

ORIGINAL ARTICLE

FTY720 (Fingolimod) sensitizes hepatocellular carcinoma cells to sorafenib-mediated cytotoxicityDilruba Ahmed¹, Petra J. de Verdier¹, Charlotta Ryk², Oscar Lunqe¹, Per Stål³ & Jenny Flygare¹¹Division of Clinical Chemistry, Department of Laboratory Medicine, Karolinska Institutet, Huddinge, Stockholm, Sweden²Urology Laboratory, Department of Molecular Medicine and Surgery, Karolinska Institutet, 171 76 Stockholm, Sweden³Division of Gastroenterology and Hepatology, Department of Medicine, Karolinska Institutet, Karolinska University Hospital Huddinge, Stockholm, Sweden**Keywords**

Cytotoxicity, FTY720, hepatocellular carcinoma, sensitization, sorafenib.

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Abstract

Hepatocellular carcinoma (HCC) is one of the leading causes of cancer-related death worldwide. The multityrosine kinase inhibitor sorafenib is used in the therapy of advanced disease. However, the effects of sorafenib are limited, and combination treatments aiming at improved survival are encouraged. The sphingosine analog FTY720 (Fingolimod), which is approved for treatment of multiple sclerosis, has shown tumor suppressive effects in cell lines and animal models of HCC. In the present study, we combined sorafenib with FTY720 in order to sensitize the HCC cell lines Huh7 and HepG2 to sorafenib treatment. Using the XTT assay we show that noncytotoxic doses of FTY720 synergistically enhanced the decrease in viability caused by treatment of both cell lines with increasing doses of sorafenib. Further studies in Huh7 revealed that combined treatment with FTY720 and sorafenib resulted in G1 arrest and enhanced cell death measured using flow cytometry analysis of cells labeled with propidium iodide (PI)/Annexin-V and PI and 4',6-diamidino-2-phenylindole-staining of nuclei. In addition, signs of both caspase-dependent and -independent apoptosis were observed, as cotreatment with FTY720 and sorafenib caused cytochrome *c* release and poly-ADP ribose polymerase-cleavage as well as translocation of Apoptosis-inducing factor into the cytosol. We also detected features of autophagy blockage, as the protein levels of LC3-II and p62 were affected by combined treatment with FTY720 and sorafenib. Together, our results suggest that FTY720 sensitizes HCC cells to cytotoxic effects induced by treatment with sorafenib alone. These findings warrant further investigations of combined treatment with sorafenib and FTY720 in vivo in order to develop more effective treatment of HCC.

Abbreviations

HCC, hepatocellular carcinoma; S1P, sphingosine 1-phosphate; PARP, poly-ADP ribose polymerase; LC3II, light chain 3II; AIF, apoptosis-inducing factor; Cox IV, cytochrome *c* oxidase; PI, propidium iodide; DAPI, 4',6-diamidino-2-phenylindole; DTT, dithiothreitol.

Introduction

Hepatocellular carcinoma (HCC) is the most common type of liver cancer and the third cause of cancer-related mortality worldwide (Jemal et al. 2011). HCC is generally diagnosed at a late stage, and the majority of HCC

patients die within 1 year after diagnosis. The only curative treatment options are resection and transplantation, but recurrence rates are high (Forner et al. 2012). HCC is highly resistant to conventional chemotherapy, and targeted therapy with the multi-kinase inhibitor sorafenib was a breakthrough in the treatment of unresectable,

advanced HCC (Llovet *et al.* 2007). Sorafenib blocks tumor cell proliferation and angiogenesis mainly by targeting Raf kinases and vascular endothelial growth factor (VEGF)/platelet-derived growth factor (PDGF) receptor tyrosine kinases (Wilhelm *et al.* 2004; Liu *et al.* 2006). Based on the results of a large randomized phase III study, sorafenib is presently the standard systemic therapy for HCC patients with advanced disease, whereas other targeted therapies for treatment of HCC are under development (Worns and Galle 2010). However, the improved median survival of HCC patients treated with sorafenib is limited to 3 months (Llovet *et al.* 2007), and therefore combination therapies with sorafenib were encouraged in the future clinical trial design for HCC (Llovet *et al.* 2008). Aiming at prolonged survival, both preclinical (Chen *et al.* 2014; Zhang *et al.* 2014) and clinical studies (Abdel-Rahman and Fouad 2014) of sorafenib-based combination treatments have been performed.

