

## Synergistic Effects of $\beta$ -Catenin Inhibitors and Sorafenib in Hepatoma Cells

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**Abstract.** *Background/Aim:* The kinase inhibitor sorafenib is the only approved drug which is effective against late-stage hepatocellular carcinoma (HCC). However, the mean survival of patients is still less than one year, making new approaches for tumor treatment essential. Oncogenic signaling through  $\beta$ -catenin is frequently overactivated in HCC and therefore a potential target for a combination therapy with sorafenib. *Materials and Methods:* Two hepatoma cell lines were treated with non-cytotoxic concentrations of sorafenib and different  $\beta$ -catenin inhibitors. The tumor-relevant end-points of proliferation, apoptosis, cell migration, and colony formation were assessed *in vitro* along with the activity of the Wingless/Int-1(WNT)/ $\beta$ -catenin and mitogen-activated protein kinase pathways. *Results:* Combined treatment with sorafenib and  $\beta$ -catenin inhibitors synergized in the inhibition of cell proliferation, migration, colony formation ability, and induction of apoptosis. *Conclusion:* *In vitro* data suggest that the combination of sorafenib and  $\beta$ -catenin inhibition might be a promising approach for HCC treatment.

Hepatocellular carcinoma (HCC) is the most frequent hepatocellular tumor in humans. Due to its often advanced state at the time of diagnosis and its intrinsic resistance against classic cytostatics, treatment options for HCC are very limited, with a mean patient survival of approximately 6 months (1). The only pharmacological treatment which has proven successful in the therapy of advanced HCC is sorafenib (2, 3), a multi-kinase inhibitor, which inhibits, amongst others, the activity of the kinase B-RAF (Rat Fibrosarcoma), an important player in the proliferative mitogen-activated protein kinase (MAPK) signaling pathway. Signaling through the MAPK

pathway is frequently overactivated in HCC (4). Sorafenib treatment, however, extends mean patient survival by only a few months (2). Thus, there is still an urgent need for additional treatment options.

The canonical Wntless/Int-1 (WNT)/ $\beta$ -catenin signaling pathway is an important driver of carcinogenesis in the liver: about one-third of HCC and 50-80% of hepatoblastoma, the most frequent pediatric hepatocellular tumor, display activating mutations in *CTNNB1*, the gene encoding the transcription factor  $\beta$ -catenin. Several reviews present data on  $\beta$ -catenin and its role in liver tumor development (5-8). Unless the pathway is physiologically activated by agonistic WNT molecules, free cytosolic  $\beta$ -catenin is rapidly phosphorylated at amino acid residues near its *N*-terminus, a process which marks the protein for proteasomal degradation. *CTNNB1* mutations mostly eliminate these phosphorylation sites, resulting in a non-degradable, constitutively active protein which accumulates and translocates to the nucleus, where it acts as a co-activator for T-cell factor (TCF) family transcription factors. Even though the exact mechanisms by which  $\beta$ -catenin activation confers a selective advantage to liver tumor cells are not fully-understood, there is compelling evidence that signaling through the canonical WNT/ $\beta$ -catenin pathway regulates tumor cell proliferation and survival (*e.g.* see 9-11). These observations, together with the high frequency of *CTNNB1* mutations in HCC, make the WNT/ $\beta$ -catenin pathway an attractive potential target. Moreover, signaling through WNT/ $\beta$ -catenin is closely-interlaced with the MAPK pathway, the latter being an important oncogenic pathway affected by sorafenib. The interrelation of these two signaling pathways, WNT/ $\beta$ -catenin and MAPK, is complex: according to some reports, both pathways converge synergistically, whereas other studies report antagonistic effects. For a recent review on  $\beta$ -catenin/MAPK crosstalk mechanisms see Zeller *et al.* (12). Sorafenib was shown to inhibit WNT/ $\beta$ -catenin signaling *in vitro* in tumor cell lines and in a xenograft model of HepG2 hepatoma cells, leading to the assumption that sorafenib might exert its effects, at least in parts, by an inhibition of the  $\beta$ -catenin pathway (13, 14). A few recent studies have revealed first evidence for

