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Sorafenib alone or as combination therapy for growth control of cholangiocarcinoma

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ABSTRACT

Background/aim: Treatment options of advanced cholangiocarcinoma (CC) are unsatisfactory and new therapeutic approaches are mandatory. Dysregulations of the mitogen-activated kinase (MAPK) pathway associated with proliferative advantages of tumors are commonly observed in CCs. The novel multi-kinase inhibitor sorafenib potently suppresses the growth of various cancers by inhibiting kinases of wild-type B-Raf, mutant^{V559E}B-Raf and C-Raf but its effects on CC remains to be explored. We therefore studied the antineoplastic potency of sorafenib in human CC cells alone and in combination with conventional cytostatics or IGF-1R inhibition.

Methods and results: Sorafenib treatment dose-dependently blocked growth-factor-induced activation of the MAPKP and inhibited the proliferation of EGI-1 and TFK-1 CC cells in a time- and dose-dependent manner. At least two mechanisms accounted for the effects observed: arrest at the G_1/G_0-transition of the cell cycle and induction of apoptosis. The cell cycle arrest was associated with upregulation of the cyclin-dependent kinase inhibitor p27^{Kip1} and down-regulation of cyclin D1. Combining sorafenib with doxorubicin or IGF-1R-inhibition resulted in (over)additive antiproliferative effects whereas co-application of sorafenib and the antimetabolites 5-FU or gemcitabine diminished the antineoplastic effects of the cytostatics.

Conclusion: Our study demonstrates that the growth of human CC cells can be potently suppressed by sorafenib alone or in certain combination therapies and may provide a promising rationale for future *in vivo* evaluations and clinical trials.

1. Introduction

Cholangiocarcinoma (CC) accounts for 3% of all gastrointestinal cancers [1] and is the second commonest primary hepatic tumor [1,2]. The incidence rates of intrahepatic CC are rising in North America, Europe, Australia and Asia [2–4]. In addition to well-described risk factors like primary sclerosing cholangitis, liver fluke infestations or hepatolithiasis [5], recent studies

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Abbreviations: AG1024, 3-bromo-5-t-butyl-4-hydroxy-benzylidenemalonitrile; CC, cholangiocarcinoma; EGF, epidermal growth factor; ERK1/2, extracellular regulated kinase 1/2; 5-FU, 5-flourouracil; IGF, insulin-like growth factor; IGF-1R, insulin-like growth factor 1 receptor; MAPK, mitogen activated kinase; MAPKP, mitogen activated kinase pathway; sorafenib, *N*-(3-trifluoromethyl-4-chlorophenyl)-*N*′-(4-(2-methylcarbamoyl-pyridin-4-yl)oxyphenyl)urea

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suggest that increased prevalences of chronic hepatitis B and chronic hepatitis C infections, human immundeficiency virus infections, the metabolic syndrome and concomitant non-alcoholic steatohepatitis, usually combined with cirrhosis, play an important role for the rising incidence of intrahepatic CC [6]. On the other hand, incidence rates of extrahepatic CC are decling internationally [2–4], most probably as a result of increasing cholecystectomy rates over the past decades [2,4].

Unfortunately, the majority of patients suffer from advanced CC at presentation. Therefore, curative surgical resection or liver transplantation can be achieved in only a minority of CC patients. Palliative surgical biliary drainage and palliative radiotherapy or chemotherapy are the treatment options of advanced CC [5]. However, overall survival is poor with less than 5% of patients surviving to 5 years [7]. Apart from biliary drainage, which improves outcome in well-selected patients with unresectable CC, palliative treatment options do not appear to improve overall survival [5]. Therefore, innovative treatment approaches are urgently needed. Recently, evidence has been accumulated that targeting the mitogen activated protein kinase (MAPK) pathway which integrates a wide array of proliferative signals initiated by receptor tyrosine kinases and G-protein coupled receptors is a promising target for cancer therapy [8,9]. MAPKs are important regulators of apoptosis, proliferation and differentiation and dysregulation of the MAPK pathway is associated with tumor development and progression [10].

Raf, which is an essential serine/threonine kinase constituent of the MAPK-pathway is activated in a wide range of human malignancies by aberrant signaling upstream of the protein (e.g. growth factor receptors and mutant Ras), or activating mutations of the Raf-protein itself [11]. The Raf kinase family is composed of three isoforms: A-Raf, B-Raf and C-Raf (Raf-1). Once activated by Ras, Raf proteins phosphorylate mitogen-activated protein kinase kinase 1/2 (MEK1/2), these in turn activate the extracellular signal-regulated kinases 1/2 (ERK1/2) which modulate the activity of transcription factors like Ets-1, c-Jun and c-Myc [12,13] resulting in increased cell proliferation, cell cycle progression and inhibition of apoptosis [14].

The novel bi-aryl urea sorafenib is an orally available multikinase inhibitor that targets kinases of wild-type B-Raf, mutant^{V559E}B-Raf and C-Raf thus blocking tumor growth [15]. Furthermore, sorafenib shows potent inhibition of receptor tyrosine kinases (RTKs) involved in angiogenesis, including human vascular endothelial growth factor receptors-2 and -3 (VEGFR-2/-3) or the platelet derived growth factor receptor-β (PDGFR-β). The mechanism of action of sorafenib is competitive inhibition of ATP-binding to the catalytic domains of the respective kinases [15].

