UNIVERSITÄTSKLINIKUM HAMBURG-EPPENDORF

Zentrum für Experimentelle Medizin,
Institut für Biochemie und Signaltransduktion

Direktor: Prof. Dr. med. Georg W Mayr

Dual Inhibition of PI3K-AKT-mTOR- and RAF-MEK-ERK signaling is synergistic in cholangiocarcinoma and reverses acquired resistance to MEK-Inhibitors

Dissertation

zur Erlangung des Grades eines Doktors der Medizin
an der Medizinischen Fakultät der Universität Hamburg.

vorgelegt von:

Dominik Nörz
aus Bad Honnef

Hamburg 2015

Angenommen von der
Medizinischen Fakultät der Universität Hamburg am: 25.01.2016

Veröffentlicht mit Genehmigung der
Medizinischen Fakultät der Universität Hamburg.

Prüfungsausschuss, der/die Vorsitzende: PD Dr. M. Jücker

Prüfungsausschuss, zweite/r Gutachter/in: Prof. Dr. L. Fischer

Prüfungsausschuss, dritte/r Gutachter/in:

1 Inhaltsverzeichnis

2 Manuskript

Dual Inhibition of PI3K-AKT-mTOR- and RAF-MEK-ERK-signaling is synergistic in cholangiocarcinoma and reverses acquired resistance to MEK-inhibitors

Florian Ewald[1*], Dominik Nörz[2*], Astrid Grottke[2], Bianca T. Hofmann[2#], Björn Nashan[1] and Manfred Jücker[2]

[1]Department of Hepatobiliary and Transplant Surgery, University Medical Center Hamburg-Eppendorf, [2]Center of Experimental Medicine, Institute of Biochemistry and Signal Transduction, University Medical Center Hamburg-Eppendorf, Martinistr. 52, 20246 Hamburg, Germany.

[#]present address: Department of General, Visceral and Thoracic Surgery, University Medical Center Hamburg-Eppendorf, Hamburg, Germany
[*] F.E. and D.N. contributed equally to this work

Running title: **Combinatorial treatment of cholangiocarcinoma**

Keywords: Cholangiocarcinoma, AKT, mTOR, MEK, combinatorial treatment

Corresponding author: **Prof. Dr. Manfred Jücker**

Center of Experimental Medicine

Institute of Biochemistry and Signal Transduction

University Medical Center Hamburg-Eppendorf

Martinistr.52, 20246 Hamburg, Germany

Phone +49-40-74105-6339

FAX +49-40-74105-2904

juecker@uke.uni-hamburg.de

Conflict of interest statement: **There is no conflict of interest**

Word count: 5282,
1 table, 5 figures, 3 supplementary files

2.1 Abstract

Until today, there is no systemic treatment available for advanced cholangiocarcinoma (CCA). Recent studies have shown a frequent upregulation of the PI3K-AKT-mTOR and RAF-MEK-ERK pathways in this type of cancer. However, considering their high extend of redundancy and cross-talk, targeting only one pathway is likely to result in therapy failure and emergence of resistances. To provide a rationale for treatment of CCA with inhibitors of these respective pathways, we analyzed the effects of AKT inhibitor MK-2206, MEK inhibitor AZD6244 (ARRY-142886) and mTOR kinase inhibitor AZD8055 on three CCA cell lines *in vitro*, concerning proliferation, cell signaling and apoptosis. Furthermore, AZD6244 resistant cell lines have been generated to investigate, how their response may be affected by prolonged treatment with only a single inhibitor. Our data demonstrates that co-targeting of both, the PI3K/AKT/mTOR and RAF-MEK-ERK pathway, as well as vertical targeting of AKT and mTOR results in strong synergistic effects on proliferation and cell survival with combination indices below 0.3. Mechanistically, the combinatorial treatment with MK-2206 in addition to AZD8055 is necessary because AKT kinase activity was quickly restored after mTOR kinase inhibition. Interestingly, acquired MEK inhibitor resistance to AZD6244 was reversed by combined treatment with AZD6244 and either MK-2206 or AZD8055. Our data suggest that a combination of inhibitors targeting those respective pathways may be a viable approach for future application in patients with cholangiocarcinoma.

Implications: AKT, mTOR and MEK are promising targets for a combinatorial treatment of cholangiocarcinoma cells even after acquisition of MEK inhibitor resistance.

2.2 Introduction

Cholangiocarcinoma (CCA) is a relatively rare but devastating disease arising from the epithelium of intra- or extrahepatic bile ducts. Although CCA accounts for only about 3% of all gastrointestinal malignancies, CCA represent the second most frequent primary hepatic cancer, and a constant increase in the incidence of CCA has been observed especially for intrahepatic cholangiocarcinoma [1,2]. Surgical resection of the lesion is the only curative treatment. However, many patients present

at advanced stages and are no longer amenable to surgical treatment, and even after careful resection, local recurrence is frequent [3]. Until today, there is no effective systemic treatment, and prognosis of patients with advanced CCA is poor [4]. Therefore, new treatment strategies are urgently needed.

Previous data shows that activation of the RAF-MEK-ERK signaling pathway occurs in approximately 58% of CCA cases and might play a central role during tumorigenesis [5-7]. Extracellular signal-regulated kinase (ERK) is a downstream kinase of many cell surface receptors, including EGFR [5]. Moreover activating mutations upstream of ERK, i.e. RAS and RAF are frequently observed in CCA [8]**.** ERK itself has a wide range of substrates, promoting cell survival and proliferation [9]. AZD6244 (ARRY-142886) is a selective, allosteric inhibitor of the MEK1/2 kinases and can be used to disrupt downstream signaling to ERK. It is considered to be a promising agent for many different cancer types, and results from phase I and II clinical trials are encouraging [10,11].

Mammalian target of rapamycin (mTOR), a member of the PI3-kinase superfamily, is a downstream target of the PI3K-AKT signaling pathway and acts as an integrator for a variety of stimuli, including mitogens, energy and nutrient levels, and affects translation, proliferation and autophagy [12]. The serine/threonine kinase AKT is a key player in the regulation of survival, migration and apoptosis [13,14]. There is a complex interaction between AKT and mTOR, given that mTORC2 phosphorylates AKT within the carboxy terminus, which is required for full activation of AKT, and AKT again controlling mTOR activity via regulation of the TSC1/2-complex [15-17]. Upregulation and activation of mTOR and AKT appears to be common in CCA and correlates with poor prognosis [7,18,19]. Allosteric inhibitors of mTOR have been in the focus of oncological research for a long time [20]. With an emerging understanding of the importance of mTORC2 signaling in tumorigenesis, compounds like the novel, highly selective, ATP competitive mTOR inhibitor AZD8055, that targets both mTOR complexes, might therefore provide a therapeutic superiority compared to rapalogs that primarily inhibit mTORC1 signaling [21,16].This might be due to inhibition of AKT, which results from disrupting mTORC2 activation. Yet, the work by Rodrik-Outmezguine indicates that even residual AKT activity in the context of mTORC1 and mTORC2 inhibition might have a significant impact on survival and proliferation of cancer cells [22]. To further address the functional role of AKT and mTOR in CCA cell lines, we analyzed the combined effects of AZD8055 with the

allosteric AKT inhibitor MK-2206 that is currently being evaluated in numerous clinical trials [23].

The RAF-MEK-ERK and the PI3K-AKT-mTOR pathways both play an important role in the control of cell proliferation and survival. There is evidence for a diligent cross-regulation between these two pathways and results from previous studies suggest a high level of functional redundancy between them. Therefore, simultaneous inhibition of both pathways appears to be reasonable and has been shown to be effective in NSCLC cell lines, xenograft cancer models and genetically engineered mouse models of cancers [24,25]. In this study, we demonstrate that combined targeting of AKT, mTOR and MEK/ERK signaling using MK-2206, AZD6244 and AZD8055 is highly efficacious and synergistic in the inhibition of CCA cell proliferation. Furthermore, we demonstrate that combined targeting of these signaling pathways reverses the acquired resistance of CCA cells against the MEK-inhibitor AZD6244. Our results suggest that dual targeting of AKT and mTOR as well as AKT and MEK might be a promising therapeutic approach in the treatment of cholangiocarcinoma.

2.3 Material and Methods

Chemicals and reagents

AZD8055 and AZD6244 were provided by AstraZeneca (London, United Kingdom). MK-2206 was obtained from AbMole BioScience (Kowloon, Hongkong). Stock solutions with a concentration of 10 mM were prepared and stored at -80 °C. Antibodies against pan AKT, AKT1, AKT2, pAKT (S473), pAKT (T308), mTOR, pmTOR (S2448), pmTOR (S2481), pERK (T202/204), ERK, pMEK (S217/221), MEK 1/2, pGSK3-beta (S9), Cyclin D3, pS6 (S240/244) and IRS-1 were purchased from Cell Signaling Technology (Danvers, MA). Antibodies against AKT2, p27 and HSC-70 were purchased from Santa Cruz. Propidium Iodide (PI) was obtained from Sigma (Taufkirchen, Germany).

Cell culture

EGI-1 [26] and TFK-1 [27] cells were obtained from DSMZ cell bank, Germany. SK-ChA-1 [28] cells were a kind gift from Dr. Knuth from the University Hospital Zürich, Department of Oncology. The cell lines have been authenticated by the DSMZ and Dr. Knuth, respectively, and used for fewer than 6 months after resuscitation. All cell

lines were maintained in RPMI, supplemented with 10% (v/v) FCS, and 1% (v/v) penicillin and streptomycin. Cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2. All cell lines were used at low passage number not exceeding 30 passages.

To generate resistant cell lines, TFK-1 and EGI-1 cells were cultured in standard medium with AZD6244 added to a final concentration of 5µM. Cells were maintained under these conditions for fewer than 6 months, until cells grew apparently normal, before experiments were carried out.

Western blot analysis

Western blot analysis was performed as described previously [29]. Protein expression was quantified using an LAS-3000 Imager from Fuji (Raytest, Straubenhardt, Germany).

Lentiviral knockdown of AKT isoforms

pLKO.1-puro vector encoding AKT1, AKT2 and non-target (scrambled, SCR) shRNA were purchased from Sigma-Aldrich (Taufkirchen, Germany). For double AKT isoform knockdown, puromycin resistance in the AKT2 and the control vector was exchanged for neomycin resistance (kind gift of Prof. Fehse, UKE Hamburg). Generation of pseudotyped lentiviruses and transduction were performed as previously described [30,29]. Cells transduced with AKT1 shRNA containing vectors were selected by addition of puromycin (Sigma-Aldrich, Taufkirchen, Germany) to culture medium with a final concentration of 1.5 µg/ml for at least one week, followed by sequential transduction with an AKT2 shRNA containing vector and selection with puromycin (final concentration 1.5 µg/ml) and G418 (final concentration 800µg/ml) containing culture medium. Controls were transduced sequentially with the control shRNA vectors.

Proliferation, apoptosis, colony formation and cell cycle analysis

Proliferation was analyzed either by flow cytometry using the BrdU APC Flow Kit (BD, Pharmingen, CA, USA) or with the colorimetric BrdU ELISA Kit (Roche, Basel, CH) as indicated in the figure legends. For FACS-based assays, cells were seeded into 10 cm dishes and allowed to attach overnight. Then, medium was replaced by medium containing the respective inhibitor or inhibitor combination, or DMSO as

control. Final DMSO concentration in culture medium was 0.1% (v/v) in all experiments. For cell labeling, BrdU was added to a final concentration of 10 µM, and cells were incubated for 12 to 16 h. For cell cycle analysis, cells were fixed in ice cold 70% ethanol for at least 6 h, washed and subsequently incubated with 5 µg PI and 5 µg RNAse A for one hour. Each experiment was performed in triplicates and has been repeated at least one time. Analysis was performed on BD Canto flow cytometer (BD Pharmingen, CA, USA). Cell cycle analysis was performed using FlowJo 7.6.5 software.

For BrdU ELISA assays, cells were seeded into 96-well plates and allowed to attach overnight. Cells were then incubated for 72 h with the respective compounds, and controls were treated with DMSO only. For apoptosis assays, cells were seeded into 96-well plates and grown in culture medium supplemented with 0.1 % FCS (v/v) before incubation with the different compounds for 24h. BrdU ELISA and Cell Death Detection ELISA plus (Roche, Basel, CH) were performed as described by the manufacturer. Each experiment was repeated at least three times in quadruplicates.