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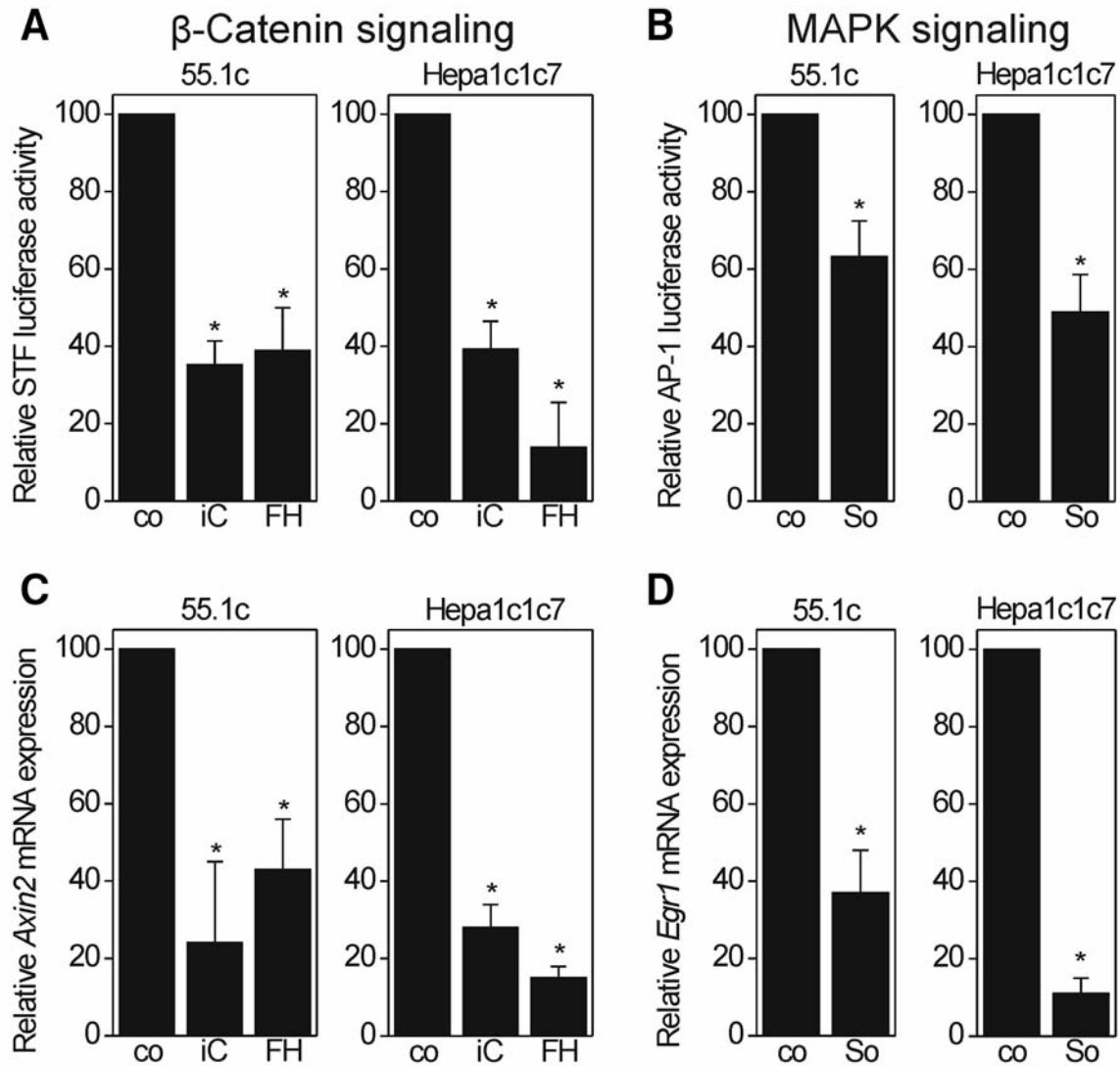


