akt can lead to resistance to MAPK inhibition (40, 41). In our study, we could also demonstrate a crosstalk between MAPK inhibition and Akt because several drugs conferred phosphorylation at Ser^473, a central regulator of cell survival. The inability of a single BRAF or MEK inhibitor to achieve substantial clinical impact indicates a crosstalk of several oncogenic signaling pathways and, thus, promotes the strategy of inhibitor combinations (42).

Although the systemic effects of these drugs are poorly understood, several biomarkers for treatment response have been identified in the sorafenib SHARP study in which VEGF and angiopoietin-2 (Ang-2), among others, were identified as strong and independent predictors for survival in patients with advanced hepatocellular carcinoma with high VEGF baseline levels as indicator for poor prognosis (3, 12, 43). In addition, HGF and soluble c-Kit, the receptor for SCGF, displayed a tendency towards a predictive response to sorafenib treatment whereby lower baseline HGF and a further decrease under therapy was associated with positive treatment response (12). To our knowledge, these biomarker studies have not yet been linked to the signaling pathways targeted by sorafenib. These biomarkers are part of the tumor microenvironment because tumor and surrounding stromal tissue secrete a variety of chemokines and growth factors, which favor tumor survival, angiogenesis, and may interfere with recruitment of immune cells (44–46). Growth factors such as VEGF and HGF and chemokines such as CXCL8 and CCL5 play an essential role for angiogenesis and tumor cell survival (9). Because the MAPK pathway partially controls expression of these factors in hepatocellular carcinoma (47–49), we anticipated that in vitro treatment of cell lines and ex vivo treatment of hepatocellular carcinoma tissue would suppress secretion of chemokines and growth factors. However, this suppressive effect varied substantially between hepatocellular carcinoma lines. Secretion of CXCL8 was suppressed by sorafenib...
and MEK inhibitors in a strongly dose-dependent manner in all cell lines, whereas suppression of VEGF, CXCL10, IL-1RA, and SCGF secretion varied between cell lines and inhibitors. In parallel, an increase in p-ERK1/2 signaling by PLX4720 resulted only in HepG2 cell in an enhancement of the growth factor and chemokine production, CXCL8, in particular. These *in vitro* data indicate that in hepatocellular carcinoma, the regulation of several chemokines and growth factors and cell proliferation is directly linked to the MAPK pathway independently from BRAf or NRas mutations and, therefore, can be inhibited by sorafenib and MEK inhibitors or, reversely, increased by PLX4720. This regulation argues for CXCL8...
as potential candidate for a functional, though indirect, readout of ERK phosphorylation in hepatoma cells that may be even further developed as a noninvasive biomarker for response to multikinase inhibitors in the blood of patients with hepatocellular carcinoma. This hypothesis is supported by the identification of an ERK1/2-dependent mechanism of CXCL8 expression in airway epithelial cells (50). Inhibition of the p38 and ERK pathways downregulated CXCL10 and CXCL8 expression in epithelial cells (51, 52). Activation of the Akt signaling pathway is involved in CXCL10 and MIF expression in the context of infection or cancer (53, 54). In strong contrast to chemokines, MIF secretion was significantly increased by BRaf as well as MEK inhibitors, which may be also part of a tumor escape mechanism because MIF has been shown to mediate direct antiapoptotic effects as well as tumor protection recognition and elimination by immune cells (55). These studies support our interpretation that chemokine and growth factor secretion is influenced by a complex signaling network where mutations can play an additional regulatory role.

Based on our in vitro experiments, we postulate 2 reaction patterns that may be able to discriminate between patients with hepatocellular carcinoma responders and nonresponders of sorafenib treatment: if expression of CXCL8, CXCL10, and VEGF is linked to the oncogenic MAPK pathway, it should be possible to measure response to sorafenib treatment in patients with hepatocellular carcinoma by decreased plasma levels of these proteins. In contrast, if these factors are not directly linked to the oncogenic MAPK pathway, treatment with sorafenib would...
not be sufficient to decrease their plasma levels, and this reaction pattern is likely to be associated with weak treatment response. Our ex vivo observations are in line with a recent biomarker study where high baseline levels of VEGF, Ang-2, and HGF correlated as independent predictors with poor survival in patients with advanced hepatocellular carcinoma (12). Although slight changes in plasma levels of HGF and soluble c-kit were observed following sorafenib treatment, none of the biomarker candidates reached statistical significance for prediction of treatment response. This variation in the individual response may be related to the hepatocellular carcinoma microenvironment, which, in turn, is likely to be influenced by the signaling and mutational network. Taken together, our observations suggest that the modulation of the tumor microenvironment mirrored by plasma levels of chemokines and growth factors may represent a feasible strategy to monitor treatment responses of patients with advanced hepatocellular carcinoma with kinase inhibitors in the future.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: C. Breunig, B.J. Mueller, C.S. Falk
Development of methodology: H. Bantel, C.S. Falk

References

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C. Breunig, L. Ulmansky, K. Hoffmann, F. Lehner, H. Bantel, C.S. Falk
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): C. Breunig, B.J. Mueller, K. Hoffmann, M.P. Manns, H. Bantel, C.S. Falk
Writing, review, and/or revision of the manuscript: C. Breunig, B.J. Mueller, K. Hoffmann, F. Lehner, M.P. Manns, H. Bantel, C.S. Falk
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): C. Breunig, L. Ulmansky, K. Wahl, H. Hoffmann, H. Bantel, C.S. Falk
Study supervision: C.S. Falk

Acknowledgments
The authors thank J. Hoheisel (DKFZ) for critical reading of the article, J. Lehmann-Mühlenhoff (Institute of Pathology at MHH) for the mutation analyses for BRAf and NRas, J. Keil, M. Stevanovic-Meyer (Transplant Immunology), T. Lerch (NCT), and M. Kühbeck (DKFZ) for excellent technical assistance.

Grant Support
This study was supported by the Helmholtz Alliance ‘Immunotherapy of Cancer’ (C.S. Falk), the Core Facility Diagnostic Centre of the IFB-Tx, ref. nr. 01E01302 and grants of the DFG/EBM projects A3 (C.S. Falk), C1 (H. Bantel, F. Lehner), B6 (M.P. Manns). C. Breunig was supported by the DKFZ International PhD Program.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received June 16, 2013; revised January 14, 2014; accepted February 2, 2014; published OnlineFirst February 26, 2014.
40. Balmanno K, Chell SD, Gillings AS, Hayat S, Cook SJ. Intrinsic resistance to the MEK1/2 inhibitor AZD6244 (ARRY-142886) is associated with weak ERK1/2 signalling and/or strong PI3K signalling in colorectal cancer cell lines. Int J Cancer 2009;125:2332–41.