Immunoprecipitation and AKT isoform specific *in vitro* kinase assay

Immunoprecipitation of AKT using a pan AKT antibody and subsequent *in vitro* kinase assay was performed as described before [30]. Whole samples were analyzed by western blot technique probed with pGSK3α/ß (S9/21) and pan AKT antibody. Subsequently, nitrocellulose membrane was incubated with secondary goat anti-mouse antibody (Santa Cruz Biotechnology, CA, USA) to detect mouse IgG levels for sample correction.

Statistical Analysis

Student's t-Test (unpaired, 2-tailed) was calculated based on the data of at least three independent experiments. Bonferroni correction for multiple testing was performed where applicable. Results were considered significant if $p<0.05$. All error bars represent SD, unless indicated otherwise. Drug interactions were analyzed based on the median effect method of Chou and Talalay [31]. CalcuSyn software (Biosoft, Cambridge, UK) was used to calculate a Combination Index (CI) for each combination point. CI values from 0.3 to 0.7 are considered to indicate synergism, CI values below 0.3 are considered to represent strong, and values below 0.1 very strong synergism. The CI values were used to draw a plot of CI values over a range

of fractions affected as described [31]. IC_{50} values, i.e. the concentration of a compound that inhibits response by 50% corresponding to the Fraction affected (Fa) of 0.5, were calculated using CurveExpert Professional 1.3 software.

2.4 Results

Combined targeting of AKT and MEK as well as AKT and mTOR is highly synergistic in CCA cell lines.

We first evaluated the activity of PI3K-AKT-mTOR and RAF-MEK-ERK signaling in the three CCA cell lines EGI-1, SK-ChA-1 and TFK-1 by Western blot analysis. Constitutive activation of both signaling pathways can readily be analyzed in all CCA cell lines, as described previously [30] (Online Resource 1). Furthermore, the efficacy of suppressing MEK-ERK- and mTOR-signaling by the allosteric MEK-inhibitor AZD6244 and the ATP-competitive mTOR inhibitor AZD8055, respectively, was confirmed by Western blot analysis (Online Resource 1). We then evaluated the effects of blocking MEK-ERK and mTOR signaling using AZD6244 and AZD8055, respectively, on proliferation of the three CCA cell lines *in vitro*. Therefore, cells were treated with a broad range of concentrations over 72 hours, until proliferation was measured by BrdU incorporation. Proliferation of all CCA cell lines was efficiently inhibited by AZD8055 with IC50 values in lower nanomolar ranges between 47nM (TFK-1) and 53nM (EGI-1). Also, all cell lines analyzed were sensitive to AZD6244, with IC50 values ranging from 0.9µM (SK-ChA-1) to 1.4µM (EGI-1), as seen in Online Resource 2. Next, to test whether inhibition of AKT would further sensitize CCA cells to inhibition of MEK/ERK, we analyzed the effect of combining AKT inhibitor MK-2206 with ADZ6244. Therefore, each cell line was treated with the respective compound alone or the combination of both drugs over a broad range of concentrations (Fig. 1). Combined targeting of AKT and MEK synergistically inhibited proliferation of all CCA cell lines, as indicated by the corresponding CI-values and CI/Fractional Effect blots (Table 1, Online Resource 3). This is in line with results previously observed in non-small cell lung cancer and thyroid cancer cell lines [25,32].

We then analyzed the effect of combined AKT and mTOR inhibition by combining MK-2206 and AZD8055 (Fig. 1). We observed strong synergistic effects, with CI values below 0.3 in all cell lines tested (Tab. 1, Online Resource 3). Although we have shown a strong synergy between AKT- and allosteric mTOR-inhibition using

MK-2206 and RAD001 in CCA before [30], we were surprised by these results because inhibition of mTORC2 by AZD8055 suppresses the phosphorylation of AKT at serine residue 473 (S473), which is crucial for full enzymatic activity of AKT [33]. Next, we speculated that combining AZD6244 with mTOR inhibitor AZD8055 should be similarly synergistic to combining AKT and MEK inhibition. However, weak synergistic effects were observed in the CCA cells lines after combined treatment with AZD6244 and AZD8055 (Table 1, Online Resource 3).

Similar results were observed in cell cycle analysis of CCA cell lines treated with the afore-mentioned compounds and combinations. As seen in Fig. 2a, treating CCA cell lines with a combination of two inhibitors significantly increased the amount of cells in G1-phase compared to each respective compound alone.

In a last step, we analyzed the induction of apoptosis in CCA cell lines after treatment with each inhibitor combination (Fig. 2b). A significant induction of apoptosis was observed in TFK-1 cells for the treatment with AZD6244 in combination with either MK-2206 or AZD8055. However, no significant induction of apoptosis was observed in EGI-1 or SK-ChA-1 cells. Therefore, it is likely that inhibition of proliferation rather than induction of apoptosis are the primary mechanisms of action of the analyzed compounds and their combinations in these cell lines.

Analysis of intracellular signaling of CCA cells after treatment

Next, the effect of MK-2206, AZD6244, AZD8055 and their combinations on the intracellular signaling of CCA cells was analyzed by Western blot. As seen in Fig. 2c, MK-2206, AZD6244 and AZD8055 clearly suppress the phosphorylation of their respective targets or downstream effectors, i.e. AKT, ERK and pS6. Treatment of CCA cells with MK-2206 and AZD8055 resulted in an upregulation of IRS-1 in EGI-1 and TFK-1, which might be part of a compensatory feedback mechanism, as proposed recently by Chandarlapaty et al [29]. Furthermore, inhibition of MEK by AZD6244 resulted in an increase in the phosphorylation of MEK at serine residues 217/221. This probably reflects a release in the feedback inhibition of RAF by ERK in cells treated with AZD6244 [34]. As seen in Fig. 2d, an increase in phosphorylation of MEK can be observed as soon as 30 minutes after incubation of cells with AZD6244, and this effect lasts for at least 48h. Notably, the increase of phosphorylation of MEK is paralleled by a weak recurrence of phosphorylated ERK.

Only inhibition of AKT by MK-2206 led to a decrease in the phosphorylation of GSK3β at serine residue 9 in TFK-1 and SK-ChA-1 cells, although AZD8055 significantly reduced the pAKT (S473) in both cell lines. However, the combination of MK-2206 and AZD8055 resulted in a stronger inhibition of pGSK3β (S9) in all cell lines. Furthermore, two compounds resulted in a stronger expression of the cell cycle inhibitor p27KIP1 compared to each inhibitor alone in CCA cell lines EGI-1 and SK-ChA-1, whereas this effect was not observed in TFK-1 cells.

Residual AKT activity after mTORC2 inhibition is facilitated by increased phosphorylation of AKT at threonine residue 308

To gain further insight into the enzymatic activity of AKT after mTORC1/2 inhibition, we further analyzed the time course of AKT phosphorylation after incubation of TFK-1 cells with AZD8055. As seen in Fig. 3a, addition of AZD8055 almost completely suppresses the phosphorylation of AKT at S473 over the whole time course of 48h. In contrast, phosphorylation of AKT at threonine residue 308 is reduced 3 to 6h after addition of AZD8055, followed by a twofold increase after 24 and 48h (Fig. 3a and 3b). Since kinase activity of AKT is regulated by both, phosphorylation at S473 and T308, these inverse effects make it hard to draw conclusion on the overall activity of AKT. Therefore, AKT kinase activity was analyzed directly in an in vitro kinase assay in TFK-1 cells. As shown in Fig. 3b, AKT kinase activity is reduced to about 5% of the initial activity 3h after the addition of AZD8055, followed by an increase to about 30% after 24h. This increase in the AKT kinase activity is associated with an increased phosphorylation of the AKT downstream substrate GSK3b indicating that the restoration of AKT activity has indeed an effect on signal transduction within the cells. This is in line with results published by Rodrik-Outmezguine et al [22], suggesting that the increased phosphorylation of AKT at T308 is sufficient for a residual enzymatic activity of AKT.

Knockdown of AKT1 and AKT2 in TFK-1 cells is highly synergistic with inhibition of mTOR

To further underline the importance of AKT signaling after mTOR inhibition, we generated TFK-1 AKT1/AKT2 knockdown cells (Fig. 4). Synergistic effects of MK-2206 and AZD8055 were confirmed when AKT1/2 knockdown cells or control cells,

were counted after incubation with AZD8055, MK-2206, or the combination of both for 72h, as indicated in Fig. 4.

Combined targeting of MEK and AKT/mTOR reverses resistance to AZD6244 in TFK-1R and EGI-1R cells

To analyze the effect of long-term exposure to AZD6244, we treated EGI-1 and TFK-1 cells with 5µM of AZD6244 until the cells grew apparently normal in the presence of the inhibitor after about 6 months. As seen in Fig. 5a, both EGI-1 and TFK-1 cells had become resistant to concentrations of AZD6244 as high as 20µM. These cell lines are hereafter called EGI-1R and TFK-1R. No differences in growth rate were observed between resistant and their corresponding parental cell line (Fig. 5a). To test whether the resistance was specific for AZD6244, cells were also treated with allosteric MEK inhibitor PD0325901 and multi-tyrosine kinase inhibitor Sorafenib. As seen in Fig 5b, cells were cross-resistant to PD0325901, but were still sensitive to treatment with Sorafenib. We then treated EGI-1R and TFK-1R cells with AZD6244, AZD8055 or MK-2206 alone or in combination (Fig. 5d). Surprisingly, combining MEK and AKT, or MEK and mTOR inhibition was highly synergistic, and completely reversed the acquired resistance against AZD6244 in these cells. Furthermore, combining MK-2206 and AZD8055 was still highly effective in these cells.

Next, we analyzed MEK/ERK signaling in wild type and resistant cells by Western blot. As seen in Figure 5e, AZD6244 dramatically reduced pERK levels in both wild type and resistant cells, however, a slightly stronger residual pERK signal was found in the resistant cell lines compared to wildtype cells. Interestingly, elevated levels of pMEK were observed in EGI-1R even after long term withdrawal of AZD6244, whereas in TFK-1R, pMEK declined significantly within 12h after AZD6244 withdrawal. After exposure to AZD6244, a slight increase in pAKT was observed in EGI-1R cells not present in wild type cells, however, this effect was again not present in TFK-1R cells.

2.5 Discussion

In this study, we aimed to reveal the effects of co-targeting the PI3K/AKT/mTOR and RAF/MEK/ERK signaling pathways, as well as vertical targeting of the PI3K/AKT/mTOR pathway in cholangiocarcinoma cell lines. Therapeutic approaches

using small molecular inhibitors as monotherapy have shown only limited benefit to patient survival and high rates of cancer resistance in the past years [35]. Our hypothesis therefore is that small molecular inhibitor combinations prevent the acquisition of resistance as seen after using single compounds, and are more likely to show clinical benefits in future clinical trials [36]. This applies in particular to the PI3K/AKT/mTOR and the RAF/MEK/ERK pathways, considering the high relevance for cancer cell proliferation and survival and the extensive cross-talk between these signaling pathways [24]. In addition, we among others have shown that dual targeting of single pathways can be highly effective and synergistic, most likely due to inhibition of feedback mechanisms [37,30].

Combining MK2206 and allosteric MEK inhibitor AZD6244 showed to be highly synergistic in all CCA cell lines analyzed. Similar results were reported previously with NSCLC cell lines using these agents [25], most likely due to increased upregulation of Bim and mitochondrial apoptosis [38]. In addition, this effect appears to be dose dependent since the synergism of these two drugs was considerably weaker at lower concentrations. When combining AZD6244 and AZD8055, weak synergistic effects were observed in the three CCA cell lines. The same agents were used in a recent study by Renshaw et al. on rhabdomyosarcoma cell lines, reporting strong synergistic effects [39] due to suppression of an ERK/mTOR/AKT feedback loop which restores tumor viability as long as only one respective pathway is blocked. However, Ho et al. demonstrated that this combination efficacy was not consistent in different uveal melanoma cell lines and depends on genetic preconditions [40].

Surprisingly, the combination of AKT kinase inhibitor MK2206 and mTOR kinase inhibitor AZD8055 was highly synergistic in all three tested CCA cell lines, even at low concentrations. We have shown that inhibition of mTORC2 activity by AZD8055 initially reduces activity of AKT by tenfold. However, AKT activity is then restored to approximately a third of its original strength within 12 hours, accompanied by a twofold increase of phosphorylation at T308. This was unexpected, since phosphorylation of AKT at S473 is crucial for AKT kinase activity [41]. The effects on the threonine 308 residue are likely caused by the relief of feedback inhibition on RTK signaling, as previously demonstrated [22]. This provides justification to combining AKT and mTOR kinase inhibitors and explains the synergistic effect we observed *in vitro*. In addition, exchanging MK-2206 with a dual knock-down of AKT1

and AKT2 affirmed that this synergistic effect is indeed caused by the inhibitors influence on AKT instead of unspecific interactions.

