BRaf and MEK inhibitors differentially regulate cell fate and microenvironment in human hepatocellular carcinoma

Running title: MAPK inhibition, signaling and microenvironment in HCC.

Christian Breunig1,2,3*, Bernadett J. Mueller2,3*, Ludmila Umansky4, Kristin Wahl6, Katrin Hoffmann6, Frank Lehner7, Michael P. Manns5, Heike Bantel5, Christine S. Falk2,3#

1 Division of Molecular Genome Analysis B050, German Cancer Research Center (DKFZ), Heidelberg, Germany
2 Institute of Transplant Immunology, OE8889, IFB-Tx, Hannover Medical School, Hannover, Germany
3 Immunomonitoring Unit, National Center for Tumor Diseases (NCT) at the DKFZ, Heidelberg, Germany
4 Department of Translational Immunology, DKFZ and NCT, Heidelberg, Germany
5 Department of Gastroenterology, Hepatology and Endocrinology, Hannover Medical School, Hannover, Germany
6 Department of General, Visceral and Transplantation Surgery, University Hospital, Heidelberg, Germany
7 Department of Abdominal and Transplantation Surgery, Hannover Medical School, Hannover, Germany
* both authors contributed equally to the manuscript.

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Correspondence: #Christine S. Falk, Prof. Dr.
Institute of Transplant Immunology, IFB-Tx, OE8889
Hannover Medical School MHH
Carl-Neuberg Str. 1
30625 Hannover
Germany
Phone +49 511 532 9745
Fax +49 511 532 8090
E-mail: falk.christine@mh-hannover.de
Statement of translational relevance

Treatment of advanced HCC patients with sorafenib or other kinase inhibitors is associated with poor clinical response. Here, we demonstrate that low-dose sorafenib treatment of HCC cells increased instead of decreased signaling. Inhibition of the MAPK 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 HCC patients because their plasma levels may correlate with HCC tissue response and allow an early discrimination between responders and non-responders. Therefore, we suggest that novel candidates for biomarkers of individual treatment responses can be further developed on the basis of our investigations.

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ABSTRACT

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

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

Results: HCC 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 HCC responded to these inhibitors with changes in their microenvironment following the patterns observed in HCC cells.

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

Hepatocellular carcinoma (HCC) represents a prevalent tumor disease with various etiologies and predominant resistance to systemic chemotherapy which leads to limited treatment options and a generally poor prognosis (1). Surgical resection or liver transplantation are treatment options with improved patient survival but due to late diagnosis, only a small proportion of patients has substantial benefit with prolonged survival (2). The small molecule inhibitor sorafenib (Nexavar®) is the only approved treatment option so far for advanced HCC patients conferring some prolongation of overall survival (3). Sorafenib inhibits B- and CRaf kinases and autophosphorylation of receptor tyrosine kinases like VEGFR1-3, FGFR1, PDGFR, c-kit and others which leads to suppression of angiogenesis, proliferation and induction of apoptosis (4, 5). Although targeting of these kinases appears to be a promising strategy for HCC treatment (6, 7), the clinical success of MAPK inhibitors is limited. In immune histology, pERK staining score is a good histological indicator for the benefit of sorafenib treatment in HCC (8).