Sorafenib has been shown to inhibit the proliferation of a variety of human cancer cell lines and xenograft models of breast, colon and pancreas carcinoma [11]. Additionally, a series of clinical studies have tested sorafenibs' antineoplastic potency in cancer patients. Phase I trials showed a favorable safety profile for 400 mg sorafenib administered twice daily in patients with advanced solid tumors [16,17]. Promising antitumor activities of sorafenib were revealed in phase II clinical studies in patients with advanced HCC [18] or melanoma [19]. Most encouraging results were seen in phases II and III trails in patients with metastatic renal cell carcinoma (RCC) [19,20]. Recently, sorafenib has received approval in the US for the treatment of advanced RCC. Moreover, preliminary data from a series of combination studies with sorafenib and a variety of conventional cytostatics for various solid tumor entities have been published [11,21].

So far, the effects of kinase inhibition by sorafenib have not been evaluated for the treatment of human cholangiocarcinoma. Hence, in the present study we characterized the antineoplastic potency of sorafenib in the two human CC cell lines EGI-1 and TFK-1 alone and in combination with the conventional cytostatics gemcitabine or 5-FU or doxorubicin or in combination with insulin-like growth factor 1 receptor inhibition by AG1024. Moreover, we provide evidence that sorafenib induces cell cycle arrest and/or apoptosis in CC cells and potently inhibits the mitogen-activated protein kinase pathway.

2. Materials and methods

2.1. Cell lines and drugs

The poorly differentiated human bile duct adenocarcinoma cell line EGI-1 ([22]; DSMZ #ACC385) and the human papillary bile duct adenocarcinoma cell line TFK-1 ([23]; DSMZ #ACC344) were cultured in RPMI 1640 medium containing 10% fetal bovine serum and 100 U/mL penicillin and 100 μg/mL streptomycin. Both cell lines are derived from patient cells prior to any exposure to chemotherapy or radiotherapy and are known to be P-glycoprotein negative [24].

Sorafenib tosylate (Nexavar^{TM}) was a kind gift from Bayer Health Care (West Haven, CT), gemcitabine hydrochloride (Gemzar^{TM}) was bought from Lilly Pharma (Gießen, Germany) and AG1024 from Calbiochem (Bad Soden, Germany). Cell culture material was from Biochrom (Berlin, Germany); all other chemicals were from Sigma (Deisenhofen, Germany), if not stated otherwise. Stock solutions were prepared in DMSO and stored at −20 °C and were diluted to the final concentration in fresh media before each experiment. In all experiments, the final DMSO concentration did not exceed 0.5%, thus not affecting cell growth. To evaluate the effects of sorafenib, gemcitabine, 5-fluorouracil (5-FU), doxorubicin or AG1024 cells were incubated with either control medium or medium containing rising concentrations of the respective drug(s).

2.2. Measurement of growth inhibition

Cell number was evaluated by crystal violet staining, as described [25]. In brief, cells in 96-well plates were fixed with 1% glutaraldehyde, then cells were stained with 0.1% crystal violet in PBS. The unbound dye was removed by washing with water. Bound crystal violet was solubilized with 0.2% Triton X-100 in PBS. Light extinction which increases linearly with the cell number was analyzed at 570 nm using an ELISA-Reader.

2.3. Drug combination studies

To check for possible additive or even overadditive antiproliferative effects, combination treatment of sorafenib plus

conventional cytostatics (gemcitabine, 5-FU or doxorubicin) or plus IGF-1R inhibition by AG1024 was studied. Increasing concentrations of the drugs were combined with 2.5 μM sorafenib (e.g. a sub-IC_{50} value). The antineoplastic activities of the combinations were compared to those of each drug alone. Concentration ranges and effectiveness of each agent was determined in prior experiments. For all experiments cell number was evaluated by crystal violet staining, as described above.

2.4. Detection of apoptosis

Changes in caspase-3 activity were assessed by measuring the cleavage of the fluorogenic substrate AC-DEVD-AMC (Calbiochem-Novabiochem, Bad Soden, Germany), as described previously [26]. In brief, cell lysates were incubated for 1 h at 37 °C with a substrate solution containing 20 μg/mL AC-DEVD-AMC, 20 mM HEPES, 10% glycerol, 2 mM DTT with a pH adjusted to 7.5. Substrate cleavage was measured fluorometrically using a VersaFluor fluorometer (filter wavelengths: excitation: 360/40 nm, emission: 460/10 nm) from Bio-Rad, Munich, Germany.

DNA fragmentation was determined by using the Cell Death Detection ELISA (Roche). After 48–72 h of incubation, cells were lysed in incubation buffer. The cytoplasmic fractions were diluted to contain 2.5×10^3 cell equivalents/mL, and the presence of mono- and oligonucleosomes was assayed using antibodies directed against DNA and histones. DNA fragments were detected by a peroxidase system with color development read at 405 nm.

2.5. Determination of cytotoxicity

Cells were seeded into 96-well microtiter plates and incubated with 1–10 μM sorafenib for 3 and 6 h. Release of the cytoplasmic enzyme lactate dehydrogenase (LDH), indicating cytotoxicity, was measured by using a colorimetric kit from Roche (Roche Diagnostics, Mannheim, Germany) as described by the manufacturer.

2.6. Cell cycle analysis

Cell cycle analysis was performed by using the method of Vindelov and Christensen [27]. Cells were trypsinized, washed, and the nuclei were isolated using CycleTest PLUS DNA Reagent Kit (Becton Dickinson, Heidelberg, Germany). DNA was stained with propidium iodide according to the manufacturers' instructions. The DNA content of the nuclei was detected by flow cytometry and analyzed using CellFit software (Becton Dickinson, Heidelberg, Germany).

2.7. Western blot analysis

Western blotting was performed as described [28]. Blots were blocked in 2.5% BSA and then incubated at 4 °C overnight with the following antibodies: ERK1/2 (1:500), p-ERK1/2 (1:500), cyclin D1 (1:100; all from Santa Cruz Biotechnology, CA) or $p27^{KIP1}$ (1:2500; Becton-Dickinson, Heidelberg, Germany). β-Actin (1:5000; Sigma, Deisenhofen, Germany) served as loading control.