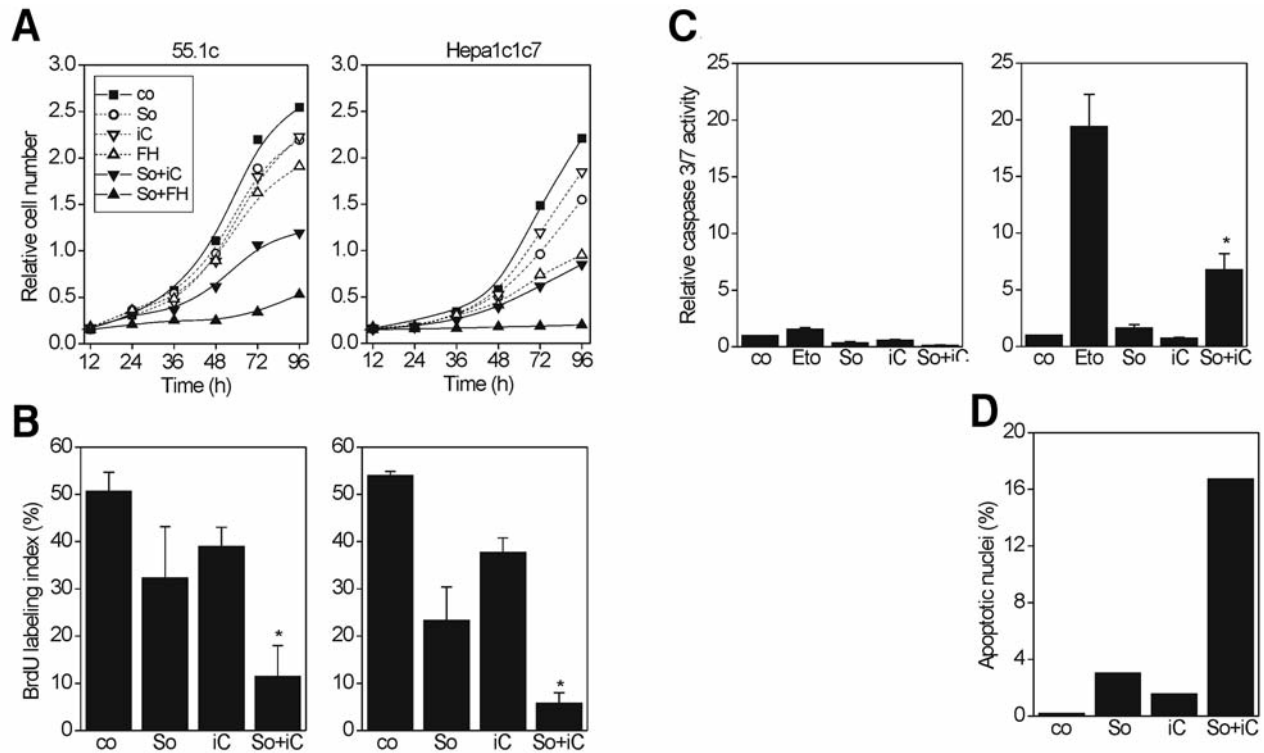
Figure 1. Target pathway inhibition by sorafenib, 2-[[[2-(4-ethylphenyl)-5-methyl-4-oxazolyl]methyl]thio]-N-(2-phenylethyl)acetamide (iCRT3) and N-(2-Methyl-4-nitro)-2,4-dichlorosulfonamide (FH535) in 55.1c and Hepa1c1c7 cells. A: Inhibition of  $\beta$ -catenin-dependent signaling [measured as luciferase activity of the 8 $\times$   $\beta$ -catenin/TCF-driven SuperTopFlash (STF) reporter] by the  $\beta$ -catenin inhibitors iCRT and FH535. B: Inhibition of mitogen-activated protein kinase (MAPK) signaling (measured as luciferase activity of a 6 $\times$  AP-1-driven Gaussia luciferase reporter gene) by sorafenib. C: Inhibition of mRNA expression of the model  $\beta$ -catenin target gene Axin2 by the  $\beta$ -catenin inhibitors iCRT and FH535. D: Inhibition of the model MAPK target gene Early growth response 1 (Egr1) by sorafenib. Data are the mean+SEM of five or more experiments (each performed in triplicate determinations). Statistical significance: \* $p$ <0.05 vs. untreated control (paired t-test). co, Solvent control; So, 2  $\mu$ M sorafenib; iC, 10  $\mu$ M iCRT3; FH, 15  $\mu$ M FH535.