Since the microenvironment plays an important role for pathogenesis and treatment of HCC, alterations in the composition of cytokines, chemokines and growth factors in HCC tissue and/or blood following treatment with small molecule inhibitors may correlate with clinical responses (8-13). In HCC, the MAPK pathway down-stream 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 HCC development. Protooncogenes like NRas or BRaf can either be mutated or constitutively activated due to 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 HCC but could not achieve clinical benefit (7). For treatment of other tumors like melanoma, the first BRaf
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 due to 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 HCC cells or tissues. Here, in order to unravel the differences between the drugs in detail, sorafenib was compared to the MEK-inhibitors U0126, AZD6244 and PD0325901, and the BRaf^{V600E} mutation-specific inhibitor PLX4720 in HCC cell lines regarding inhibition of MAPK and PI3K signaling pathways, cell proliferation and viability, apoptosis induction and chemokine/growth factor secretion. In order to demonstrate that the alterations by MAPK inhibitors with HCC cells reflect the situation in HCC tissue, the chemokine and growth factor secretion was investigated in HCC and non-malignant liver tissue. We could demonstrate that MAPK inhibitors significantly alter the microenvironment in HCC cell lines which could even be confirmed in ex vivo-treated HCC tissues. These findings might, therefore, open new strategies for monitoring of treatment response to novel kinase inhibitors in HCC patients by following the changes in tumor microenvironment.
MATERIALS AND METHODS

HCC cell lines and proliferation assays

All HCC cell lines were obtained from ATCC via the laboratory of M. Müller-Schilling (Department of Gastroenterology and Hepatology, University Hospital Regensburg). HepG2 cells (hepatoblastoma, mutant NRas<sup>Q61L</sup>) were cultured in DMEM medium, 10mM HEPES, 100µg/ml gentamycin, 2mM L-glutamine and 10% fetal calf serum (Invitrogen, Germany). Hep3B cells (hepatocellular carcinoma) were cultured in MEM medium, 10mM HEPES, 100µg/ml gentamycin, 1x nonessential amino acids, 2mM L-glutamine and 10% FCS. Huh-7 cells (hepatocellular carcinoma) were cultured in DMEM, 100U/ml penicillin/streptomycin and 10% FCS. For proliferation assays, cells were stained 10min with 5µM eFluor670 (eBioScience, USA), adjusted to 1x10<sup>5</sup> cells and seeded into 6-well plates. After 24h, medium was replaced by medium containing inhibitors for additional 96h. For cell viability assays, 1x10<sup>4</sup> cells were seeded in a 96-well plate overnight and treated with different concentrations of the inhibitors. At the end of 72h, incubation 10µl of WST-1 (Roche, Germany) reagent was added to each well at the end of 72h incubation period for an hour and absorbance was measured at 440nm.

Quantification of apoptosis

Quantification of apoptosis was performed by measuring DNA fragmentation of propidium iodide-stained nuclei (subG1 peak). After treatment with inhibitors, HCC cells were incubated with 300µl Nicoletti solution (0.1% sodium citrate, 0.1% Triton X-100, pH 7.4) containing 20µg/ml propidium iodide (Sigma, Germany) and measured by flow cytometry.

Quantification of phosphorylated signaling proteins

HCC cells treated with inhibitor were lysed using cell lysis solution (Bio-Rad, Hercules, USA). Protein concentrations were determined with the BCA protein assay (Thermo Scientific, Waltham, USA) according to the manufacturer's manual and adjusted to 100µg/ml with pervanadate-containing assay buffer (Bio-Rad, USA). To quantify phospho- and total...
proteins, 5µg 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**

HCC cells (1x10^5 per 6-well plate or 1x10^4 per 96-well plate) were treated with 0.2, 1, 5, 6.5, 10µM or 7.5µg/ml sorafenib (Nexavar® Bayer AG,Germany), PLX4720, PD0325901, AZD6244, sunitinib, GW5074 (all Selleck Chemicals,USA), U0126 (Promeg,USA), or with control solvent DMSO (Sigma-Aldrich,USA).

**Ex vivo treatment of liver tissue samples**

HCC explants (n=6) were obtained from patients (45-77 years; mean age 63.2±4.3; 83.3% male) with chronic HCV infection (n=2), chronic 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 HCC patients was described (22). HCC or healthy liver tissue was cut into 125mm^3 pieces under sterile conditions and incubated in 24-well plates with medium control or 7.5µg/ml sorafenib. Pairs of tumor and tumor-free liver tissues from HCC patients (n=2) were treated with or without 6.5µM sorafenib, 5µM PLX4720, AZD6244, U0126 or PD0325901 for 8 h at 37°C. Supernatants were collected and tissue lysates were generated as described. All HCC 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 HCC cells (48h treatment) or tissues (8h treatment) were quantified by the Luminex-based multiplex technique according to manufacturer's instructions (Bio-Rad, USA). Standard curves and concentrations were calculated with Bio-Plex Manager 6.0. The detection range of all proteins was between 2pg/ml and 40µg/ml.