2.8. Statistical analysis

If not stated otherwise, means of at least three independent experiments ±S.E.M. or S.D. are shown. Significance between controls and treated samples was calculated by Student's two sided *t*-test. Caspase-3 measurements were evaluated using the two sided Welch *t*-test. *p*-Values were considered to be significant at <0.05.

3. Results

3.1. Growth inhibitory effects of sorafenib on CC cells

Growth inhibition by sorafenib was studied by measuring cell proliferation. Sorafenib time- and dose-dependently inhibited the proliferation of EGI-1 (Fig. 1A) and TFK-1 (Fig. 1B) cells. The IC_{50} value of sorafenib, determined after 72 h of incubation, was 3.3 μM for EGI-1 cells and 4.1 μM for TFK-1 cells, respectively. Interestingly, already after 24 h of sorafenib treatment (10 μM) EGI-1 cells displayed an remarkable growth inhibition of up to 95% compared to control.

Additionally cytotoxicity of sorafenib treatment was evaluated by measuring LDH release of the cells into the culture medium. Incubating EGI-1 and TFK-1 cells with 1–10 μM sorafenib for up to 6 h did not result in a significant increase of LDH release in both cases (data not shown), indicating that sorafenib does not directly affect cell membrane integrity and does not have immediate toxic effects even at high concentrations.

3.2. Sorafenib and cell cycle regulation

To test whether an induction of cell cycle arrest contributed to the antiproliferative potency of sorafenib in cholangiocarcinoma cells, we performed cell cycle analyses. Incubating TFK-1 with rising sorafenib concentrations (1–10 μM) for 24 h led to a dose-dependent arrest of the cells in the G_1/G_0 phase of the cell cycle (up to +20% compared to control), thereby decreasing the proportion of cells in the S-phase. The G_1/G_0-arrest of TKI-1 cells was significant above concentrations of 2.5 μM of sorafenib. At the same time the proportion of cells in the G_2/M phase of the cell cycle showed a slight increase indicating a partial additional block in the G_2/M phase due to sorafenib treatment (Fig. 2B). EGI-1 cells (Fig. 2A) required a higher concentration of sorafenib (10 μM) for being arrested significantly in the G_1/G_0-phase.

3.3. Sorafenib modulates the expression of cell cycle regulators

To further characterize sorafenib's effects on the cell cycle, we performed Western blots to reveal the underlying molecular mechanisms. Treating TFK-1 cells for up to 72 h with 5 μM of sorafenib (e.g. approximately the IC_{50}-concentration) resulted in a suppression of cyclin D1, which is an essential promoter for the transition from the G_1- to the S-phase. At the same time, the expression of the cyclin-dependent kinase inhibitor (CDKI) $p27^{Kip1}$ markedly increased (Fig. 3B). In accordance with the results obtained

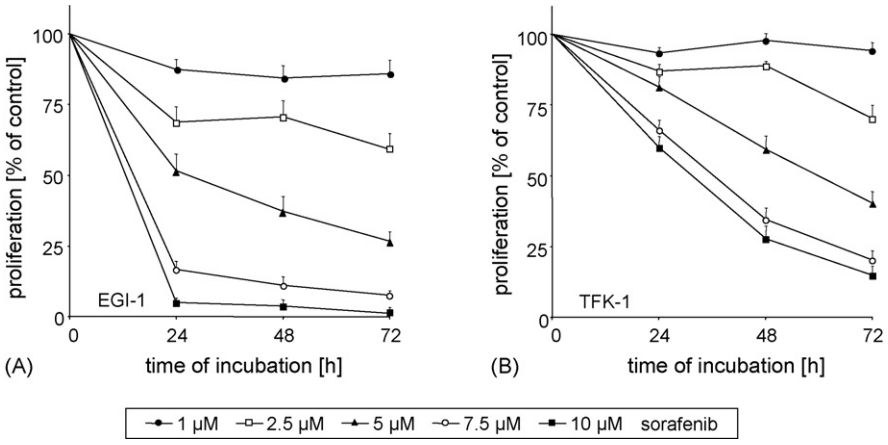

Fig. 1 – Antiproliferative effects of sorafenib in cholangiocarcinoma cells. Sorafenib caused a time- and dose-dependent growth inhibition of human EGI-1 (A) and TFK-1 cholangiocarcinoma cells (B). After 72 h of continuous incubation with rising concentrations of sorafenib cell numbers of EGI-1 (A) and TFK-1 cells (B) decreased by up to 99% and 87% as determined by crystal violet staining. Data are given as percentage of controls (mean ± S.E.M. of at least four-independent experiments). Statistical significance ($p < 0.05$) of growth inhibition was shown for all drug concentrations >1 μM.

in the experiments on the modulatory effects of sorafenib on the cell cycle regulation of EGI-1 cells, we found no changes of the expression of $p27^{Kip1}$and just a slight decrease in cyclin D1 expression (Fig. 3A).