synergistic effects of sorafenib and  $\beta$ -catenin inhibition in tumor cells: destruxin B, a compound which, amongst other effects, inhibits WNT signaling, synergizes with sorafenib in the inhibition of hepatoma cell proliferation (15). Furthermore, liver tumor cell proliferation *in vitro* was also diminished in another recent study with sorafenib and the  $\beta$ -catenin inhibitor FH535 (16). Of note, inhibition of *K-RAS* (Rat sarcoma), upstream in the MAPK pathway, synergizes with  $\beta$ -catenin antagonists in the inhibition of colon cancer cells (17).

In the present study, we investigated the consequences of concomitant treatment of hepatoma cells with  $\beta$ -catenin antagonists and sorafenib. The data show a synergy of treatments with respect to a variety of tumor-relevant end-points.

### Materials and Methods

**Cell lines and treatment.** Murine Hepa1c1c7 and 55.1c cells (18) were cultured in D-MEM/F:12 medium with 10% fetal bovine serum and antibiotics (all purchased from Invitrogen, Karlsruhe,



**Figure 2.** Sorafenib and  $\beta$ -catenin inhibitors synergize in the inhibition of tumor cell growth in vitro in mouse hepatoma cells from lines 55.1c (left column) and Hepa1c1c7 (right column). **A:** Cell growth in the presence and absence of sorafenib,  $\beta$ -catenin inhibitors 2-[[[2-(4-ethylphenyl)-5-methyl-4-oxazolyl]methyl]thio]-N-(2-phenylethyl)acetamide (iCRT3) or N-(2-Methyl-4-nitro)-2,4-dichlorosulfonamide (FH535), or a combination of both was monitored by sulforhodamine B staining for 96 h. Data from a representative experiment (out of four; each performed in octuple determinations) are shown as the mean of eight technical replicates. **B:** Influence of treatment for 24 h on cell proliferation, as measured by BrdU incorporation. Data are the mean  $\pm$  SEM of  $n=3$  experiments. At least 1000 nuclei were counted per culture condition and experiment. **C:** Treatment-dependent induction of apoptosis, as measured by determination of caspase 3/7 activity. Data are the mean  $\pm$  SEM of five experiments (each measured in duplicates). Etoposide was used as a positive control for apoptosis induction. **D:** Validation of caspase activity data by Hoechst33258 staining of apoptotic nuclei in Hepa1c1c7 cells. At least 500 nuclei were counted per treatment group. Statistical significance: \* $p<0.05$  combined vs. both single treatments and combined treatment vs. untreated control (paired *t*-test). co, Solvent control; Eto, 10  $\mu$ M etoposide; So, 2  $\mu$ M sorafenib; iC, 10  $\mu$ M iCRT3; FH, 15  $\mu$ M FH535.

Germany). Cells were incubated in the presence or absence of sorafenib (Enzo, Lörrach, Germany), N-(2-Methyl-4-nitro)-2,4-dichlorosulfonamide (FH535; Merck, Darmstadt, Germany), 2-[[[2-(4-ethylphenyl)-5-methyl-4-oxazolyl]methyl]thio]-N-(2-phenylethyl)acetamide (iCRT3; Merck), or etoposide (Sigma, Taufkirchen, Germany). Controls received the appropriate amount of the solvent dimethylsulfoxide (DMSO) (max. 0.25% v/v). Cells were characterized for activating mutations in the *Ha-ras*, *B-raf*, and *Ctnnb1* proto-oncogenes (19,20).

**Cytotoxicity testing.** A total of 9,000 cells/well were seeded on 96-well plates and allowed to adhere for 24 h, followed by treatment with the test substances for 24 h. Neutral red uptake (NRU), Alamar Blue assays, and cellular ATP determinations were performed using standard methodology as described previously (21). An absence of toxicity was assumed at a viability of >80% in the most sensitive assay.