To analyze the efficacy of combined targeting of MEK and AKT, or MEK and mTOR after prolonged treatment or relapse, a model of acquired resistance against MEK inhibitor AZD6244 was used. Both tested cell lines were able to overcome their sensitivity to AZD6244 and their growth rate remained largely unchanged compared to their progenitors. The resistance is most likely mediated by amplification of RAS or RAF, as shown by Little et al. [42]. Furthermore, increased abundance of BRAFV600E was shown to be sufficient to cause resistance to AZD6244 in colorectal and melanoma cell lines [43].

This is in line with our results, showing a cross resistance against other allosteric MEK inhibitors, but not to Sorafenib. However, none of the resistant cell lines seem to have adapted to AZD6244 treatment by restoring ERK activity. Only EGI-1R shows slightly increased pERK levels, compared to the parental cell line, while under AZD6244 treatment. These results strongly suggest that other signaling pathways functionally replace ERK signaling in the MEK resistant cells.

Interestingly, even though EGI-1R and TFK-1R were completely resistant to AZD6244, combining AZD6244 with MK-2206 or AZD8055 substantially reversed this resistance, resulting in strong synergistic inhibition of proliferation.

Similar findings have been reported by Atefi et al. who showed that in melanoma cell lines primary or acquired resistance to MEK and BRAF inhibitors is associated with high AKT activity and can be reversed by AKT or mTOR inhibition [44]. Turke et al. described a feedback mechanism through HER3 which leads to activation of AKT after MEK inhibitor treatment of EGFR/Her2 driven tumor cells [45]. In addition, a study by Meng and colleagues compared AZD6244 sensitive and intrinsically resistant NSCLC cell lines. Phospho-AKT Levels appeared to be significantly higher in resistant cell lines and inhibition of AKT activity lead to restored susceptibility to AZD6244 [46].

Presumably, high AKT activity could be a prognostic factor for agents affecting the RAF/MEK/ERK pathway. Intrinsic or acquired resistances to RAF/MEK/ERK pathway inhibitors can possibly be overcome or prevented by combined therapy with an AKT inhibitor, if high AKT activity is observed.

In conclusion, we have demonstrated the susceptibility of CCA cell lines to inhibitors of the PI3K/AKT/mTOR and RAF/MEK/ERK pathways. Combining the AKT inhibitor

MK-2206 with either the MEK inhibitor AZD6244 or the mTOR kinase inhibitor AZD8055 has proved to be consistently efficacious in all three tested cell lines. The vertical approach, combining MK-2206 and AZD8055 has shown to have the strongest impact, particularly at low inhibitor concentrations and is apparently superior to pathway co-targeting with MK-2206 and AZD6244 in the CCA cell lines analyzed in our study. Although two of our cell lines have been able to acquire resistances against AZD6244 after prolonged treatment, they were even then still susceptible to combined treatment withAZD6244 and either MK-2206 or AZD8055. Accordingly, inhibitor combinations including a MEK inhibitor can still be viable, even if the respective tumor is not susceptible to MEK inhibitor monotherapy. Our data supports results from other groups that have shown the superior antitumor potential of combined inhibition of both the PI3K/AKT/mTOR and RAF/MEK/ERK signaling pathways. In addition, the strong synergistic effect of AKT and mTOR inhibitors observed at low inhibitor concentrations may be clinically important because treatment protocols with lower doses of inhibitors may reduce the side effects in the patients.

In summary, our study demonstrates that dual targeting with inhibitors against either AKT and MEK or AKT and mTOR have strong synergistic effects on the proliferation and survival of cholangiocarcinoma cells. Therefore, these combinations might be a promising treatment option for CCA patients and their effectiveness should be analyzed in future clinical applications for the treatment of cholangiocarcinoma.

2.6 Abbreviations

mTOR: mammalian target of rapamycin; PI3K: phosphatidylinositol 3-kinase; PI3KCA: catalytic subunit of phosphatidylinositol 3-kinase; IRS-1: insulin receptor substrate 1; BrdU: 5-bromo-2'-deoxyuridine; DMSO: dimethyl sulfoxide; PBS: phosphate buffered saline; CCA: cholangiocarcinoma, CI: combination index.

2.7 Authors' contributions

FE, DN, BN and MJ designed the study. FE, DN, AG and BH performed the experiments and interpreted the experimental findings. FE, DN and MJ drafted the

manuscript and wrote the final version of the manuscript. All authors read and approved the final manuscript.

2.8 Acknowledgements

We are grateful to Dr. Knuth from the University Hospital Zürich, Department of Oncology for providing SK-ChA-1 cells. Florian Ewald is a fellow of the University Cancer Center Hamburg (UCCH).