3.4. *Induction of apoptosis by sorafenib*

To study the potency of sorafenib to induce apoptosis in CC cell lines we investigated sorafenib-induced activation of caspase-3, a key enzyme of the apoptotic pathway. Cells were treated with escalating sorafenib concentrations (1–10 μM) and subsequently caspase-3 activity was determined after 3, 6 and 24 h of drug incubation. Both TFK-1 and EGI-1 cells displayed a significant increase in caspase-3 activity, but the augmentation of enzyme activity was less pronounced in TFK-1 cells, with a maximum of +53% as compared to control after 24 h for the highest sorafenib concentration of 10 μM (Fig. 4B). In EGI-1 cells caspase-3 activity increased dose-dependently due to sorafenib treatment (Fig. 4A). Already 3 h after the beginning of drug treatment, concentrations ≥2.5 μM led to a significant increase in enzyme activity of up to nearly +300% as compared to controls. With prolonged incubation time,

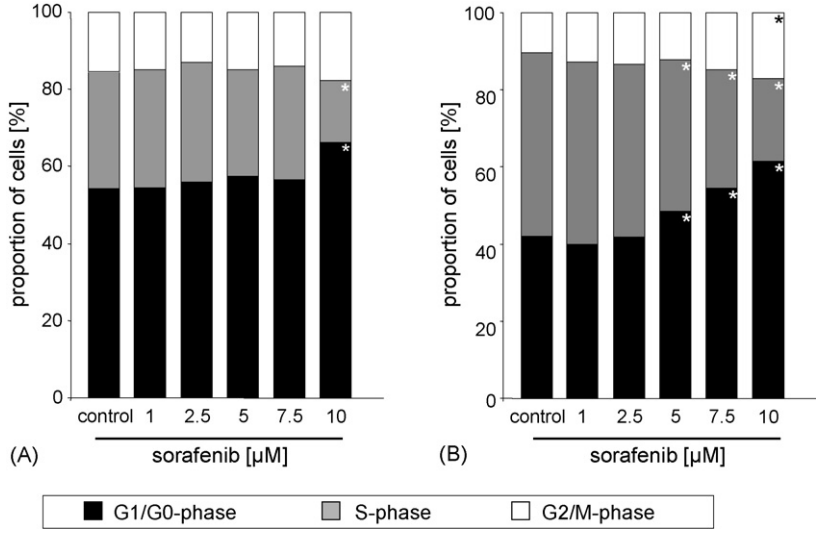

Fig. 2 – Induction of cell cycle arrest in the G_1/G_0 phase by sorafenib in cholangiocarcinoma cells. Twenty-four hours of incubation of TFK-1 cells (B) with sorafenib led to a dose-dependent accumulation of the cells in the G_1/G_0 phase of the cell cycle. Accordingly, the number of cells in the S-phase decreased. In addition, the proportion of cells in the G_2/M-phase slightly increased, indicating a partial additional block in the G_2/M-phase. Cell cycle alterations were less pronounced in EGI-1 cells (A), showing significance only for the highest concentration investigated (10 μM sorafenib). Means of three independent experiments for each cell line are shown. *Statistical significance ($p < 0.05$) compared to untreated controls.

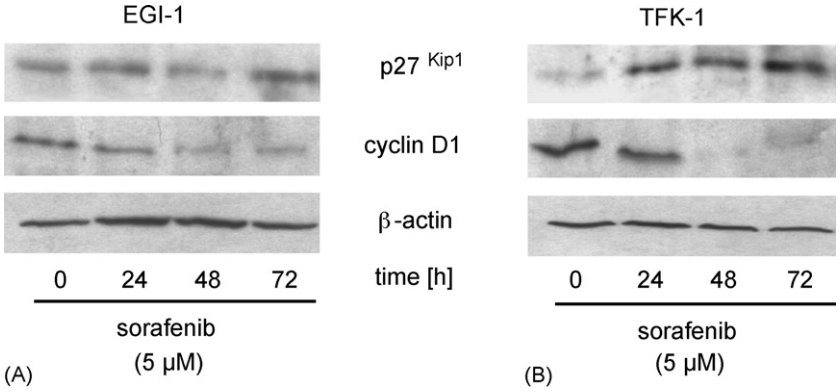

Fig. 3 – Modulation of the expression of cell cycle regulators by sorafenib. Modulation of protein expression by sorafenib was analyzed by Western blotting. CC cells were treated with sorafenib (5 μM) for up to 72 h. The cell cycle inhibitor $p27^{KIP1}$ was upregulated by sorafenib in TFK-1 cells (B) but not in EGI-1 cells (A). The expression of the cell cycle promoter cyclin D1 was found to be decreased in TFK-1 cells due to sorafenib treatment, but less so in EGI-1 cells. One representative out of three independent experiments is shown for each protein investigated.

caspase-3 activity in response to lower drug concentrations (2.5–7.5 μM) was further increased.

To confirm sorafenib-induced apoptosis of CC cells, we additionally measured the fragmentation of DNA into mono- and oligonucelosomes, which represents a hallmark of apoptosis. Sorafenib dose-dependently induced DNA fragmentation of CC cells. After 24 h of incubation, we observed an increase in DNA fragmentation of more than 512 ± 27% in EGI-1 cells (Fig. 4C). Albeit at a lower level, comparable results were obtained for TFK cells, showing an increase in DNA fragmentation of up to 173 ± 11% (not shown). The extent of DNA fragmentation corresponded to the extent of caspase-3 activation of either cell line.

3.5. *Growth factor-induced ERK1/2-activation in cholangiocarcinoma cells*

To further shed light on the growth inhibitory effects of sorafenib in CC cells, we investigated the activation of ERK1/2, a key protein of the mitogen-activated protein kinase (MAPK)-pathway known to be involved in mitogenic and antiapoptotic signaling.