Statistics

All statistical calculations were performed with the GraphPad Prism 5.01 program (La Jolla, USA). 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 non-parametric data sets. Significances of multiplex assays were calculated using 2-way ANOVA statistics on the basis of mean and median values, standard deviations and numbers of replicates between 25 and 100 beads. Indicated p-values are defined as *p<0.05, **p<0.01, ***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 Ser\(^{217/221}\) and ERK1/2 at Thr\(^{185/202}\)Tyr\(^{187/204}\) 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 three HCC lines, HepG2, Hep3B and Huh-7 (figure 1). Substantial differences were detected in p-MEK1 and p-ERK1/2 and in the individual kinetics of cells and inhibitors depending on the concentration (figure 1, online suppl. figure 1A). Sorafenib only inhibited MEK1 and ERK1/2 phosphorylation in the three cell lines at 5µM and higher concentrations (figure 1A, 1B and online suppl. figure 1A). However, increased p-MEK and p-ERK1/2 levels were observed at low concentrations of 0.2 and 1µM in HepG2 and Hep3B but not in Huh-7 cells (figure 1A-C). Thus, this sorafenib effect at low doses may be cell-specific but independent of mutated BRaf or NRas since only HepG2 carries a NRas\(^{Q61L}\) 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 wildtype BRaf seems to mediate dimerization with CRaf which leads to phosphorylation of MEK1 and ERK1/2. Since 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 (online suppl. figure 1C). In contrast to BRaf inhibitors, MEK inhibitors had dose-dependent suppressive effects on ERK1/2 phosphorylation in all three cell lines. Due to interference of ADZ6244 and PD0325901 with the ATP binding site, MEK1 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 (online suppl. figure 1C), since 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 1µM had no substantial effect on the total amount of these proteins (online suppl. figure 3A-C). However, at higher concentrations of 5 or 6.5µM, some inhibitors had a strong impact at later time points (online suppl. figure 3). Since protein degradation can be a sign of drug toxicity, cell viability, proliferation and induction of apoptosis were also analyzed at different inhibitor concentrations (figure 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**

Since other signaling pathways are linked to the MAPK cascade, the drug effect was tested regarding phosphorylation of the kinases c-Jun, Akt and the transcription factor ATF2 (figure 2, online suppl. figure 2). In HepG2 cells treated at high concentrations of 5 and 6.5µM sorafenib for more than 6h, phosphorylation of c-JunSer63 and ATF2Thr71 was strongly increased and weak stabilization was detected at later time points by PLX4720, AZD6244 and PD0325901 (figure 2, online suppl. figure 2). 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 while PLX4720 treatment had minor effects on p-c-Jun. In Huh-7 cells, treatment with sorafenib and 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 5µM sorafenib treatment after 6h in all three HCC cell lines and returned to baseline levels at 24h (figure 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 (0.2µM) 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 (online suppl. figure 2, 3). Since 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 anti-proliferative and pro-apoptotic 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 5µM in all three HCC cell lines (figure 3A, online suppl. figure 4A, B). 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 HCC cells.