2.9 Figures

Figure 1

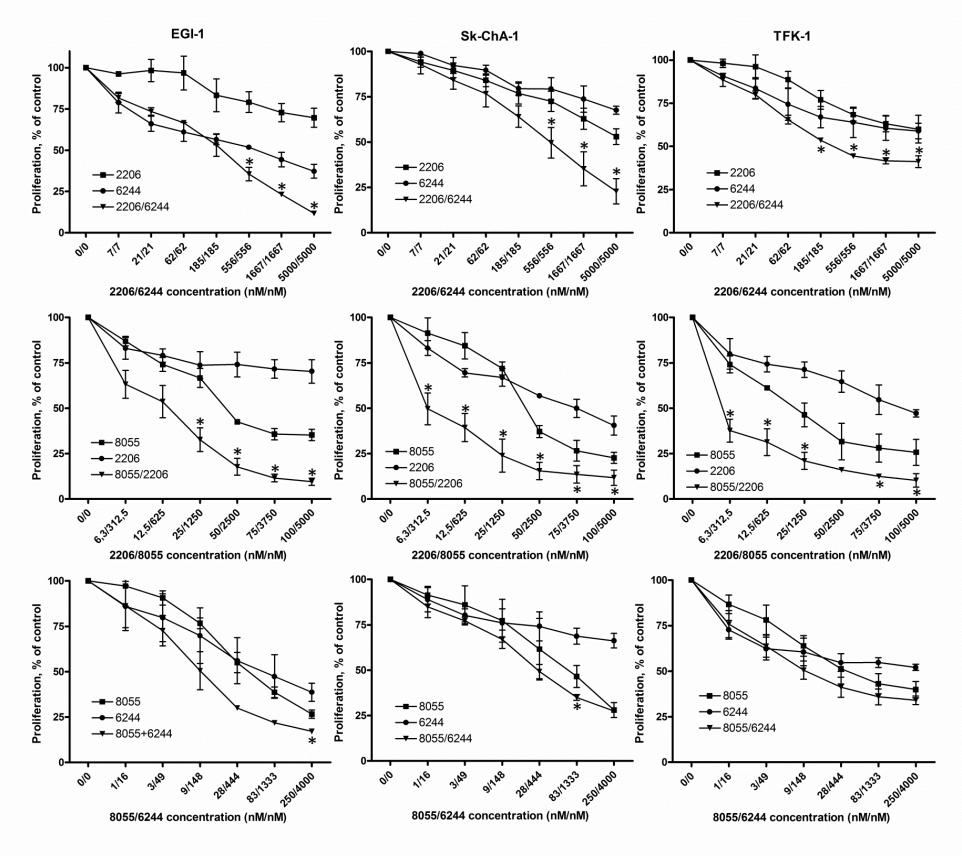


Figure 2

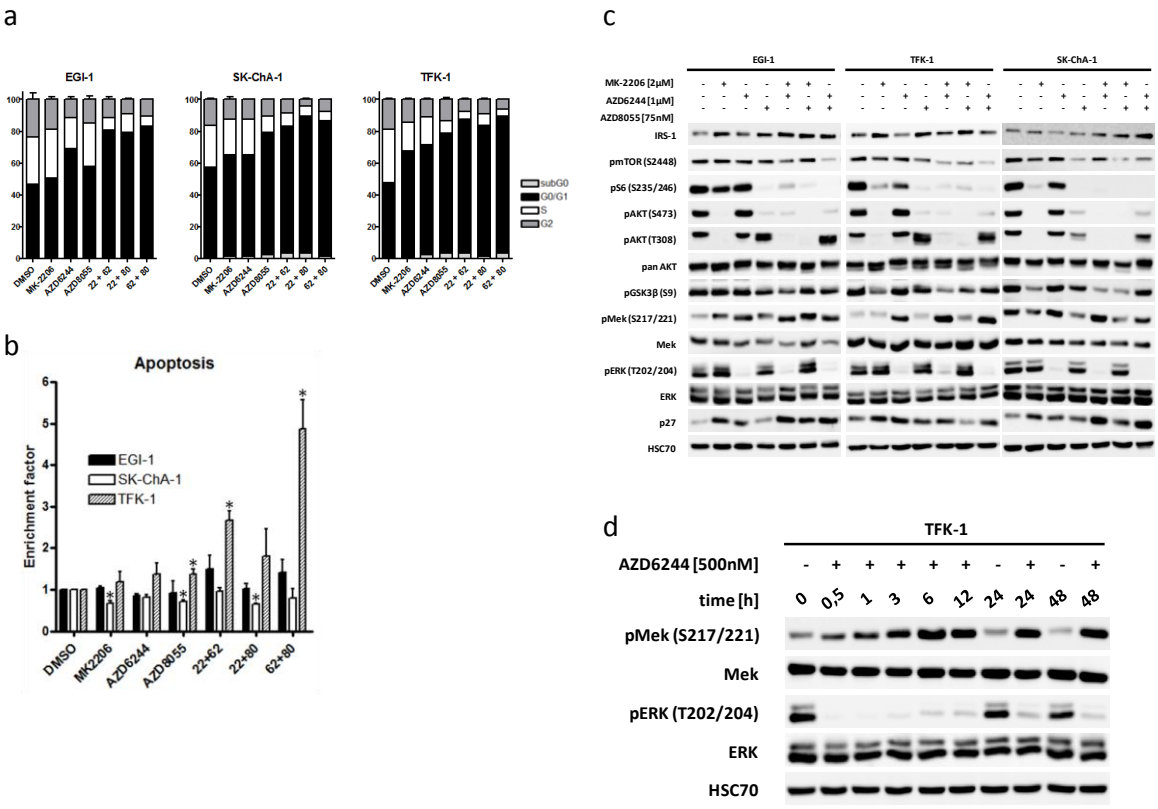


Figure 3

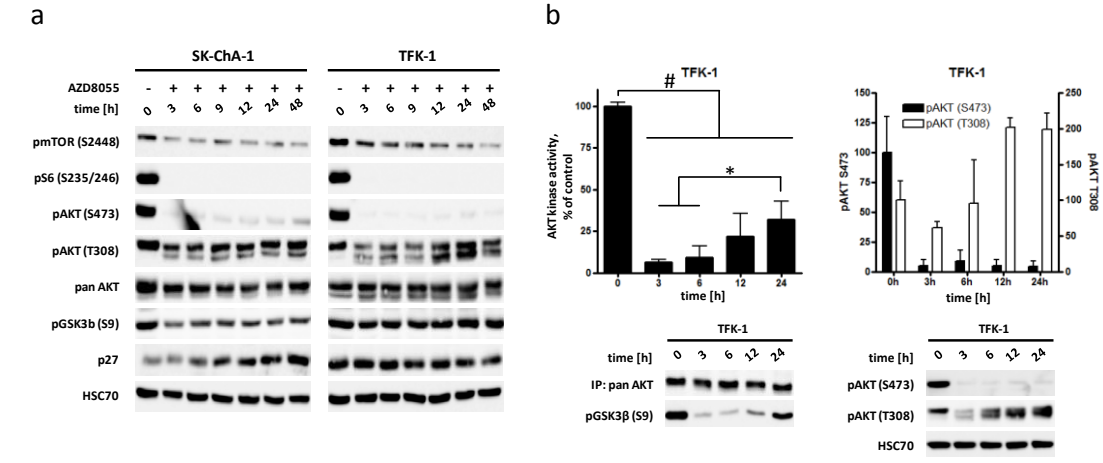


Figure 4

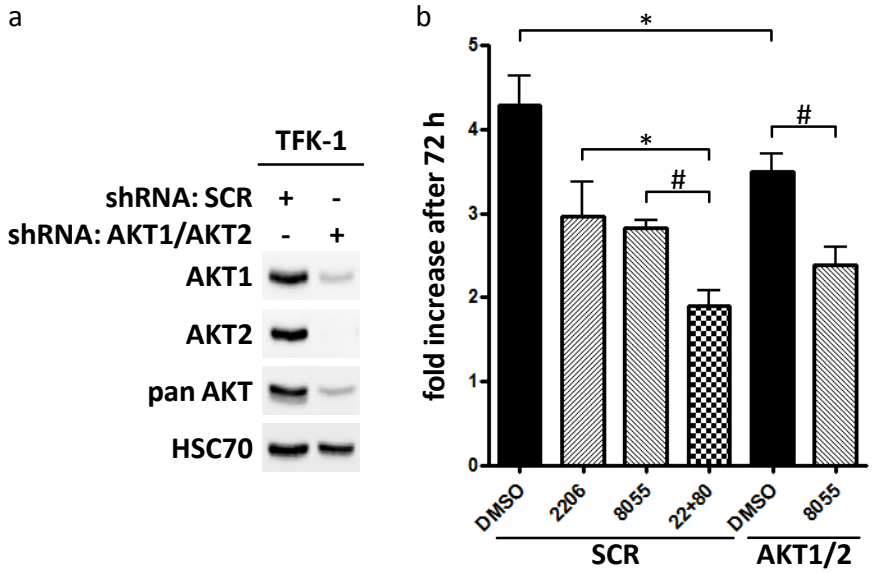
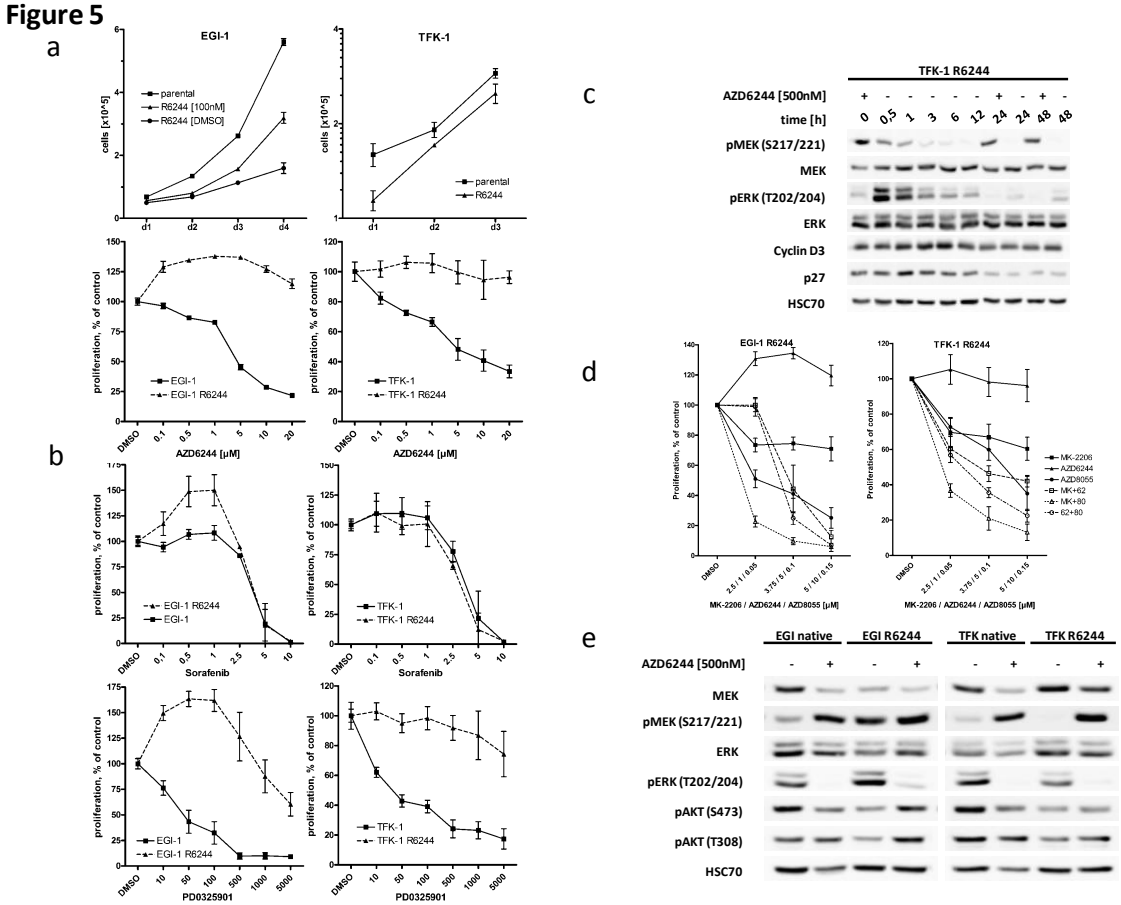


Figure 5



2.10 Tables

Table 1 Dual targeting with inhibitors against AKT (MK-2206), mTOR (AZD8055) and MEK (AZD6244) has synergistic effects on proliferation of cholangiocarcinoma cell lines

Inhibitor	Cell line	Fa 25%	Fa 50%	Fa 75%
MK-2206 + AZD6244	EGI-1	2,872	0,288	0,037
	Sk-ChA-1	0,392	0,201	0,103
	TFK-1	0,314	0,259	0,467
MK-2206 + AZD8055	EGI-1	0.434	0.275	0.236
	Sk-ChA-1	0.319	0.266	0.333
	TFK-1	0.168	0.187	0.238
AZD6244 + AZD8055	EGI-1	0.561	0.383	0.400
	Sk-ChA-1	0.523	0.523	0.703
	TFK-1	4.749	0.396	0.683

Table 1 CCA cell lines were treated with various concentrations of the indicated inhibitors. Shown are the values of the combinatorial indices (CI) measured at different percentages of fractions affected (Fa). CI values from 0.3 to 0.7 are considered to indicate synergism, and CI values below 0.3 are considered to represent strong synergism.

2.11 Figure legend

Fig. 1 Combined treatment of CCA cell lines with increasing concentrations of MK-2206, AZD6244 and AZD8055 synergistically suppresses proliferation

CCA cells were seeded into 96-well plates and incubated with increasing concentrations of inhibitors against AKT (MK-2206), MEK (AZD6244) or mTOR (AZD8055) or a combination of two inhibitors. Controls were treated with DMSO. Proliferation was analyzed after 72h by BrdU incorporation. Each data point represents mean of three independent experiments, normalized to controls; bars, SD. *, $p < 0.05$

Fig. 2 Effects of combined treatment on cell cycle, apoptosis and intracellular signaling

(a) Cell cycle analysis of CCA cell lines after 24h treatment with 2µM MK-2206, 1µM AZD6244, 100nM AZD8055 or a combination of two inhibitors. Control cells were treated with DMSO. Columns: mean of one representative experiment performed in triplicates; bars, SD. (b) CCA cells were seeded into 96-well plates and treated with the afore mentioned compounds and concentrations for 24 hours, before apoptosis was measured by Cell Death Detection ELISA. Controls were treated with DMSO. Combinations of AZD6244 with either MK-2206, or AZD8055 significantly increased apoptosis in two of the three tested CCA cell lines. Each data point represents three independent experiments, performed in triplicates; bars: SD. *, $p < 0.05$. (c) Cells were treated with 2µM MK-2206, 1µM AZD6244, 75nM AZD8055, or a combination of two of these compounds. PI3K-AKT-mTOR and RAF-MEK-ERK signaling pathway activity was analyzed by western blot with antibodies directed against the indicated proteins. HSC70 served as loading control. (d) TFK-1 cells were treated with 500nM AZD6244 for up to 48h and cell lysates were prepared at the indicated time points. Controls were treated with DMSO. RAF-MEK-ERK pathway activity was analyzed by western blot with phospho specific antibodies directed against MEK (pMEK) and ERK (pERK)

Fig. 3 Inhibition of mTOR causes upregulation of phospho-AKT at T308 and increased residual AKT activity in CCA cell lines

(a) Sk-Cha-1 and TFK-1 cells were treated with 75nM AZD8055 for up to 48h and cell lysates were prepared at the indicated time points. PI3K-AKT-mTOR pathway signaling was analyzed by western blot. HSC70 was used as loading control. (b) TFK-1 cells were treated with 75nM AZD8055 for 0, 3, 6, 12 and 24 hours. Phospho-AKT levels were analyzed by western blot. AKT *in vitro* kinase assays were performed after quantitative pan-AKT immunoprecipitation. GSK3α/β fusion protein was used as AKT substrate and phosphorylation at S9/21 detected by western blot. Bars: SD. *, $p < 0.05$; #, $p < 0.01$

Fig. 4 Knock-down of AKT1 and AKT2 is synergistic with mTOR inhibition

(a) Knock-down of AKT1 and AKT2 in TFK-1 cells was analyzed by western blot. HSC70 was used as loading control. (b) TFK-1 SCR and TFK-1 AKT1/2 knock-down cells were incubated with 2µM MK-2206 and 100nM AZD8055 or a combination of both for 72h before cells were counted. Control cells were treated with DMSO.

Experiments were performed in triplicates and data were normalized to respective controls. Bars: SD. *, $p < 0,05$; #, $p < 0,01$

Fig. 5 Combined treatments with inhibitors against MEK and AKT or MEK and mTOR remain synergistic in CCA cells with an acquired MEK inhibitor resistance

(a) EGI-1 and TFK-1 cells were seeded in triplicates in 5cm plates and incubated in normal culture medium. Their AZD6244 resistant derivatives were treated with 5µM AZD6244, or DMSO. Cells were harvested and counted after 24, 48 and 72 hours. Bars: SD. For proliferation analysis, cells were seeded in triplicates into 96-well plates and incubated with increasing concentrations of AZD6244, as indicated. Controls were treated with DMSO. Proliferation was analyzed after 72 hours by BrdU incorporation. Bars: SD. (b) EGI-1 and TFK-1, as well as their AZD6244 resistant derivatives were seeded into 96-well plates and treated with increasing concentrations of Sorafenib, or PD0325901. Controls were treated with DMSO. Proliferation was analyzed after 72h by BrdU incorporation. Each data point represents three independent experiments, each performed in triplicates. Bars: SD. (c) TFK-1R cells were treated with normal culture medium without AZD6244 for up to 48 hours. Cell lysates were prepared at the indicated time points. Controls were treated with 500nM AZD6244. RAF-MEK-ERK pathway activity was analyzed by western blot. HSC70 was used as loading control. (d) EGI-1R and TFK-1R were seeded into 96-well plates and treated with increasing concentrations of MK-2206, AZD6244, AZD8055, or a combination of two inhibitors. Controls were treated with DMSO. Proliferation was analyzed after 72h by BrdU incorporation. Each data point represents mean of three independent experiments. Bars: SD. (e) EGI-1R and TFK-1R cells were treated with 500nM AZD6244 or DMSO. PI3K-AKT-mTOR and RAF-MEK-ERK pathway activity was analyzed by western blot. HSC70 served as loading control

2.12 References

1. Khan SA, Thomas HC, Davidson BR, Taylor-Robinson SD (2005) Cholangiocarcinoma. Lancet 366 (9493):1303-1314. doi:S0140-6736(05)67530-7 [pii] 10.1016/S0140-6736(05)67530-7