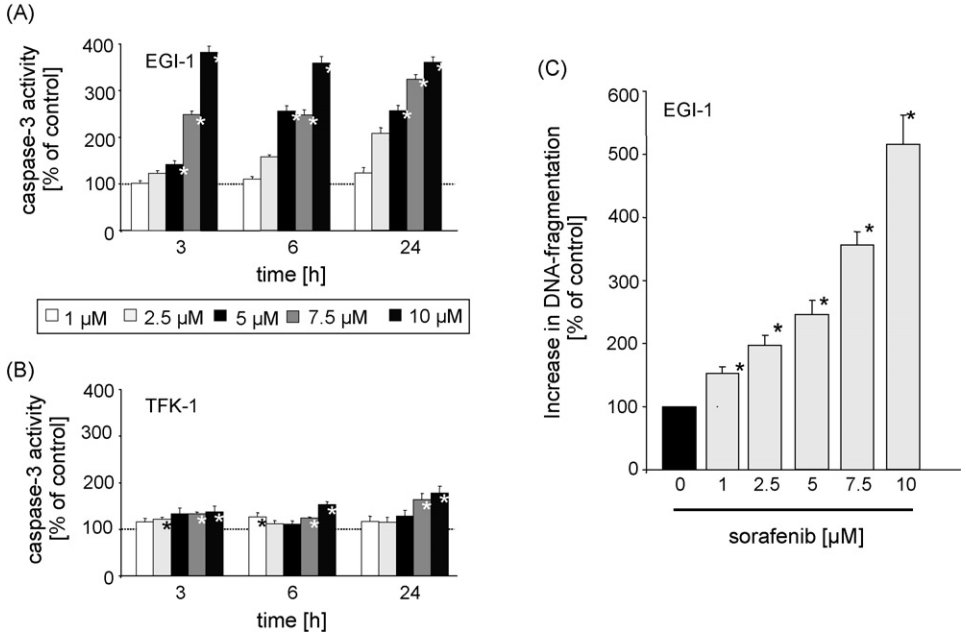

Fig. 4 – Sorafenib-induced apoptosis. Sorafenib time- and dose-dependently induced a strong caspase-3 activation in EGI-1 cells (A) but to a lesser extent in TFK-1 cells (B). Cells were treated with escalating concentrations (1–10 μM) of sorafenib for 3, 6 and 24 h. Sorafenib induced a dose-dependent increase in apoptosis-specific DNA fragmentation of CC cells after 24 h of incubation (C). Data are shown as mean ± S.E.M. of at least five independent experiments for each cell line. *Statistical significance ($p < 0.05$) compared to controls which were set at 100%.

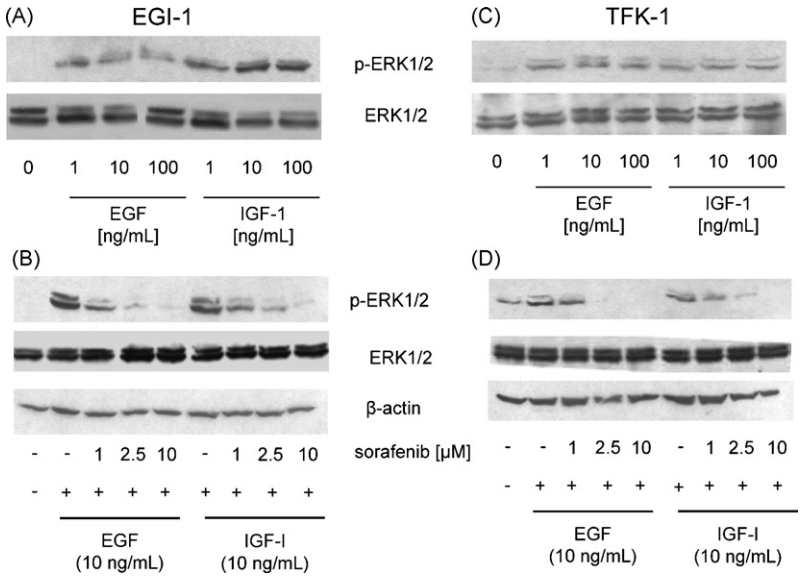

Fig. 5 – Sorafenib inhibits EGF and IGF-1-induced ERK1/2-activation in cholangiocarcinoma cells. EGF or IGF-1-treatment (1–100 ng/mL; 15 min) of serum-starved EGI-1 (A) or TFK-1 (C) cholangiocarcinoma cells led to an activation of the mitogenic MAPK-pathway as demonstrated by an increase in ERK1/2-phosphorylation. Serum-free conditions were chosen in order to focus on the effects of the respective growth factor. One representative out of three independent experiments is shown for each protein investigated. Sorafenib dose-dependently inhibited growth factor-induced activation of the mitogen activated protein kinase pathway. Sorafenib-pretreatment (1, 2.5 or 10 μM; 30 min) inhibited both EGF (10 ng/mL)-and IGF-1 (10 ng/mL)-induced ERK1/2-activation in EGI-1 (B) and TFK-1 (D) cells. One representative out of three independent experiments is shown (+: addition of the respective substance; −: absence of the respective substance).

In order to demonstrate the influence of the epidermal growth factor (EGF) and the insulin-like growth factor 1 (IGF-1) on the activation of the MAPK pathway in cholangiocarcinoma cells, serum-starved CC cells were incubated for 15 min with escalating concentrations (1, 10 or 100 ng/mL) of EGF or IGF-1. Western blotting of whole cell lysates revealed activation of ERK1/2 in response to both EGF or IGF-1. EGF and IGF-1 activated TFK-1 cell ERK1/2 to a similar extent, and this activation could not be augmented by increasing the concentration of the respective growth factors (Fig. 5B). In EGI-1 cells ERK1/2 activation was dose-dependent and more pronounced after IGF-1 treatment (Fig. 5A).