The differences in sensitivity to apoptosis were also reflected by cell viability and proliferation using CFSE (figure 3B) or WST-1 assays (figure 3C). Cell proliferation was suppressed in all three HCC cell lines by high-dose sorafenib treatment. In addition, HepG2 cell proliferation was significantly inhibited by U0126 and PD0325901 in a dose-dependent manner while 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 while 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 since the influence of the drugs was either dose-dependent, i.e. 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 (online suppl. figure 4C). 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 24h 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 HCC 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 HCC cells

The release of chemokines and growth factors like CXCL8 (IL-8), VEGF and others can lead to “remodeling” of the tumor microenvironment which then favors a pro-angiogenic “tumor escape” situation (9, 23). Since 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 HCC lines showed baseline secretion of chemokines, IL-1 receptor antagonist (IL-1RA) and growth factors with individual variation (figure 4A, 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 figure 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 (figure 4B). These differences in response to MEK inhibitors were even more pronounced regarding the chemokines CXCL12 and CCL5 (data not shown) and the macrophage migration inhibitory factor (MIF) that was strongly enhanced in MEK inhibitor-treated HCC cells, HepG2 and Hep3B (figure 4B). The hepatocyte growth factor HGF was not expressed by HCC 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 HCC 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 (23-25). Since chemokine and growth factor secretion by HCC 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, 26). Fresh HCC liver tissue (n=6) was obtained from patients with HCC and healthy liver tissue (n=8) from patients with liver metastases of nonhepatic origin. Molecular analyses of mutations in codon 600 of BRaf, codons 12 and 13 of NRas revealed that all HCC samples carried wild-type sequences of these kinases (data not shown). Defined tissue pieces were treated with inhibitors as described (22, 26). Treatment with sorafenib reduced secretion of CXCL8, CXCL10, CCL5 and IL-1RA in both supernatant and tissue of most patients (figures 5, 6). The strongest reduction was observed with CXCL8 in 5 patients indicating that in these patients, CXCL8 expression was addicted to the MAPK pathway. Since secretion of CXCL12 was not modulated, a general suppressive
effect on protein expression can be excluded (figure 5A). A more variable response to sorafenib was observed for VEGF, HGF and SCGF secretion ranging from reduction to an increase in HCC tissue of one patient. No influence of sorafenib was seen for M-CSF or MIF expression. In comparison to HCC tissue, the microenvironment of healthy liver tissue was not altered by sorafenib since concentrations of chemokines and growth factors remained unchanged in supernatants of healthy liver (figure 5B). This striking difference between HCC and tumor-free liver tissue strongly supports our hypothesis that chemokine and growth factor expression in HCC tissue is driven by an oncogenic MAPK pathway, independently from BRaf or NRas mutations.

From two additional HCC patients, sufficient tumor and corresponding non-malignant liver tissue pairs were available for treatment with all five drugs (figure 6). With the low baseline secretion of chemokines and growth factors, HCC and non-tumor tissue of patient 7 resembled rather the HepG2 pattern while the high concentrations in HCC and tumor-free tissue of patient 8 rather followed the Hep3B pattern. HCC tissue of patient 7 showed lower baseline expression of HGF, M-CSF, MIF and CCL5 compared to tumor-free tissue suggesting that HCC 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 HCC 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 HCC and tumor-free tissue regarding 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 HCC cell lines (figure 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 HCC (3, 15, 27, 28). In contrast to other cancers, no oncogene addition loops have been identified so far for HCC (23). Nevertheless, intensive research has been performed on components of the MAPK pathway as therapeutic target (29). The MEK/ERK signaling cascade can be inhibited by the multi-kinase inhibitor sorafenib, as well as MEK inhibitors like 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 advanced HCC patients 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 advanced HCC patients, high p-ERK staining in HCC tissue was also associated with increased TTP (30) 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 HCC cells with sorafenib can increase MEK1 and ERK1/2 phosphorylation most likely by a similar mechanism to PLX4720, i.e. dimerization of B- and Craf without simultaneous inhibition of kinase activity. This observation implies that in HCC 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 HCC cell lines, MEK inhibitors displayed a high, dose-dependent efficacy in inhibition of pERK1/2 phosphorylation compared to sorafenib which is in concordance to other reports (17, 31). MEK inhibitors did not block the phosphorylation site of MEK which resulted in an accumulation of p-MEK1 as demonstrated in previous studies (17, 31). 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 5µM. 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 B-Raf$^{V600E}$. However, in BRaf wildtype, NRas-mutant cells, dimerization results in increased MEK1 and ERK1/2 phosphorylation and, thus, promotes the MAPK pathway, proliferation and metastasis formation (19, 32, 33). Moreover, in vemurafenib-resistant melanoma cells, aberrantly spliced BRaf$^{V600E}$ 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 HCC cells, the first step of BRaf/CRaf dimerization is obviously mediated by low-dose sorafenib treatment that is insufficient to mediate the second step, i.e. 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 two HCC 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 (33). As expected, neither induction of apoptosis nor decreased cell viability or proliferation was observed in PLX4720 treated HCC cells. In contrast to HepG2 and Hep3B cells, Huh-7 cells were generally less sensitive to drug-treatment regarding signaling, proliferation, and apoptosis induction which might be due to point mutation of p53 at codon 220 (34). This relative insensitivity to drug treatment indicates that Huh7 cells and eventually other liver cancer cells are relative independent of the ERK pathway concerning proliferation and viability.