**Transfection and reporter assay.** A total of 40,000 cells/cm<sup>2</sup> were seeded on 24-well plates 24 h prior to transfection. Using Lipofectamine 2000 (Invitrogen), cells were transfected with the 8x

$\beta$ -Catenin/TCF-driven SuperTopFlash (STF) Firefly luciferase reporter (18), or with a 6x AP-1-driven Gaussia luciferase reporter (22). Firefly luciferase was normalized to Renilla luciferase transcribed from the co-transfected plasmid pRL-CMV (Promega, Mannheim, Germany), which expresses Renilla luciferase under the control of the constitutive cytomegaly virus promoter. Gaussia luciferase was normalized to cell vitality, as determined by the Alamar Blue assay. Luciferase activity assays were conducted according to Braeuning & Vetter (23).

**Gene expression analysis.** RNA isolation and reverse transcription were carried out as previously described (23). Quantitative real-time polymerase chain reaction (PCR) analyses were performed using the FastStart DNA Master SYBR Green I kit (Roche, Mannheim, Germany) on a LightCycler instrument. Primer pairs were as follows: *Axin2\_f* 5'-CGACGCACTGACCGACGATT-3', *Axin2\_r* 5'-TCCAGA CTATGGCGGCTTTCC-3'; *Early growth response 1 (Egr1)\_f* 5'-CGAGCGAACAACCCTATGAG-3', *Egr1\_r* 5'-CGCAGCCGAGT AGATGGGAC-3'; 18s rRNA\_f 5'-CGGCTACCACAT CCAAGGAA-3', 18s rRNA\_r 5'-GCTGGAATTACCGCGGCT-3'. Expression of 18s rRNA was used for normalization according to Pfaffl (24).

**Proliferation assay.** Proliferation was monitored using the sulforhodamine B assay (25): 5,000 cells/well were seeded on 96-well plates; treatment started 12 h later. The assay was run in octuple determinations. A medium change was performed after 48 h.

**BrdU incorporation assay.** Cells were seeded at 20,000 cells/cm<sup>2</sup> on 3.5 cm plates 24 h prior to treatment. Following 22 h of treatment with different substances, BrdU (Applichem, Darmstadt, Germany) was added at a concentration of 100 µg/ml for 2 h. Cells were fixed on ice in acetone:methanol and stained using an antibody against BrdU (1:30 dilution; Dako, Glostrup, Denmark) in combination with a horseradish peroxidase-conjugated anti-mouse secondary antibody (1:100; Sigma) and 3,3'-diaminobenzidine/H<sub>2</sub>O<sub>2</sub> as substrates. BrdU-negative nuclei were visualized by hematoxylin staining.

**Analysis of apoptosis.** Cells were seeded at 20,000 cells/cm<sup>2</sup> on 3.5 cm plates 24 h prior to treatment. After 22 h of treatment, cells were harvested and lysed for determination of caspase activity or used for Hoechst33258 staining. Activity of caspase 3/7 was determined as previously described (26) using the fluorogenic substrate DEVD-AFC (synthetic peptide Asp-Glu-Val-Asp, conjugated to the fluorophore 7-amino-4-trifluoromethylcoumarine; Biomol, Hamburg, Germany). Caspase activity was normalized to protein content, as determined by the Bradford assay. For Hoechst staining, cells were fixed by 3% formaldehyde and stained with 10 µg/ml Hoechst33258 for 20 min. Stained nuclei were analyzed by fluorescence microscopy.

**Colony formation assay.** Following treatment with test substances, cells were trypsinized and vitality was analyzed by trypan blue staining. One hundred viable cells were seeded per 10 cm dish and cultivated for eight days in standard medium without test substances. Macroscopically visible colonies were counted after staining with 0.01% crystal violet.

**Soft agar assay.** Colony formation in 0.5% soft agar was analyzed according to Sinnberg *et al.* (27). Five hundred cells were seeded per cavity of a 24-well plate. Starting 24 h after seeding, cells were incubated with test substances for eight days. Medium was changed after three and six days. Colonies were counted under a light microscope.

**Migration assay.** Scratch assays were performed according to Liang *et al.* (28). In brief, cells were grown to confluence and scratches were applied to the cell layer with a pipet tip. Scratch width was then monitored for up to 36 h.