3.6. *Inhibition of growth factor-induced ERK1/2 activation by sorafenib*

EGF or IGF-1-induced activation of the mitogenic ERK1/2 was blocked by pretreating the cells with sorafenib. CC cells were treated for 30 min with 1, 2.5 or 10 μM of sorafenib and subsequently stimulated with EGF or IGF-1 (10 ng/mL) for 15 min. Again, activation of ERK1/2 was determined by Western blotting. Sorafenib-untreated EGI-1 or TFK-1 cells (control) displayed a pronounced activation of ERK1/2 due to growth factor-stimulation. Sorafenib dose-dependently inhibited ERK1/2-phosphorylation in both cell lines investigated. In TFK-1 cells, sorafenib concentrations above 1 μM completely inhibited ERK1/2-activation, whereas in EGI-1 cells the MAPKP could not be blocked completely even at high sorafenib doses of 10 μM (Fig. 5C and D).

3.7. *Antineoplastic potency of sorafenib in combination with cytostatics*

In light of encouraging results with sorafenib in combination with conventional cytostatics in previous studies [11], we studied possible (over)additive antineoplastic effects of sorafenib plus cytostatics in CC cells. Cells were treated with a sub-IC_{50} concentration of sorafenib (2.5 μM) alone or in combination with rising concentrations of 5-FU (0–500 nM) or gemcitabine (0–100 nM) or doxorubicin (0–100 nM) for 72 h. Upon treatment with sorafenib alone a growth inhibitory effect of 39% (EGI-1) and 30% (TFK-1) was observed after 72 h of continuous exposure to the drug. When incubated with 5-FU, EGI-1 cells dose-dependently displayed a strong growth inhibition of up to 85% (500 nM 5-FU), whereas the growth of TFK-1 cells was unaffected by 5-FU. Combining 5-FU with sorafenib did not result in (over)additive effects. In contrast, the growth inhibitory effect of 5-FU on EGI-1 cells was rather attenuated by coincubation with sorafenib (Fig. 6A: EGI-1; B: TFK-1). Similar results were obtained when co-administering sorafenib and gemcitabine: EGI-1 cholangiocarcinoma cells were highly sensitive towards gemcitabine treatment resulting in a reduction of cell number of up to 96% (10 nM gemcitabine), while TFK-1 cells appeared to be less sensitive towards the drug with a reduction of cell number of 75% for a 10-fold gemcitabine concentration of 100 nM. Coincubation with sorafenib diminished the antineoplastic effects of gemcitabine in both CC cell lines (Fig. 6C: EGI-1; D: TFK-1).

Treatment of the CC cells with rising concentrations of the topoisomerase II inhibitor doxorubicin resulted in a