2. Welzel TM, McGlynn KA, Hsing AW, O'Brien TR, Pfeiffer RM (2006) Impact of classification of hilar cholangiocarcinomas (Klatskin tumors) on the incidence of intra- and extrahepatic cholangiocarcinoma in the United States. J Natl Cancer Inst 98 (12):873-875. doi:98/12/873 [pii]
10.1093/jnci/djj234
3. Jarnagin WR, Fong Y, DeMatteo RP, Gonen M, Burke EC, Bodniewicz BJ, Youssef BM, Klimstra D, Blumgart LH (2001) Staging, resectability, and outcome in 225 patients with hilar cholangiocarcinoma. Ann Surg 234 (4):507-517; discussion 517-509
4. Jarnagin WR, Shoup M (2004) Surgical management of cholangiocarcinoma. Semin Liver Dis 24 (2):189-199. doi:10.1055/s-2004-828895
5. Yoon JH, Gwak GY, Lee HS, Bronk SF, Werneburg NW, Gores GJ (2004) Enhanced epidermal growth factor receptor activation in human cholangiocarcinoma cells. J Hepatol 41 (5):808-814. doi:S0168-8278(04)00331-9 [pii]
10.1016/j.jhep.2004.07.016
6. Wang C, Maass T, Krupp M, Thieringer F, Strand S, Worns MA, Barreiros AP, Galle PR, Teufel A (2009) A systems biology perspective on cholangiocellular carcinoma development: focus on MAPK-signaling and the extracellular environment. J Hepatol 50 (6):1122-1131. doi:S0168-8278(09)00156-1 [pii]
10.1016/j.jhep.2009.01.024
7. Schmitz KJ, Lang H, Wohlschlaeger J, Sotiropoulos GC, Reis H, Schmid KW, Baba HA (2007) AKT and ERK1/2 signaling in intrahepatic cholangiocarcinoma. World J Gastroenterol 13 (48):6470-6477
8. Hezel AF, Deshpande V, Zhu AX (2010) Genetics of biliary tract cancers and emerging targeted therapies. J Clin Oncol 28 (21):3531-3540. doi:JCO.2009.27.4787 [pii]
10.1200/JCO.2009.27.4787
9. McCubrey JA, Steelman LS, Chappell WH, Abrams SL, Wong EW, Chang F, Lehmann B, Terrian DM, Milella M, Tafuri A, Stivala F, Libra M, Basecke J, Evangelisti C, Martelli AM, Franklin RA (2007) Roles of the Raf/MEK/ERK pathway in cell growth, malignant transformation and drug resistance. Biochim Biophys Acta 1773 (8):1263-1284. doi:S0167-4889(06)00315-6 [pii]
10.1016/j.bbamcr.2006.10.001

10. Davies BR, Logie A, McKay JS, Martin P, Steele S, Jenkins R, Cockerill M, Cartlidge S, Smith PD (2007) AZD6244 (ARRY-142886), a potent inhibitor of mitogen-activated protein kinase/extracellular signal-regulated kinase kinase 1/2 kinases: mechanism of action in vivo, pharmacokinetic/pharmacodynamic relationship, and potential for combination in preclinical models. Mol Cancer Ther 6 (8):2209-2219. doi:6/8/2209 [pii]
10.1158/1535-7163.MCT-07-0231
11. Bekaii-Saab T, Phelps MA, Li X, Saji M, Goff L, Kauh JS, O'Neil BH, Balsom S, Balint C, Liersemann R, Vasko VV, Bloomston M, Marsh W, Doyle LA, Ellison G, Grever M, Ringel MD, Villalona-Calero MA (2011) Multi-institutional phase II study of selumetinib in patients with metastatic biliary cancers. J Clin Oncol 29 (17):2357-2363. doi:JCO.2010.33.9473 [pii]
10.1200/JCO.2010.33.9473
12. Laplante M, Sabatini DM (2012) mTOR signaling in growth control and disease. Cell 149 (2):274-293. doi:S0092-8674(12)00351-0 [pii]
10.1016/j.cell.2012.03.017
13. Engelman JA (2009) Targeting PI3K signalling in cancer: opportunities, challenges and limitations. Nat Rev Cancer 9 (8):550-562. doi:nrc2664 [pii]
10.1038/nrc2664
14. Hirai H, Sootome H, Nakatsuru Y, Miyama K, Taguchi S, Tsujioka K, Ueno Y, Hatch H, Majumder PK, Pan BS, Kotani H (2010) MK-2206, an allosteric Akt inhibitor, enhances antitumor efficacy by standard chemotherapeutic agents or molecular targeted drugs in vitro and in vivo. Mol Cancer Ther 9 (7):1956-1967. doi:1535-7163.MCT-09-1012 [pii]
10.1158/1535-7163.MCT-09-1012
15. Inoki K, Corradetti MN, Guan KL (2005) Dysregulation of the TSC-mTOR pathway in human disease. Nat Genet 37 (1):19-24. doi:ng1494 [pii]
10.1038/ng1494
16. Sparks CA, Guertin DA (2010) Targeting mTOR: prospects for mTOR complex 2 inhibitors in cancer therapy. Oncogene 29 (26):3733-3744. doi:onc2010139 [pii]
10.1038/onc.2010.139
17. Liu P, Gan W, Inuzuka H, Lazorchak AS, Gao D, Arojo O, Liu D, Wan L, Zhai B, Yu Y, Yuan M, Kim BM, Shaik S, Menon S, Gygi SP, Lee TH, Asara JM, Manning BD, Blenis J, Su B, Wei W (2013) Sin1 phosphorylation impairs mTORC2 complex

integrity and inhibits downstream Akt signalling to suppress tumorigenesis. Nat Cell Biol 15 (11):1340-1350. doi:ncb2860 [pii]
10.1038/ncb2860
18. Wang Z, Zheng T, Wu Q, Wang J, Wu C (2012) Immunohistochemical analysis of the mTOR pathway in intrahepatic cholangiocarcinoma. Neoplasma 59 (2):137-141
19. Chung JY, Hong SM, Choi BY, Cho H, Yu E, Hewitt SM (2009) The expression of phospho-AKT, phospho-mTOR, and PTEN in extrahepatic cholangiocarcinoma. Clin Cancer Res 15 (2):660-667. doi:15/2/660 [pii]
10.1158/1078-0432.CCR-08-1084
20. Markman B, Dienstmann R, Tabernero J (2010) Targeting the PI3K/Akt/mTOR pathway--beyond rapalogs. Oncotarget 1 (7):530-543. doi:101012 [pii]
21. Lv X, Ma X, Hu Y (2013) Furthering the design and the discovery of small molecule ATP-competitive mTOR inhibitors as an effective cancer treatment. Expert Opin Drug Discov. doi:10.1517/17460441.2013.800479
22. Rodrik-Outmezguine VS, Chandarlapaty S, Pagano NC, Poulikakos PI, Scaltriti M, Moskatel E, Baselga J, Guichard S, Rosen N (2011) mTOR kinase inhibition causes feedback-dependent biphasic regulation of AKT signaling. Cancer Discov 1 (3):248-259. doi:2159-8290.CD-11-0085 [pii]
10.1158/2159-8290.CD-11-0085
23. Pal SK, Reckamp K, Yu H, Figlin RA (2010) Akt inhibitors in clinical development for the treatment of cancer. Expert Opin Investig Drugs 19 (11):1355-1366. doi:10.1517/13543784.2010.520701
24. Mendoza MC, Er EE, Blenis J (2011) The Ras-ERK and PI3K-mTOR pathways: cross-talk and compensation. Trends Biochem Sci 36 (6):320-328. doi:S0968-0004(11)00050-8 [pii]
10.1016/j.tibs.2011.03.006
25. Meng J, Dai B, Fang B, Bekele BN, Bornmann WG, Sun D, Peng Z, Herbst RS, Papadimitrakopoulou V, Minna JD, Peyton M, Roth JA (2010) Combination treatment with MEK and AKT inhibitors is more effective than each drug alone in human non-small cell lung cancer in vitro and in vivo. PLoS One 5 (11):e14124. doi:10.1371/journal.pone.0014124
26. Scherdin G, Garbrecht M, Klouche M (1987) In vitro interaction of á-difluoromethylornithine (DFMO) and human recombinant interferon-α (rIFN-α) on human cancer cell lines. Immunobiology 175 (1-2):1-143

27. Saijyo S, Kudo T, Suzuki M, Katayose Y, Shinoda M, Muto T, Fukuhara K, Suzuki T, Matsuno S (1995) Establishment of a new extrahepatic bile duct carcinoma cell line, TFK-1. Tohoku J Exp Med 177 (1):61-71
28. Knuth A, Gabbert H, Dippold W, Klein O, Sachsse W, Bitter-Suermann D, Prellwitz W, Meyer zum Buschenfelde KH (1985) Biliary adenocarcinoma. Characterisation of three new human tumor cell lines. J Hepatol 1 (6):579-596
29. Grabinski N, Bartkowiak K, Grupp K, Brandt B, Pantel K, Jucker M Distinct functional roles of Akt isoforms for proliferation, survival, migration and EGF-mediated signalling in lung cancer derived disseminated tumor cells. Cell Signal 23 (12):1952-1960. doi:S0898-6568(11)00200-2 [pii]
10.1016/j.cellsig.2011.07.003
30. Ewald F, Grabinski N, Grottke A, Windhorst S, Norz D, Carstensen L, Staufer K, Hofmann BT, Diehl F, David K, Schumacher U, Nashan B, Jucker M (2013) Combined targeting of AKT and mTOR using MK-2206 and RAD001 is synergistic in the treatment of cholangiocarcinoma. Int J Cancer. doi:10.1002/ijc.28214
31. Chou TC, Talalay P (1984) Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. Adv Enzyme Regul 22:27-55
32. Kandil E, Tsumagari K, Ma J, Abd Elmageed ZY, Li X, Slakey D, Mondal D, Abdel-Mageed AB (2013) Synergistic inhibition of thyroid cancer by suppressing MAPK/PI3K/AKT pathways. J Surg Res 184 (2):898-906. doi:S0022-4804(13)00246-1 [pii]
10.1016/j.jss.2013.03.052
33. Liao Y, Hung MC (2010) Physiological regulation of Akt activity and stability. Am J Transl Res 2 (1):19-42
34. Fritsche-Guenther R, Witzel F, Sieber A, Herr R, Schmidt N, Braun S, Brummer T, Sers C, Bluthgen N (2011) Strong negative feedback from Erk to Raf confers robustness to MAPK signalling. Mol Syst Biol 7:489. doi:msb201127 [pii]
10.1038/msb.2011.27
35. Yamanaka K, Petrulionis M, Lin S, Gao C, Galli U, Richter S, Winkler S, Houben P, Schultze D, Hatano E, Schemmer P (2013) Therapeutic potential and adverse events of everolimus for treatment of hepatocellular carcinoma - systematic review and meta-analysis. Cancer Med 2 (6):862-871. doi:10.1002/cam4.150
36. Engelman JA, Chen L, Tan X, Crosby K, Guimaraes AR, Upadhyay R, Maira M, McNamara K, Perera SA, Song Y, Chirieac LR, Kaur R, Lightbown A, Simendinger J,

Li T, Padera RF, Garcia-Echeverria C, Weissleder R, Mahmood U, Cantley LC, Wong KK (2008) Effective use of PI3K and MEK inhibitors to treat mutant Kras G12D and PIK3CA H1047R murine lung cancers. Nat Med 14 (12):1351-1356. doi:nm.1890 [pii]
10.1038/nm.1890
37. Grabinski N, Ewald F, Hofmann BT, Staufer K, Schumacher U, Nashan B, Jucker M (2012) Combined targeting of AKT and mTOR synergistically inhibits proliferation of hepatocellular carcinoma cells. Mol Cancer 11:85. doi:1476-4598-11-85 [pii]
10.1186/1476-4598-11-85
38. Meng J, Fang B, Liao Y, Chresta CM, Smith PD, Roth JA (2010) Apoptosis induction by MEK inhibition in human lung cancer cells is mediated by Bim. PLoS One 5 (9):e13026. doi:10.1371/journal.pone.0013026
39. Renshaw J, Taylor KR, Bishop R, Valenti M, De Haven Brandon A, Gowan S, Eccles SA, Ruddle R, Johnson LD, Raynaud FI, Selfe J, Thway K, Pietsch T, Pearson AD, Shipley J (2013) Dual blockade of the PI3K/AKT/mTOR (AZD8055) and RAS/MEK/ERK (AZD6244) pathways synergistically inhibits rhabdomyosarcoma cell growth in vitro and in vivo. Clin Cancer Res. doi:1078-0432.CCR-13-0850 [pii]
10.1158/1078-0432.CCR-13-0850
40. Ho AL, Musi E, Ambrosini G, Nair JS, Deraje Vasudeva S, de Stanchina E, Schwartz GK (2012) Impact of combined mTOR and MEK inhibition in uveal melanoma is driven by tumor genotype. PLoS One 7 (7):e40439. doi:10.1371/journal.pone.0040439
PONE-D-12-04817 [pii]
41. Sarbassov DD, Guertin DA, Ali SM, Sabatini DM (2005) Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. Science 307 (5712):1098-1101. doi:307/5712/1098 [pii]
10.1126/science.1106148
42. Little AS, Balmanno K, Sale MJ, Newman S, Dry JR, Hampson M, Edwards PA, Smith PD, Cook SJ (2011) Amplification of the driving oncogene, KRAS or BRAF, underpins acquired resistance to MEK1/2 inhibitors in colorectal cancer cells. Sci Signal 4 (166):ra17. doi:4/166/ra17 [pii]
10.1126/scisignal.2001752
43. Corcoran RB, Dias-Santagata D, Bergethon K, Iafrate AJ, Settleman J, Engelman JA (2010) BRAF gene amplification can promote acquired resistance to MEK

inhibitors in cancer cells harboring the BRAF V600E mutation. Sci Signal 3 (149):ra84. doi:3/149/ra84 [pii]
10.1126/scisignal.2001148
44. Atefi M, von Euw E, Attar N, Ng C, Chu C, Guo D, Nazarian R, Chmielowski B, Glaspy JA, Comin-Anduix B, Mischel PS, Lo RS, Ribas A (2011) Reversing melanoma cross-resistance to BRAF and MEK inhibitors by co-targeting the AKT/mTOR pathway. PLoS One 6 (12):e28973. doi:10.1371/journal.pone.0028973 PONE-D-11-08524 [pii]
45. Turke AB, Song Y, Costa C, Cook R, Arteaga CL, Asara JM, Engelman JA (2012) MEK inhibition leads to PI3K/AKT activation by relieving a negative feedback on ERBB receptors. Cancer Res 72 (13):3228-3237. doi:0008-5472.CAN-11-3747 [pii]
10.1158/0008-5472.CAN-11-3747
46. Meng J, Peng H, Dai B, Guo W, Wang L, Ji L, Minna JD, Chresta CM, Smith PD, Fang B, Roth JA (2009) High level of AKT activity is associated with resistance to MEK inhibitor AZD6244 (ARRY-142886). Cancer Biol Ther 8 (21):2073-2080. doi:9844 [pii]