**Statistical analysis.** Student's t-test (GraphPad Prism software, version 5; GraphPad, La Jolla, CA, USA) was used for the calculation of statistical significance. Differences were considered significant at p<0.05. Statistically significant changes are indicated by asterisks in the diagrams.

**Results**

Two mouse hepatoma cell lines representing the frequent HCC phenotype of constitutive β-catenin activation and high activity of the MAPK cascade were selected for studying the effects of the combination of sorafenib and inhibitors of Wnt/β-catenin signaling. 55.1c and Hepa1c1c7 cells both

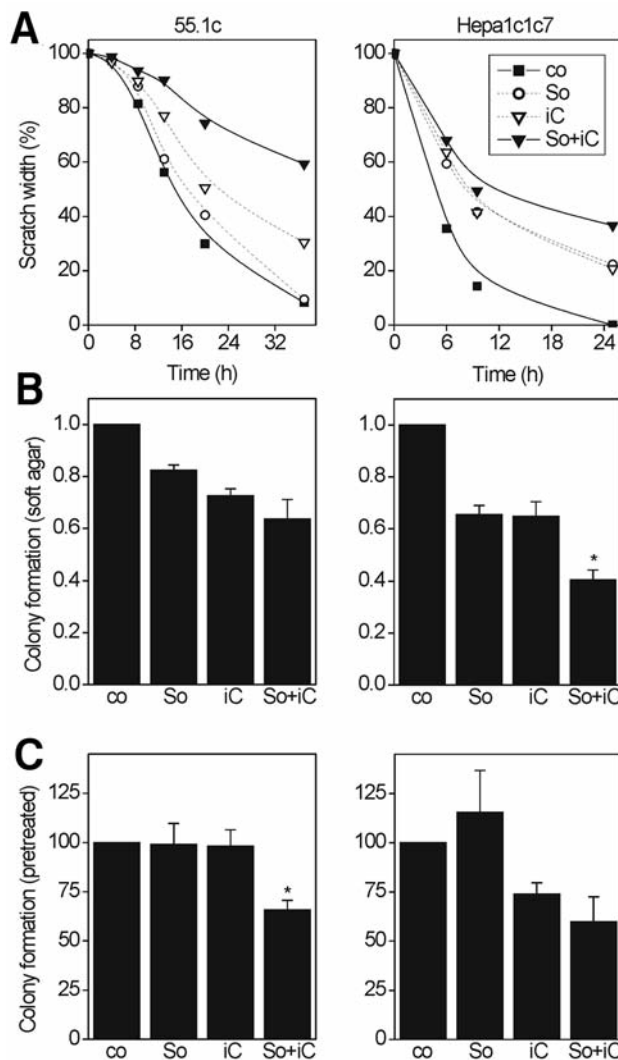


Figure 3. Influence of sorafenib and β-catenin inhibition on cell migration and tumorigenicity of 55.1c (left column) and Hepa1c1c7 (right column) cells. A: Migration was determined by scratch assays in the absence and presence of sorafenib or 2-[[[2-(4-ethylphenyl)-5-methyl-4-oxazolyl]methyl]thio]-N-(2-phenylethyl)acetamide (iCRT3), as indicated. Data from a representative experiment (out of n≥5) are shown for each cell line. B: Colony formation ability on soft agar in relation to treatment. Data are the mean+SEM of four experiments (each in sextuple determinations). C: Colony formation ability of cells pre-treated with sorafenib and/or iCRT3 for 24 h on normal culture dishes. Data are the mean+SEM of three experiments. Statistical significance: \*p<0.05 combined vs. both single treatments and combined treatment vs. untreated control (paired t-test). co, Solvent control; So, 2 µM sorafenib; iC, 10 µM iCRT3.

display high constitutive activity of β-catenin due to the mutational activation of *Ctnnb1* by deletion of exon 3 (29), as well as that of the Ras/Raf/MAPK signaling pathway due to activating mutations in codon 61 of *Ha-ras* (Hepa1c1c7) or codon 637 of *B-raf* (55.1c) (not shown).