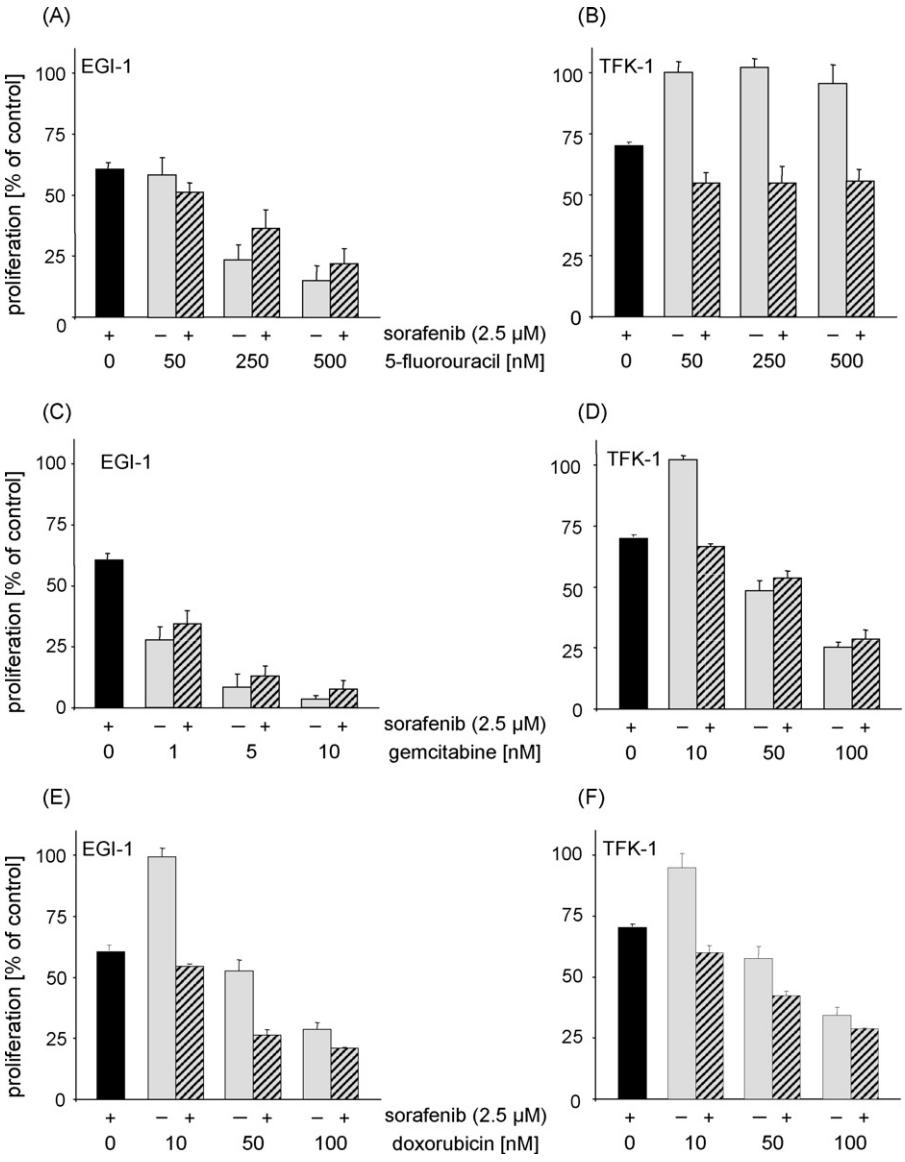

Fig. 6 – Antiproliferative effects of sorafenib plus cytostatics. EGI-1 (A, C, and E) and TFK-1 cholangiocarcinoma cells (B, D, and F) were treated for 72 h with 2.5 μM sorafenib plus rising concentrations of 5-fluorouracil (A and B; 0–500 nM) or gemcitabine (C and D; EGI-1: 0–10 nM, TFK-1: 0–100 nM) or doxorubicin (E and F; 0–100 nM). Black bars indicate the antiproliferative effects of 2.5 μM sorafenib on its own, grey bars show the effects of the respective cytostatic alone and hatched bars indicate the antiproliferative effects of combination treatment (2.5 μM sorafenib plus different concentrations of the respective cytostatic agent) obtained by crystal violet staining. Data are given as percentage of controls which were set at 100%. Mean ± S.D. of at least three independent experiments.

dose-dependent reduction of cell number. Moreover, over-additive growth inhibitory effects were observed when doxorubicin was combined with 2.5 μM sorafenib in EGI-1 (Fig. 6E) as well as in TFK-1 cells (Fig. 6F).

3.8. *Antineoplastic potency of sorafenib in combination with the IGF-1R-TK inhibitor AG1024*

To check the potency of inhibiting multiple targets of mitogenic cell signaling for more efficacious treatment, we combined MAPK pathway blockade by sorafenib (2.5 μM) with IGF-1R-TK inhibition by AG1024 (0–10 μM). AG1024 mono-treatment only showed a moderate inhibition of cell growth for both EGI-1 and TFK-1 cells, whereas strong additional effects were detected when AG1024 was co-administered with sorafenib (Fig. 7A: EGI-1; B: TFK-1).

4. Discussion

Treatment options of advanced cholangiocarcinoma (CC) are unsatisfactory, and the prognosis of patients suffering from advanced CC is poor. Thus, novel therapeutic approaches are much needed.

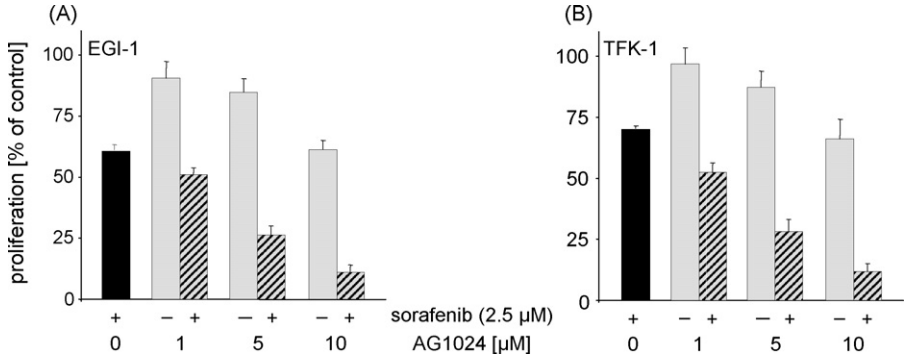

Fig. 7 – Antiproliferative effects of sorafenib plus AG1024. EGI-1 (A) and TFK-1 cholangiocarcinoma cells (B) were treated for 72 h with 2.5 μM sorafenib plus increasing concentrations of the IGF-1R-TK inhibitor AG1024 (1–10 μM). Black bars indicate the antiproliferative effects of 2.5 μM sorafenib on its own, grey bars show the effects of AG1024 alone and hatched bars indicate the antiproliferative effects of combination treatment (2.5 μM sorafenib plus different concentrations AG1024) obtained by crystal violet staining. Data are given as percentage of controls which were set at 100%. Mean ± S.E.M. of three independent experiments.

The novel bi-aryl urea sorafenib is an orally available multi-kinase inhibitor that targets kinases of wild-type B-Raf, mutant^{V559E}B-Raf and C-Raf [15]. Sorafenib has shown its potency to inhibit the growth of a variety of human cancer cells [11] but has not been tested for the treatment of CC so far. With a mutation rate of more than 60%, the MAPK pathway displays by far the most common genetic alterations in CC [29]. Activating Ras mutations are described for 56% and B-Raf mutations for up to 22% of all CCs, qualifying sorafenib for future CC therapy regimen. Here, we provide evidence that sorafenib may be a promising anticancer agent for CC treatment alone and in combination with conventional cytostatics. Additionally, we show promising *in vitro* results for a combination of sorafenib with inhibitors of insulin-like growth factor 1 receptor (IGF-1R) signaling.

Sorafenib inhibited cholangiocarcinoma cell growth in a time- and dose-dependent manner in both EGI-1 and TFK-1 cholangiocarcinoma cells. The antiproliferative effects observed were due to an arrest of the cell cycle and an induction of apoptosis. The respective contribution of both effects differed among the two cell lines investigated: While in TFK-1 cells, sorafenib dose-dependently led to a strong arrest in the G_1/G_0 phase of the cell cycle, the induction of apoptosis was only moderate. The cell cycle arrest observed in TFK-1 cells was associated with an induction of the expression of the cell cycle inhibitor p27^{Kip1} and a suppression of the cell cycle promoter cyclin D1. By contrast, EGI-1 cells displayed a powerful induction of apoptosis due to sorafenib treatment, whereas the cell cycle of EGI-1 cells remained nearly unaffected. This points to a dual mechanism of tumor suppression by sorafenib which likely improves its *in vivo* efficacy even in multiclonal CC cell populations that contain cells that have developed resistance to single pathway inhibition.