3 Letter of Acceptance

3.1 Letter of Acceptance

Von: em.drug.0.3ce97f.26f23ac7@editorialmanager.com [mailto:em.drug.0.3ce97f.26f23ac7@editorialmanager.com] Im Auftrag von Investigational New Drugs
Gesendet: Montag, 4. August 2014 19:45

An: Florian Ewald
Betreff: Decision on your manuscript #DRUG-D-14-00140

Dear Dr. Florian Ewald:

We are pleased to inform you that your manuscript, "Dual Inhibition of PI3K-AKT-mTOR- and RAF-MEK-ERK-signaling is synergistic in cholangiocarcinoma and reverses acquired resistance to MEK-inhibitors" has been accepted for publication in Investigational New Drugs.

For queries regarding your accepted paper, please contact the Production Editor Rosario Gramatica (Rosario.EGramatica@springer.com).

Please remember to always include your manuscript number, #DRUG-D-14-00140, whenever inquiring about your manuscript. Thank you.

Best regards,

The Editorial Office
Investigational New Drugs

3.2 Publikation

Von: Springer [mailto:SpringerAlerts@springeronline.com]
Gesendet: Dienstag, 25. November 2014 00:47
An: juecker@uke.uni-hamburg.de
Betreff: Your article in Investigational New Drugs has been published in the completed journal issue

Final publication of your article
25.11.2014
visit us at springer.com

Your article has been published in the completed journal issue

Dear Springer Author,

Good news for you: your article Dual Inhibition of PI3K-AKT-mTOR- and RAF-MEK-ERK-signaling is synergistic in cholangiocarcinoma and reverses acquired resistance to MEK-inhibitors has now been published in the following paginated issue of Investigational New Drugs: Volume 32, Issue 6 (2014), Page 1144-1154

This information can be used for the citation of your article. Please also add your article's Digital Object Identifier (DOI) in the citation in order to enable readers to easily find your article in print and online.

Your paper is available electronically on SpringerLink:
http://www.springerlink.com/openurl.asp?genre=article&id=doi:10.1007/s10637-014-0149-7
We encourage you to forward this email to your co-authors and colleagues or mention your article and its DOI on your website or your social media profiles.

Thank you again for your contribution. We hope that your experience of publishing with Springer was a satisfying one and look forward to your future contributions! For more information about publishing with us, visit our author website at

springer.com/authors, where you will find valuable services. If you have any questions, just contact our Author Helpdesk

Best regards,

Your Springer Marketing Team

4 Zusammenfassende Darstellung

4.1 Einleitung

Das Cholangiozelluläre Karzinom (CCA) ist eine vergleichsweise seltene Tumorerkrankung mit einer Inzidenz zwischen 1-2/100 000 Neuerkrankungen pro Jahr. Sie geht vom Epithel der intra-, oder extrahepatischen Gallenwege aus und ist damit die zweithäufigste primär hepatische Tumorerkrankung nach dem hepatozellulären Karzinom [1]. Während Inzidenz und Mortalität des intrahepatischen CCA in den vergangenen Jahren stetig anstiegen, zeigte sich die Mortalität des extrahepatischen CCA eher rückläufig [2, 3]. Aufgrund der späten klinischen Manifestation werden die meisten CCA leider erst in bereits fortgeschrittenen Stadien diagnostiziert, wenn die einzige kurative Behandlungsoption, die chirurgische Resektion, nicht mehr zur Verfügung steht [4]. Die 5-jahres Überlebensraten bei fortgeschrittenem CCA sind ausgesprochen schlecht, und bis heute konnte keine zuverlässig wirksame systemische Therapie etabliert werden [5].
Eine Arbeit von Sia et al. hat gezeigt, dass sich das intrahepatische CCA in zwei molekularbiologische Subgruppen unterteilen lässt. Bei dem „inflammatorischen-Typ“ sind Anomalien in der Signaltransduktion von Entzündungsmediatoren das vorherrschende Merkmal. Im Gegensatz dazu zeigt der „proliferative Typ“ eine Überaktivierung von Rezeptor-Tyrosinkinasen und des RAS-MEK-ERK Signalwegs und zeichnet sich durch einen aggressiveren klinischen Verlauf aus [6]. Die Hochregulation von einschlägigen Signalwegen, die mit Proliferation und Tumorgenese assoziiert sind, wurde beim CCA schon in der Vergangenheit gezeigt

[7]. Dementsprechend könnte der Einsatz moderner Kinaseinhibitoren für diese Tumorerkrankung ein vielversprechender Behandlungsansatz sein.

Eine unkontrollierte Zellteilung, bzw. Proliferation, hervorgerufen unter anderem durch Veränderungen in den entsprechenden intrazellulären Signalwegen, ist ein fundamentaler Bestandteil der Tumorgenese [8]. Der RAS-MEK-ERK Signalweg wird in nicht maligne transformierten Zellen durch Rezeptor-Tyrosinkinasen auf der Zelloberfläche aktiviert und hat große Bedeutung bei der Regulation von Proliferation und Zellüberleben. Dieser Signalweg gilt als einer der Wichtigsten für Krebserkrankungen, und dementsprechend häufig finden sich hier aktivierende Mutationen in transformierten Zellen [9]. Der selektive allosterische MEK-Inhibitor AZD6244 (ARRY-142886) ermöglicht eine Unterbrechung dieser Signalkaskade und führt zur Inaktivierung von ERK [10]. Sein Potential für den künftigen Einsatz bei Krebspatienten wird in klinischen Studien untersucht und AZD6244 ist bereits für die Behandlung des Aderhautmelanoms durch die FDA zugelassen [11, 12]. Die Serin/Threonin-Kinase AKT, auch Proteinkinase B (PKB) genannt, nimmt eine zentrale Stellung im PI3K-AKT-mTOR Signalweg ein, welcher ebenfalls für Wachstum, Proliferation und Zellüberleben von Bedeutung ist. Ihre Aktivität wird weitestgehend durch die Phosphorylierung an den Aminosäureresten Threonin 308 und Serin 473 bestimmt [13]. Das „mammalian target of rapamycin“ (mTOR) wird durch viele verschiedene Einflussfaktoren, wie z.B. Energie- und Nährstofflevel reguliert, und kontrolliert unter anderem die Proteinsynthese über die Regulation von 4EBP-1 und die p70-S6-Kinase [14]. Zwischen AKT und mTOR bestehen komplexe Interaktionsmechanismen, welche eine gegenseitige Regulation ermöglichen. Beispielsweise führt die Inhibition von TSC1/2 durch AKT zu einer Aktivierung von mTOR. Des weiteren hat mTORC2 direkten Einfluss auf die Aktivität von AKT, indem es dieses am Serinrest 473 phosphoryliert [15-17]. Der Einsatz allosterischer Inhibitoren von mTOR (idR. Rapamycin-Derivate) für die Behandlung von Tumorerkankungen wird bereits seit mehreren Jahren untersucht. Obwohl das Rapamycin-Derivat RAD001 (Affinitor, Everolimus) erfolgreich bei der Behandlung von Nierenzellkarzinomen eingesetzt wird, waren die bisherige Ergebnisse, unter anderem beim hepatozellulären Karzinom, jedoch ernüchternd [18, 19]. Neue, ATP kompetetive mTOR-Inhibitoren wie AZD8055 könnten ein größeres Potential für die Tumortherapie besitzen, da diese Substanzen beide mTOR Komplexe (mTORC1 und mTORC2) hemmen, im Gegensatz zu Rapamycin-Derivaten, welche vor allem

mTORC1 inhibieren [20]. Des weiteren besteht die Möglichkeit diesen Signalweg über eine Hemmung von AKT zu beeinflussen, welches oberhalb von mTOR eine Schlüsselposition einnimmt. Hierfür könnte unter anderem der allosterische AKT Inhibitor MK-2206 eingesetzt werden [21].
Zwischen den RAS-MEK-ERK- und PI3K-AKT-mTOR Signalwegen besteht ein hohes Maß an Interaktion und Redundanz [22]. Um Feedback- und Kompensations-Mechanismen zu unterbinden ist es wahrscheinlich, dass beide Signalwege simultan inhibiert werden müssen. Ein solches Vorgehen hat sich bereits in prä-klinischen Studien zum nicht-kleinzelligen Bronchialkarzinom, sowohl in vitro, als auch in vivo, als wirkungsvoll erwiesen [23].
In der vorliegenden Arbeit sollte die Wirkung von Kombinationsbehandlungen mit Inhibitoren gegen MEK (AZD6244), AKT (MK-2206) und mTOR (AZD8055) bei drei CCA-Zelllinien untersucht werden (TFK-1, EGI-1 und Sk-Cha-1). Es wird gezeigt, dass sowohl die Hemmung von AKT und MEK, als auch AKT und mTOR effektiv und synergistisch gegen die Proliferation dieser Zelllinien wirkt. Des weiteren wird gezeigt, dass eine Kombinationsbehandlung mit diesen Inhibitoren dazu in der Lage ist, eine erworbene Resistenz gegen AZD6244 zu überwinden.

4.2 Material und Methoden

Für diese Arbeit wurden die folgenden drei etablierten Cholangiokarzinom-Zelllinien eingesetzt: EGI-1, TFK-1 und Sk-Cha-1. Zur Erstellung von AZD6244 resistenten-Zelllinien wurden EGI-1 und TFK-1 jeweils für mindestens 6 Monate, unter Behandlung mit AZD6244 in einer Konzentration von 5µM, kultiviert.
Der Einfluss der einzelnen Kinaseinhibitoren, und Inhibitor-Kombinationen auf die Proliferation der einzelnen Zelllinien wurde durch BrdU-ELISA-Untersuchungen ermittelt. Des weiteren erfolgten Analysen des nukleären DNA-Gehalts mit Hilfe von Durchflusszytometrie zur Zellzyklus-Analyse.
Die Expression und Aktivität der relevanten Signaltransduktionswege wurde mit Hilfe von Western-Blots ermittelt. Wichtige Proteine und Phosphorylierungsstellen waren unter anderem AKT, mit den Phosphorylierungsstellen S473 und T308, MEK (pMek S217/221), ERK (pERK T202/204) und pS6 (S235/246), als Endstrecke des AKT-mTOR Signalwegs. Zur Ermittlung der AKT-Kinaseaktivität wurde zunächst eine

Immunpräzipitation mit pan-AKT-Antikörpern in den entsprechenden Zell-Lysaten durchgeführt. Im Anschluss erfolgte ein in-vitro Kinase Assay und Western Blot auf das genutzte AKT Substrat GSK3α/ß.
Bei den Doppel-Knock-Down-Versuchen erfolgte die sequentielle Transduktion von gegen AKT1, oder AKT2 gerichteter shRNA, kombiniert mit einer Antibiotikaresistenz, mithilfe eines lentiviralen Transduktionssystems und anschließender Selektion der erfolgreich transduzierten Zellklone durch Behandlung mit Puromycin und G418.
Die Evaluation von Inhibitorinteraktionen und synergistischen Effekten erfolgte durch Anwendung der „median effect method“ von Chou und Talalay [24].