The comparison of MEK inhibitors revealed different downstream effects on other signaling pathways, for example the JNK pathway that regulates transcription factors like c-Jun and ATF2 involved in differentiation, cell migration and apoptosis (35). 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 since in a murine model, activated c-Jun has been shown to protect from liver damage and to promote tumorigenesis (36). 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 (37) and high p-Akt was associated with poor prognosis of HCC patients (38). In addition, several studies have shown that increased p-Akt can lead to resistance to MAPK inhibition (39, 40). In our study, we could also demonstrate a crosstalk between MAPK inhibition and Akt since several drugs conferred phosphorylation at Ser473, 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 (41).

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 advanced HCC patients with high VEGF baseline levels as indicator for poor prognosis (3, 42, 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 (43). 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 since 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 like VEGF and HGF and chemokines like CXCL8 and CCL5 play an essential role for angiogenesis and tumor cell survival (9). Since the MAPK pathway partially controls expression of these factors in HCC (47-49), we anticipated that in vitro treatment of cell lines and ex vivo treatment of HCC tissue would suppress secretion of chemokines and growth
factors. However, this suppressive effect varied substantially between HCC 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 HCC, 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 an functional, though indirect, readout of ERK phosphorylation in hepatoma cells that may be even further developed as non-invasive biomarker for response to multikinase inhibitors in the blood of HCC patients. 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 down-regulated 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 since MIF has been shown to mediate direct anti-apoptotic 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 two reaction patterns that may be able to discriminate between HCC patient responders and non-responders 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 HCC patients 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 advanced HCC patients (43). 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 HCC 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 HCC with kinase inhibitors in the future.

**Acknowledgements**

We thank Joerg Hoheisel for critically reading of the manuscript, Jürgen Lehmann-Mühlenhoff (Institute of Pathology at MHH) for the mutation analyses for BRaf and NRas, Jana Keil, Maja Stevanovic-Meyer (Transplant Immunology), Tina Lerchl (NCT), and Moritz Küblbeck (DKFZ) for excellent technical assistance.
References


FIGURE LEGENDS

Figure 1. Modulation of MEK1 and ERK1/2 phosphorylation by MAPK inhibitors. Phospho-MEK1 and p-ERK1/2 at 0, 6, 24, 48 and 72h were detected in lysates of HCC 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 5µM (black circle) sorafenib, PLX4720, U0126, AZD6244 or PD0325901. Values represent phosphoplex data of mean fluorescence intensities (MFI) of 30-60 beads for each kinase at each time point and significance levels using 2 way ANOVA statistics are listed in online suppl. table 1.