Additionally, we investigated the effects of sorafenib on the activation of one of the major mitogenic pathways, the MAPK pathway. The MAPK pathway is a key signaling mechanism that regulates many cellular functions such as cell growth, transformation and apoptosis [10]. One of the essential components of this pathway is the serine/threonine kinase Raf. Raf relays the extracellular signal from the receptor/Ras complex to a cascade of cytosolic kinases by phosphorylating MEK (MAPK/ERK kinase) and finally ERK1/2 (extracellular signal regulated kinase) [30]. Regulation of both Ras and Raf is crucial in the proper maintenance of cell growth as oncogenic mutations in these genes lead to high transforming activity [30].

Here, we could demonstrate that growth factor induced activation of ERK1/2 is potently inhibited by sorafenib. As activated ERK1/2 serves as a regulator of gene expression of various proteins, such as cell cycle inhibitors like p27^{Kip1} [31] and inhibits apoptosis by suppressing caspase activation [32] and the expression of several antiapoptotic proteins [10], this explains sorafenib's pronounced effects on the cell cycle progression and apoptosis. However, the exact mechanisms of sorafenib-induced cell cycle arrest and apoptosis and the reasons for the differences in the respective contribution of both mechanisms to the antitumor activity of sorafenib in different cell lines remain to be elucidated in further investigations. Concerning sorafenib-induced apoptosis Yu and co-workers recently published an interesting study, showing that sorafenib-induced apoptosis of cancer several cells including cholangiocarcinoma cells occurred via down-regulation of the anti-apoptotic Bcl-2 member Mcl-1. Interestingly, at least in lung cancer cells this downregulation was independent of MAPK signaling [38]. It will be of future interest to examine if this observation reflects a general or rather cell model specific finding, which also holds true for cholangiocarcinoma.

For non-cholangiocarcinomas, preliminary data from various combination studies with sorafenib and a variety of conventional cytostatics for several solid tumor entities have been published [11,21] and a series of clinical trials is being conducted at the moment, e.g. targeting non-small cell lung cancer (sorafenib/carboplatin/paclitaxel) or recurrent gastric cancer (sorafenib/docetaxel/cisplatin) (www.clinicaltrials.gov).

We also focused on possible (over)additive effects of combinations of sorafenib with the antimetabolites 5-FU or

gemcitabine or with the topoisomerase-II-inhibitor doxorubicin, three cytostatics commonly used for palliative chemotherapy of CC [5]. Pronounced synergistic effects were seen when sorafenib was combined with doxorubicin. Recently, results of a phase-I-trial of sorafenib in combination with doxorubicin in patients with solid tumors refractory to conventional chemotherapy demonstrated good tolerability, no significantly increased toxicity and promising efficacy [33] thus justifying further clinical investigations of this combination for CC treatment, too. Potentiation of antitumor effects may have important clinical implications. Instead of increasing cytostatics to supratoxic levels it may be possible to enhance antitumor activity by addition of sorafenib.

In contrast, sorafenib failed to enhance the antineoplastic efficacy of 5-FU or gemcitabine. This may be due to the mode of action of the two agents: 5-FU and gemcitabine are antimetabolites, thus exerting their cytostatic action in the S-phase of the cell cycle. As we could show that the sorafenib-induced arrest of the cell cycle in the G_1/G_0-phase is associated with a decrease in the proportion of cells in the S-phase, sorafenib treatment reduces the number of antimetabolite-sensitive cells.

For the MAPKP integrates a wide array of proliferative signals initiated by receptor tyrosine kinases (e.g. of growth factor receptors such as EGFR or IGF-1R) or G-protein coupled receptors [8,9,34] we expanded the concept of mitogenic signaling-inhibition by blocking two targets of the mitogenic signaling simultaneously. For the first time we hereby focused on the inhibition of the insulin-like growth factor 1 receptor by the tyrphostine AG1024 in combination with sorafenib multi-kinase inhibition. The IGF-1R being activated by either IGF-1 or IGF-2 contributes to the growth, survival, adhesion and motility of cancer cells. IGF-1R signaling is mediated through MAPK, phosphatidylinositol-3-kinase (PI3K) and stress-activated protein kinase (SAPK) [35]. Here we could show IGF-1-induced activation of the MAPKP could be potently blocked by sorafenib in CC cells. Favorable antineoplastic effects were obtained when IGF-1R inhibition by AG1024 was combined with sorafenib. Our results suggest that a combination regimen may yield greater anticancer activity than approaches that address only a single mitogenic target and should be investigated more extensively in future studies. The reason for this observation may be the fact that inhibition of multiple Raf-isoforms is necessary for a potent inhibition of the MAPKP. This is supported by the report that knocking out both Raf-1 and B-Raf expression is required to completely inhibit MAPKP activation upon B cell receptor activation [36] and the observation that there is no inhibition of growth factor-mediated MAPKP activation in mouse embryonic fibroblasts from Raf-$1^{-/-}$ embryos [37]. Thus, additional blocking of a target upstream of Raf enhances sorafenib's antiproliferative efficacy.

To conclude, our study provides first evidence that the growth of human cholangiocarcinoma cells can be potently suppressed by sorafenib. At least two mechanisms account for the effects observed: an arrest of the cell cycle in the G_1/G_0-phase and an induction of apoptosis. The respective contribution of both mechanisms differed among the two cell lines investigated, the underlying reasons for this observation remain to be elucidated in future studies. Sorafenib's inhibitory effects on the mitogenic MAPKP-signaling of CC cells with subsequent changes in the expression and/or activation of apoptotic factors and/or cell cycle regulators may explain the effects observed.

In addition to monotherapeutic approaches of CC by sorafenib, this study may also provide a rationale for sorafenib's suitability for combination treatment with cytostatics or inhibitors of growth factor signaling. Taken together our encouraging *in vitro* data show that sorafenib might be a promising weapon to fight CC and merit further *in vivo* evaluation.

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