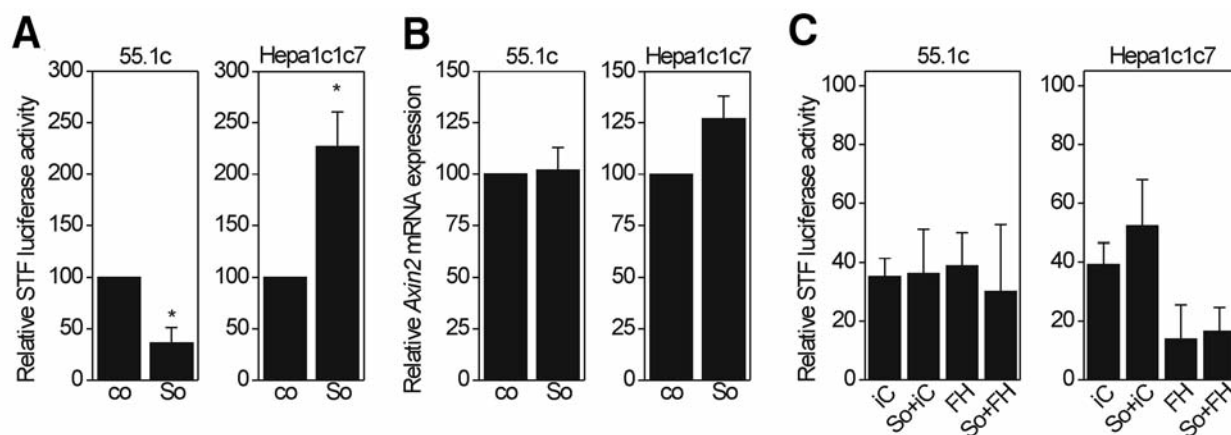


Figure 4. Effects of sorafenib on the  $\beta$ -catenin signaling pathway, as measured by luciferase activity of the  $8\times$   $\beta$ -catenin/TCF-driven SuperTopFlash (STF) reporter (A), and by mRNA expression of the model  $\beta$ -catenin target gene Axin2 (B). Data are the mean+SEM of three or more experiments (each performed in triplicate determinations). C: Influence of single or combined treatment with sorafenib and  $\beta$ -catenin inhibitors on the STF reporter. Statistical significance: \* $p < 0.05$  vs. untreated control (paired t-test). co, Solvent control; So, 2  $\mu$ M sorafenib; iC, 10  $\mu$ M iCRT3; FH, 15  $\mu$ M FH535.

Initially, the highest non-cytotoxic concentrations were selected for each substance (sorafenib,  $\beta$ -catenin inhibitors iCRT3 and FH535), based on the results of NRU, Alamar Blue, and ATP level testing after 24 h of incubation (not shown). At the selected concentrations used in all further assays, a considerable inhibition of either WNT/ $\beta$ -catenin ( $\beta$ -catenin inhibitors) or MAPK-dependent (sorafenib) signaling was visible at the level of reporter gene transactivation and target gene expression (Figure 1).

Cells were incubated with a combination of sorafenib and a  $\beta$ -catenin inhibitor, either iCRT3 or FH535, for up to 96 h to monitor cell growth. Sulforhodamine B staining revealed a synergistic inhibition of cell growth by sorafenib/ $\beta$ -catenin inhibitor combinations in both cell lines (Figure 2A). Similar synergy was observed when single- or double-treated cells were analyzed for proliferation by BrdU incorporation (Figure 2B). A significant synergistic induction of apoptosis upon combined treatment for 24 h was seen in Hepa1c1c7, but not in 55.1c cells (Figure 2C). These results were validated by Hoechst33258 staining of apoptotic nuclei (Figure 2D). Of note, 55.1c cells are generally quite refractory to apoptosis induction (compare the induction of caspase 3/7 activity by the positive control etoposide). In principle, an overadditive induction of apoptosis by double treatment was also inducible in 55.1c, but only at higher concentrations of sorafenib and the  $\beta$ -catenin inhibitors, which already were cytotoxic when applied alone (not shown). Altogether, the data indicate that depending on the cell line and the inhibitor concentrations, both reduced proliferation and induction of apoptotic cell death play a role in the inhibition of tumor cell growth by combinations of sorafenib and  $\beta$ -catenin inhibitors.