4.3 Ergebnisse

Die Kombination des AKT-Inhibitors MK-2206 mit dem MEK-Inhibitor AZD6244 zeigte zwar einen deutlichen, aber dosisabhängigen synergistischen Effekt auf die Proliferation der drei CCA Zelllinien. Einen deutlich stärkeren Synergismus, vor allem bei niedrigen Konzentrationen, zeigte sich bei der Kombination von MK-2206 mit dem mTOR Kinase-Inhibitor AZD8055. Obwohl ein Synergismus bei der Kombinationsbehandlung eines AKT-Inhibitors mit einem allosterischen mTOR Inhibitor bereits in einer vorrangegangen Arbeit beschrieben worden ist [25], waren diese Ergebnisse unerwartet, da AZD8055, anders als der allosterische mTOR-Inhibitor RAD001, sowohl mTORC1 als auch mTORC2 hemmt und somit einen wichtigen Feedback-Mechanismus zwischen mTORC2 und AKT unterbricht. Bei der Western-Blot-Analyse zeigt sich, dass durch die Behandlung mit AZD8055, wie erwartet, die mTORC2-vermittelte Phosphorylierung von AKT an S473 effektiv unterdrückt wurde. Die Phosphorylierung an T308 jedoch stieg innerhalb von 24 Stunden auf etwa das Doppelte des Ursprungswertes an. Im *in vitro* Kinase Assay wurde bestätigt, dass dieser Anstieg mit einer Wiederherstellung der Kinase-Aktivität von AKT auf ca. 30% ihres ursprünglichen Werts einherging.
Weiterhin führte die Kombination von AZD6244 und AZD8055 lediglich zu schwachen synergistischen Effekten.
Um zu untersuchen, wie die Zelllinien auf eine langfristige Behandlung mit AZD6244 reagieren, wurden EGI-1- und TFK-1-Zellen für sechs Monate mit 5 µM AZD6244 kultiviert, bis sie hierunter annähernd normal wuchsen. Diese Zellen zeigten

anschließend unter AZD6244-Behandlung bis zu einer Konzentration von 20 µM keine Beeinträchtigung der Proliferation. Diese resistenten Sublinien werden im folgenden als EGI-1R und TFK-1R bezeichnet. Es zeigte sich eine Kreuzresistenz gegen einen anderen allosterischen MEK-Inhibitor (PD0325901), nicht jedoch gegen den Multi-Kinaseinhibitor Sorafenib. Bei der Behandlung dieser resistenten Zelllinien mit einer Kombination aus AZD6244 und MK-2206 waren erneut deutliche synergistische Effekte auf die Proliferation zu beobachten, trotz der stabilen Resistenz gegen AZD6244. In der Western-Blot-Analyse zeigten sowohl die resistenten-, als auch die parentalen Zelllinien eine drastische Suppression von pERK unter Behandlung mit AZD6244. Bei den resistenten Zelllinien war jedoch ein geringfügig stärkeres pERK1/2 Signal nachweisbar. Dies spricht für eine Reaktivierung von ERK1/2 unter Therapie mit AZD6244, wie es bereits für andere Zellen mit einer erworbenen RAF- oder MEK-Inhibitor Resistenz gezeigt worden ist.

4.4 Diskussion

Beim Verständnis der Tumorbiologie, insbesondere bei den für die Tumorentstehung und Metastasierung relevanten Signalwegen, wurden in den vergangenen Jahren erhebliche Fortschritte erzielt, die unter anderem zu der Entwicklung von neuen, gezielten molekularen Therapieansätzen führten [26]. Das beste Beispiel für den Erfolg eines genauen Verständnisses der molekularen Tumorbiologie ist die Einführung von Imatinib (Gleevec) zur Behandlung der chronischen myeloischen Leukämie (CML) [27]. Doch bereits beim Einsatz von Imatinib zeigte sich, dass Leukämiezellen dazu in der Lage sind, einer gezielten molekularen Therapie, z.B. durch Mutationen in den Zielstrukturen dieser Inhibitoren, auszuweichen und erworbene Resistenzen zu entwickeln [28]. Ein weiteres, gut untersuchtes Beispiel hierfür ist der Einsatz von BRAF-Inhibitoren beim malignen Melanom [29]. Bekannte Mechanismen einer solchen Resistenzentstehung sind hier z.B. die Amplifikation von Driver-Onkogenen, oder neue, aktivitätssteigernde Mutationen bei Kinasen des selben Signalwegs, unterhalb des gehemmten Zielproteins [30, 31]. Eine Monotherapie mit einem einzelnen Inhibitor wird vermutlich nur in wenigen, ausgewählten Tumorentitäten nachhaltige Erfolge erzielen können. Besonders die RAS-MEK-ERK-, und PI3K-AKT-mTOR Signalwege erfordern, aufgrund ihrer hohen

Redundanz und gegenseitigen Vernetzung, wahrscheinlich eine Blockade mehrerer Zielproteine, um den Zellen einfache Ausweichmechanismen zu nehmen und eine schnelle Resistenzentwicklung zu erschweren [32]. Außerdem kann es sinnvoll sein, mehrere Zielproteine innerhalb desselben Signalwegs zu blockieren, um Feedbackmechanismen auszuschalten [33].

Die Kombination des AKT-Inhibitors MK-2206, und des MEK-Inhibitors AZD6244 zeigte in allen drei CCA-Zelllinien deutliche, mit der Inhibitor-Konzentration zunehmende, synergistische Effekte auf die Proliferation. In einer früheren Arbeit wurde die Wirkung dieser Kombination bereits in NSCLC-Zelllinien beschrieben, und auf die Induktion von mitochondrialer Apoptose durch Hochregulation von Bim zurückgeführt [23]. Die Kombination des mTOR-Inhibitors AZD8055 mit dem MEK-Inhibitor AZD6244 zeigte jedoch nur geringe synergistische Effekte, obwohl prinzipiell dieselben Signalwege beteiligt waren. In einer vorrangegangenen Arbeit hatte sich diese Kombination zwar in Rhabdomyosarkom-Zelllinien als wirksam erwiesen, jedoch konnte in einer zweiten Studie gezeigt werden, dass die Wirksamkeit in Aderhautmelanom-Zelllinien stark von dem genetischen Hintergrund der Tumorzellen abhängig ist, insbesondere von Mutationen im GNAQ-Gen [34, 35].

Interessanterweise zeigten sich die stärksten synergistischen Effekte bei der Kombination von dem AKT-Inhibitor MK-2206 und dem mTOR-Inhibitor AZD8055, und das bereits bei niedrigen Konzentrationen. Ursächlich dafür ist vermutlich der hier beobachtete Feedback-Mechanismus, der zu einer gesteigerten Phosphorylierung von AKT bei T308 führt, wodurch es wiederum zu einer Wiederherstellung von ca. einem Drittel der ursprünglichen Kinase-Aktivität von AKT kommt. Dieses Phänomen ist bereits in einer früheren Arbeit beschrieben worden, und ist wahrscheinlich auf die Aufhebung einer Feedback-Inhibition auf das Signaling von Rezeptor-Tyrosinkinasen zurückzuführen [36]. Dementsprechend ist die kombinierte AKT und mTORC1/2 Hemmung ein legitimer Ansatz. Das Prinzip einer „vertikalen Hemmung“, durch Inhibitorkombinationen, welche mehrere Zielenzyme des selben Signalwegs blockieren, wurde prä-klinisch bereits in anderen Tumorentitäten erfolgreich angewendet, wie z.B. beim malignen Melanom [33].

Um die Effektivität einer Kombination der oben genannten Inhibitoren nach langfristiger Behandlung, oder auch einem Tumorrezidiv zu untersuchen, wurde ein Modell für eine erworbene Resistenz gegen AZD6244 genutzt. Sowohl TFK-1 als auch EGI-1-Zellen waren nach sechsmonatiger Kultivierung unter AZD6244-

Behandlung dazu in der Lage, selbst hohe Konzentrationen des Inhibitors ohne Einbußen beim Wachstum zu tolerieren. In vorrangegangen Arbeiten wurde gezeigt, dass eine solche Resistenz vermutlich durch eine Amplifikation von RAS, oder RAF verursacht wird. Außerdem wurde gezeigt, dass ein verstärktes Aufkommen von BRAFV600E allein bereits dazu in der Lage ist, eine Resistenz gegen AZD6244 zu erwirken [37]. In der Western-Blot Analyse zeigte sich jedoch bei keiner der beiden AZD6244-resistenten Zelllinien eine signifikante Wiederherstellung der ERK Aktivität.
Trotz der stabilen Resistenz gegen AZD6244 führte eine Kombinationsbehandlung von TFK-1R und EGI-1R mit AZD6244 und MK-2206 erneut zu synergistischen Effekten auf die Proliferation, d.h. die erworbene Resistenz wurde zu einem gewissen Maß aufgehoben. Ähnliches wurde bereits bei Melanom-Zellen beobachtet. Den BRAF- und-MEK Inhibitoren kommt beim Melanom eine besondere Bedeutung zu, da insbesondere BRAF Mutationen dort sehr häufig sind (40 - 60% aller Patienten, davon 90% $BRAF^{V600E}$-Mutationen), und dementsprechend Inhibitoren gegen den RAS-MEK-ERK Signalweg als vielversprechende neue Behandlungsoption gelten [38]. Eine andere Studie hat verschiedene, intrinsisch AZD6244-resistente und sensible NSCLC-Zelllinien (Non-small cell lung cancer) miteinander verglichen und festgestellt, dass hohe phospho-AKT Level mit AZD6244-Resistenz assoziiert sind, und eine Hemmung von AKT zu einer Wiederherstellung der Sensibilität gegen AZD6244 führt [39]. Dies lässt die Annahme zu, dass phospho-AKT, bzw. die Aktivität von AKT in Zukunft als Biomarker herangezogen werden könnte, um die Wirksamkeit von Inhibitoren des RAS-MEK-ERK Signalwegs einzuschätzen. Anhand dieser Daten lässt sich schließen, dass der PI3K-AKT-mTOR Signalweg eine wichtige Ausweichmöglichkeit für Tumorzellen darstellt, wenn MEK- oder BRAF inhibiert werden.
In dieser Arbeit wurde die Wirksamkeit von Inhibitoren des PI3K-MEK-mTOR- und des RAF-MEK-ERK Signalwegs bei CCA Zelllinien demonstriert. Die Kombination des AKT-Inhibitors MK-2206 und des MEK-Inhibitors AZD6244, oder des mTOR-Kinase Inhibitors AZD8055 hat sich konsistent als wirkungsvoll in allen drei getesteten Zelllinien erwiesen. Der Ansatz einer vertikalen Hemmung von AKT und mTOR zeigte sich in den hier verwendeten Zelllinien überlegen, im Vergleich zu einer parallelen Blockade zweier Signalwege durch die Inhibition von AKT und MEK. Zwar konnten zwei Zelllinien eine Resistenz gegen den MEK-Inhibitor AZD6244 erlangen, jedoch waren sie anschließend nach wie vor für eine Kombinationsbehandlung mit

AZD6244 und MK-2206, oder AZD8055 sensibel. Dies lässt den Schluss zu, dass Inhibitorkombinationen noch immer wirksam und sinnvoll sein können, obwohl bereits eine Resistenz gegen Einen der verwendeten Wirkstoffe besteht. Außerdem könnte der beobachtete Synergismus von AKT- und mTOR-Inhibitor klinisch relevant sein, da sich bereits bei niedrigen Konzentrationen ein starker synergistischer Effekt beobachten lässt, und somit durch den Einsatz geringerer Dosen Nebenwirkungen vermieden werden könnten.

Kombinationen von Hemmstoffen gegen AKT und MEK, oder AKT und mTOR könnten für Patienten mit Cholangiokarzinom in Zukunft eine vielversprechende Behandlungsoption darstellen. Daher sollte deren klinische Anwendbarkeit für die Therapie des CCA weiter untersucht werden.

In Folgeprojekten dieser Arbeit wurde unter anderem die Wirksamkeit der beschriebenen Inhibitorkombinationen in Zelllinien des hepatozellulären Karzinoms bestätigt. In einem weiteren Projekt wurden AZD6244 resistente Sublinien von mehreren Zelllinien verschiedener Tumorentitäten generiert. Dabei zeigte sich unter anderem, dass Inhibitor-entzug bei Zellen mit einer erworbenen Resistenz gegen MEK-Inhibitoren zu einer erheblich gesteigerten Migration und Invasion führen kann.