Figure 2. Effects of MAPK inhibitors on c-Jun, Akt and ATF2 phosphorylation. Kinetics of p-c-Jun (above), p-Akt (middle) and p-ATF2 (below) phosphorylation in HepG2 (A) and Hep3B (B) or Huh-7 (C) cell lines. MFI values of DMSO (gray circle), 0.2 (black cube), 1 (black triangle) or 5µM (black circle) sorafenib, PLX4720, U0126, AZD6244 or PD0325901 treatment after 6, 24, 48 and 72h are displayed. The data represent phosphoplex data mean fluorescence intensities of 30-60 beads for each kinase and time point.

Figure 3. Apoptosis induction, cell proliferation and viability after MAPK inhibitor treatment. (A) Apoptosis was determined in HepG2, Hep3B and Huh-7 cells after treatment with 0.2 (light gray bar), 1 (dark gray bar) or 5µM (black bar) inhibitor or DMSO (white bar) at 48h by measuring the subG1 peak using propidium iodide (n=3-7 ±SD). (B) Cell division was determined by eFluor670 labeling of HepG2, Hep3B and Huh-7 cells after 96h treatment with the different inhibitor concentrations and detected by flow cytometry (n=3 ±SD). (C) The WST-1 metabolic assay was used to detect the viability/proliferation of the three cell lines after treatment with the respective inhibitors for 72h. Each bar represents the mean values ± SDs from three to six replicates.

Figure 4. Secretion of chemokines and growth factors by HCC cell lines after MAPK inhibition. HepG2 Hep3B and Huh-7 cells were treated in vitro with 0.2 (light gray bar), 1
(dark gray bar) or 5µM (black bar) sorafenib, PLX4720, U0126, AZD6244, PD0325901, or DMSO control (white bar). After 48h incubation, supernatants were analyzed for CXCL8, VEGF, CXCL10 (A) IL-1RA, MIF and SCGF (B) secretion using the multiplex technology. Each bar represents the mean ± SDs from duplicate experiments.

**Figure 5.** Chemokine and growth factor alteration of HCC and tumor-free liver tissue after sorafenib treatment. Fresh HCC tumor tissue (n=6,A) and tumor-free tissue (n=8,B) was treated *in vitro* with medium (M) or 7.5 µg/ml sorafenib (S) for 8h. Supernatants (SN) and 25µg total protein of tissue lysates were analyzed for chemokines CXCL8, CXCL10, CXCL12, CCL5 and IL-1RA or growth factors VEGF, HGF, SCGF, M-CSF and MIF (*conc.>*out of range). Each line indicates an individual patient sample (n=6). HCC samples carried wildtype sequences of BRaf codon 600 and NRas codon 12 and 13.

**Figure 6.** Chemokine and growth factor release of HCC and surrounding non-tumor tissue after MAPK inhibition. Fresh HCC tumor (black) and surrounding tumor-free liver (gray) tissue of two patients (pat.) were treated for 8h *in vitro* with 6.5µM sorafenib, 5µM PLX4720, U0126, AZD6244, PD0325901 or DMSO. Supernatant (SN) was collected and the concentrations (pg/ml) of CXCL8, CXCL10, CXCL12, CCL5, IL-1RA, VEGF, HGF, SCGF, M-CSF and MIF were measured by the multiplex technology.
Figure 1

A HepG2

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C Huh-7

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Figure 2

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MFI vs. time (h)
Figure 3

A

% subG1 peak

HepG2  Hep3B  Huh-7

B

eFluor 670 (MFI)

D  Sora  PLX  U0126  AZD  PD

C

cell viability (WST-1)

D  Sora  PLX  U0126  AZD  PD
Figure 5

B

CXCL8  CXCL10  CXCL12  CCL5  IL-1RA

VEGF  HGF  SCGF  M-CSF  MIF

pg/ml

SN

M  S

1  1000  10000  100000

10  100  1000  10000

pg/ml

SN

M  S

1  1000  10000  100000

10  100  1000  10000

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