We were next interested to examine whether the combination treatment would also affect the migratory ability of the cells. Thus, scratch assays were conducted in the absence and presence of the different inhibitors. A synergistic reduction of migration was observed in both cell lines, with a more pronounced effect in 55.1c (Figure 3A). Data obtained with sorafenib and iCRT3 are depicted; comparable results were obtained with FH535 (not shown). In addition, the effects of sorafenib treatment and  $\beta$ -catenin inhibition on tumorigenicity were assessed in two different assays: firstly, the ability of cells to form colonies in soft agar was analyzed in the presence of sorafenib and concomitant  $\beta$ -catenin inhibition. As evident from Figure 3B, colony growth on soft agar was diminished by treatment with the individual substances alone. Simultaneous incubation of cells with sorafenib plus a  $\beta$ -catenin inhibitor further reduced the colony formation, an effect which was, however, statistically significant only in Hepa1c1c7 cells. Next, the ability of surviving pre-treated cells to divide after cessation of treatment and to form colonies on normal cell culture dishes was assessed. In 55.1c cells, combination treatment inhibited the colony formation rate, while the single compounds did not produce any effect (Figure 3C). In Hepa1c1c7, colony formation was also lowest in double-treated cells, but not significantly different from treatment with the  $\beta$ -catenin inhibitor alone.

Recent publications indicate that sorafenib is capable of inhibiting signaling through the WNT/ $\beta$ -catenin pathway (13, 14). We, therefore, checked for effects of sorafenib on the activity of the  $\beta$ -catenin-dependent STF luciferase reporter, as well as on an endogenous  $\beta$ -catenin target gene, Axin2. As shown in Figure 4A, sorafenib inhibited  $\beta$ -catenin

reporter activity in 55.1c cells, whereas it had an opposite effect in cell line Hepa1c7. At the target mRNA level, no remarkable effects were detectable (Figure 4B), indicating that no major alterations of WNT/ $\beta$ -catenin signaling activity are caused by sorafenib in the cell lines analyzed. Suppression of  $\beta$ -catenin signaling by a combination of  $\beta$ -catenin inhibitors and sorafenib was not different from pathway inhibition by  $\beta$ -catenin inhibitors alone (Figure 4C).

## Discussion

In synopsis, the data presented here demonstrate that the combination of sorafenib and one of the pharmacological WNT/ $\beta$ -catenin inhibitors iCRT3 and FH535 synergizes *in vitro* in hepatoma cell lines with respect to important tumor-relevant parameters, namely proliferation, cell death, migration, and colony formation ability. Our results are in line with recent published data on synergistic effects of sorafenib and one of the  $\beta$ -catenin inhibitors, FH535, on liver tumor cell proliferation (16), as well as with a study reporting a synergism of destruxin B and sorafenib (15). The present study, however, goes beyond previously published results by analyzing new end-points, such as migration, colony formation, and apoptosis in different hepatoma cell lines. Importantly, our present results demonstrate marked synergistic effects of combination treatment at substance concentrations which exert no or only minor effects when administered alone. Previous studies have shown a reduction of  $\beta$ -catenin activity in hepatoma cells by sorafenib (13, 14) and it has been suggested that at least some effects of the drug might be related to an inhibition of oncogenic WNT/ $\beta$ -catenin signaling. Present data show that sorafenib, at the concentration applied, does not remarkably interfere with the expression of target genes of the  $\beta$ -catenin pathway. Activation of a  $\beta$ -catenin-dependent reporter gene, which in our experience is more sensitive to variations of the pathway, is differentially affected by sorafenib in the two cell lines, regardless of similar consequences on cell proliferation and other parameters. Moreover,  $\beta$ -catenin pathway activity was comparable in cells treated with a  $\beta$ -catenin alone or with a combination of a  $\beta$ -catenin inhibitor and sorafenib. This indicates that the synergistic effects observed in the present study are not solely dependent on additive inhibitory effects on the  $\beta$ -catenin pathway and that the Wnt/ $\beta$ -catenin pathway is not the major downstream signaling pathway for the cellular effects exerted by sorafenib. Altogether, the present *in vitro* data constitute a promising basis for further investigation of  $\beta$ -catenin inhibitor/sorafenib combination approaches *in vivo*.

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