4.5 Quellenangabe

1. Khan SA, Thomas HC, Davidson BR, Taylor-Robinson SD: **Cholangiocarcinoma.** *Lancet* 2005, **366:**1303-1314.
2. Khan SA, Taylor-Robinson SD, Toledano MB, Beck A, Elliott P, Thomas HC: **Changing international trends in mortality rates for liver, biliary and pancreatic tumours.** *J Hepatol* 2002, **37:**806-813.
3. Patel T: **Worldwide trends in mortality from biliary tract malignancies.** *BMC Cancer* 2002, **2:**10.
4. Khan SA, Davidson BR, Goldin RD, Heaton N, Karani J, Pereira SP, Rosenberg WM, Tait P, Taylor-Robinson SD, Thillainayagam AV, et al: **Guidelines for the diagnosis and treatment of cholangiocarcinoma: an update.** *Gut* 2012, **61:**1657-1669.
5. Razumilava N, Gores GJ: **Cholangiocarcinoma.** *Lancet* 2014, **383:**2168-2179.
6. Sia D, Hoshida Y, Villanueva A, Roayaie S, Ferrer J, Tabak B, Peix J, Sole M, Tovar V, Alsinet C, et al: **Integrative molecular analysis of intrahepatic cholangiocarcinoma reveals 2 classes that have different outcomes.** *Gastroenterology* 2013, **144:**829-840.

7. Schmitz KJ, Lang H, Wohlschlaeger J, Sotiropoulos GC, Reis H, Schmid KW, Baba HA: **AKT and ERK1/2 signaling in intrahepatic cholangiocarcinoma.** *World J Gastroenterol* 2007, **13:**6470-6477.

8. Hanahan D, Weinberg RA: **Hallmarks of cancer: the next generation.** *Cell* 2011, **144:**646-674.

9. McCubrey JA, Steelman LS, Chappell WH, Abrams SL, Wong EW, Chang F, Lehmann B, Terrian DM, Milella M, Tafuri A, et al: **Roles of the Raf/MEK/ERK pathway in cell growth, malignant transformation and drug resistance.** *Biochim Biophys Acta* 2007, **1773:**1263-1284.

10. Davies BR, Logie A, McKay JS, Martin P, Steele S, Jenkins R, Cockerill M, Cartlidge S, Smith PD: **AZD6244 (ARRY-142886), a potent inhibitor of mitogen-activated protein kinase/extracellular signal-regulated kinase kinase 1/2 kinases: mechanism of action in vivo, pharmacokinetic/pharmacodynamic relationship, and potential for combination in preclinical models.** *Mol Cancer Ther* 2007, **6:**2209-2219.

11. Bekaii-Saab T, Phelps MA, Li X, Saji M, Goff L, Kauh JS, O'Neil BH, Balsom S, Balint C, Liersemann R, et al: **Multi-institutional phase II study of selumetinib in patients with metastatic biliary cancers.** *J Clin Oncol* 2011, **29:**2357-2363.

12. Carvajal RD, Sosman JA, Quevedo JF, Milhem MM, Joshua AM, Kudchadkar RR, Linette GP, Gajewski TF, Lutzky J, Lawson DH, et al: **Effect of selumetinib vs chemotherapy on progression-free survival in uveal melanoma: a randomized clinical trial.** *JAMA* 2014, **311:**2397-2405.

13. Vivanco I, Sawyers CL: **The phosphatidylinositol 3-Kinase AKT pathway in human cancer.** *Nat Rev Cancer* 2002, **2:**489-501.

14. Laplante M, Sabatini DM: **mTOR signaling in growth control and disease.** *Cell* 2012, **149:**274-293.

15. Inoki K, Corradetti MN, Guan KL: **Dysregulation of the TSC-mTOR pathway in human disease.** *Nat Genet* 2005, **37:**19-24.

16. Sparks CA, Guertin DA: **Targeting mTOR: prospects for mTOR complex 2 inhibitors in cancer therapy.** *Oncogene* 2011, **29:**3733-3744.

17. Liu P, Gan W, Inuzuka H, Lazorchak AS, Gao D, Arojo O, Liu D, Wan L, Zhai B, Yu Y, et al: **Sin1 phosphorylation impairs mTORC2 complex integrity and inhibits downstream Akt signalling to suppress tumorigenesis.** *Nat Cell Biol* 2013, **15:**1340-1350.

18. Markman B, Dienstmann R, Tabernero J: **Targeting the PI3K/Akt/mTOR pathway--beyond rapalogs.** *Oncotarget* 2010, **1:**530-543.

19. Zhu AX, Kudo M, Assenat E, Cattan S, Kang YK, Lim HY, Poon RT, Blanc JF, Vogel A, Chen CL, et al: **Effect of everolimus on survival in advanced hepatocellular carcinoma after failure of sorafenib: the EVOLVE-1 randomized clinical trial.** *JAMA* 2014, **312:**57-67.

20. Lv X, Ma X, Hu Y: **Furthering the design and the discovery of small molecule ATP-competitive mTOR inhibitors as an effective cancer treatment.** *Expert Opin Drug Discov* 2013, **8:**991-1012.

21. Pal SK, Reckamp K, Yu H, Figlin RA: **Akt inhibitors in clinical development for the treatment of cancer.** *Expert Opin Investig Drugs* 2010, **19:**1355-1366.

22. Mendoza MC, Er EE, Blenis J: **The Ras-ERK and PI3K-mTOR pathways: cross-talk and compensation.** *Trends Biochem Sci* 2011, **36:**320-328.

23. Meng J, Dai B, Fang B, Bekele BN, Bornmann WG, Sun D, Peng Z, Herbst RS, Papadimitrakopoulou V, Minna JD, et al: **Combination treatment with MEK and AKT inhibitors is more effective than each drug alone in human non-small cell lung cancer in vitro and in vivo.** *PLoS One* 2010, **5:**e14124.

24. Chou TC, Talalay P: **Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors.** *Adv Enzyme Regul* 1984, **22:**27-55.

25. Ewald F, Grabinski N, Grottke A, Windhorst S, Norz D, Carstensen L, Staufer K, Hofmann BT, Diehl F, David K, et al: **Combined targeting of AKT and mTOR using MK-2206 and RAD001 is synergistic in the treatment of cholangiocarcinoma.** *Int J Cancer* 2013, **133:**2065-2076.

26. Huang M, Shen A, Ding J, Geng M: **Molecularly targeted cancer therapy: some lessons from the past decade.** *Trends Pharmacol Sci* 2013, **35:**41-50.

27. Druker BJ, Sawyers CL, Kantarjian H, Resta DJ, Reese SF, Ford JM, Capdeville R, Talpaz M: **Activity of a specific inhibitor of the BCR-ABL tyrosine kinase in the blast crisis of chronic myeloid leukemia and acute lymphoblastic leukemia with the Philadelphia chromosome.** *N Engl J Med* 2001, **344:**1038-1042.

28. Gorre ME, Mohammed M, Ellwood K, Hsu N, Paquette R, Rao PN, Sawyers CL: **Clinical resistance to STI-571 cancer therapy caused by BCR-ABL gene mutation or amplification.** *Science* 2001, **293:**876-880.

29. Das Thakur M, Salangsang F, Landman AS, Sellers WR, Pryer NK, Levesque MP, Dummer R, McMahon M, Stuart DD: **Modelling vemurafenib resistance in melanoma reveals a strategy to forestall drug resistance.** *Nature* 2013, **494:**251-255.

30. Little AS, Smith PD, Cook SJ: **Mechanisms of acquired resistance to ERK1/2 pathway inhibitors.** *Oncogene* 2013, **32:**1207-1215.

31. Wagle N, Emery C, Berger MF, Davis MJ, Sawyer A, Pochanard P, Kehoe SM, Johannessen CM, Macconaill LE, Hahn WC, et al: **Dissecting therapeutic resistance to RAF inhibition in melanoma by tumor genomic profiling.** *J Clin Oncol* 2011, **29:**3085-3096.

32. Shimizu T, Tolcher AW, Papadopoulos KP, Beeram M, Rasco DW, Smith LS, Gunn S, Smetzer L, Mays TA, Kaiser B, et al: **The clinical effect of the dual-targeting strategy involving PI3K/AKT/mTOR and RAS/MEK/ERK pathways in patients with advanced cancer.** *Clin Cancer Res* 2012, **18:**2316-2325.

33. Werzowa J, Koehrer S, Strommer S, Cejka D, Fuereder T, Zebedin E, Wacheck V: **Vertical inhibition of the mTORC1/mTORC2/PI3K pathway shows synergistic effects against melanoma in vitro and in vivo.** *J Invest Dermatol* 2010, **131:**495-503.

34. Renshaw J, Taylor KR, Bishop R, Valenti M, De Haven Brandon A, Gowan S, Eccles SA, Ruddle RR, Johnson LD, Raynaud FI, et al: **Dual blockade of the**

PI3K/AKT/mTOR (AZD8055) and RAS/MEK/ERK (AZD6244) pathways synergistically inhibits rhabdomyosarcoma cell growth in vitro and in vivo. *Clin Cancer Res* 2013, **19:**5940-5951.

35. Ho AL, Musi E, Ambrosini G, Nair JS, Deraje Vasudeva S, de Stanchina E, Schwartz GK: **Impact of combined mTOR and MEK inhibition in uveal melanoma is driven by tumor genotype.** *PLoS One* 2012, **7:**e40439.

36. Rodrik-Outmezguine VS, Chandarlapaty S, Pagano NC, Poulikakos PI, Scaltriti M, Moskatel E, Baselga J, Guichard S, Rosen N: **mTOR kinase inhibition causes feedback-dependent biphasic regulation of AKT signaling.** *Cancer Discov* 2011, **1:**248-259.

37. Corcoran RB, Dias-Santagata D, Bergethon K, Iafrate AJ, Settleman J, Engelman JA: **BRAF gene amplification can promote acquired resistance to MEK inhibitors in cancer cells harboring the BRAF V600E mutation.** *Sci Signal* 2010, **3:**ra84.

38. Atefi M, von Euw E, Attar N, Ng C, Chu C, Guo D, Nazarian R, Chmielowski B, Glaspy JA, Comin-Anduix B, et al: **Reversing melanoma cross-resistance to BRAF and MEK inhibitors by co-targeting the AKT/mTOR pathway.** *PLoS One* 2011, **6:**e28973.

39. Meng J, Peng H, Dai B, Guo W, Wang L, Ji L, Minna JD, Chresta CM, Smith PD, Fang B, Roth JA: **High level of AKT activity is associated with resistance to MEK inhibitor AZD6244 (ARRY-142886).** *Cancer Biol Ther* 2009, **8:**2073-2080.

5 Erklärung des Eigenanteils an der Publikation

Der hier präsentierte Artikel wurde von Florian Ewald, mir, Björn Nashan und Manfred Jücker konzepiert. Die beschriebenen Experimente wurden zum größten Teil von mir und Florian Ewald durchgeführt und interpretiert. An einzelnen Experimenten waren Astrid Grotke und Bianca Hoffmann beteiligt. Das Manuskript wurde durch mich, Florian Ewald und Manfred Jücker verfasst. Alle angegebenen Autoren des Artikels haben das finale Manuskript gelesen und der Veröffentlichung zugestimmt.

6 Danksagung

Ich bedanke mich bei Hr. PD Dr. Jücker und Hr. Prof. Nashan für die Bereitstellung und Unterstützung bei der Durchführung des Projekts.

Weiterhin möchte ich mich bei Hr. Dr. Ewald für die gute Zusammenarbeit bedanken, sowie bei A. Grotke, B. Hoffmann und W. Wegner für die Mithilfe bei der Durchführung von Experimenten im Rahmen dieser Arbeit.

7 Lebenslauf

Name: Dominik Sebastian Nörz
Geburtsdatum: 26.09.1988

(Detaillierter Lebenslauf aus Datenschutzgründen entfernt)

8 Eidesstattliche Versicherung

Ich versichere ausdrücklich, dass ich die Arbeit selbständig und ohne fremde Hilfe verfasst, andere als die von mir angegebenen Quellen und Hilfsmittel nicht benutzt und die aus den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen einzeln nach Ausgabe (Auflage und Jahr des Erscheinens), Band und Seite des benutzten Werkes kenntlich gemacht habe.
Ferner versichere ich, dass ich die Dissertation bisher nicht einem Fachvertreter an einer anderen Hochschule zur Überprüfung vorgelegt oder mich anderweitig um Zulassung zur Promotion beworben habe.
Ich erkläre mich einverstanden, dass meine Dissertation vom Dekanat der Medizinischen Fakultät mit einer gängigen Software zur Erkennung von Plagiaten überprüft werden kann.

Unterschrift: .....