There is accumulating evidence for the involvement of sphingosine 1-phosphate (S1P) and sphingosine kinase 1 (SK1) in cancer (Pyne and Pyne 2010). SK1 converts tumor suppressive sphingosine to S1P, which promotes proliferation, migration, angiogenesis and metastasis. The sphingosine analog FTY720 (Fingolimod, Gilenya™ Stein, Switzerland) is an immunosuppressant that has recently been approved by the U.S. Food and Drug Administration and the European Medicines Agency for the treatment of relapsing multiple sclerosis. In addition to its immunomodulatory effects, FTY720 acts as a competitive inhibitor to SK1 and targets the enzyme for degradation (Tonelli *et al.* 2010), thereby preventing the conversion of sphingosine to S1P. Sphingosine kinase 2 converts FTY720 to (S)-FTY720 phosphate, which inhibits the effects of S1P signaling by acting directly as a functional antagonist to the S1P1 receptor (Brinkmann *et al.* 2002). FTY720 also exerts additional S1P receptor-independent antitumor effects (Brinkmann *et al.* 2001; Pyne and Pyne 2010; Pitman *et al.* 2012). Combined treatment with sorafenib and a synthetic inhibitor to SK2 has earlier shown antitumor activity in HCC xenografts (Beljanski *et al.* 2011).

FTY720 has shown antitumor and antiangiogenic effects in animal models of breast cancer, prostate cancer, lung cancer and malignant melanoma (Azuma *et al.* 2002; Chua *et al.* 2005; Schmid *et al.* 2005; LaMontagne *et al.* 2006; Zhou *et al.* 2006; Pereira *et al.* 2013; Saddoughi *et al.* 2013). Moreover, FTY720 has been shown to selectively induce cytotoxicity in hepatoma cell lines, and to suppress tumor growth with no apparent side-effects in a mouse model of HCC (Lee *et al.* 2004). Whereas the cytostatic and antiangiogenic effects of sorafenib counteract tumor development, sorafenib has also been shown to promote tumor invasiveness and metastasis in a mouse model of HCC (Zhang *et al.* 2012). In contrast, treatment with FTY720

suppressed liver tumor metastasis after liver transplantation and resection in rat models of HCC (Ushitora *et al.* 2009; Li *et al.* 2012). FTY720 has also been shown to enhance the effects of chemotherapeutic treatment of cell lines and animal models representing breast cancer, lymphoma, neuroblastoma, and prostate cancer cells (Pchejetski *et al.* 2010; Alinari *et al.* 2011; Mousseau *et al.* 2012; Li *et al.* 2013; Marvaso *et al.* 2014). In view of these observations, we hypothesized that sorafenib and FTY720 could act synergistically to suppress survival and growth of HCC cells, and therefore investigated the cytotoxic effects of combined treatment of these two compounds on HCC cell lines.

Materials and Methods

Sorafenib tosylate (BAY 54-9085) was provided by Bayer HealthCare Pharmaceuticals (Montville, NJ), FTY720 (Fingolimod) was purchased from Cayman Chemical Ann Arbor, Michigan, USA. (MI). Stock solutions of 100 mmol/L sorafenib and of 20 mmol/L FTY720 dissolved in Dimethyl sulfoxide (DMSO) were aliquoted and deep-frozen. Primary antibodies against poly-ADP ribose polymerase (PARP) and light chain 3II (LC3II) were from Cell Signaling (Cell Signaling Technology Inc., Danvers, MA). p62 from Sigma-Aldrich LLC. (St. Louis, MO), Apoptosis-inducing factor (AIF) from Santa Cruz Biotechnology, Inc. (Texas, USA), cytochrome *c* oxidase (Cox IV) from Novus Biologicals, LLC, (Littleton, Colorado, USA), Cytochrome *c* (Cyt *c*) from BD Bioscience (San Jose, CA), α -Tubulin and β -Actin from Sigma-Aldrich Co. LLC. were used. Secondary fluorescent antibodies, goat anti-rabbit and goat anti-mouse, were purchased from LI-COR® (Nebraska, USA). Cell culture medium and supplements were purchased from Invitrogen (Eugene, OR).

Cell culture

The human hepatocellular-carcinoma cell line Huh7 was purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany) and HepG2 cell line was purchased from ATCC. Huh7 cells were cultured in Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, penicillin 100 U/mL and streptomycin 100 μ g/mL at 37°C in an atmosphere containing 5% CO₂, whereas HepG2 cells were cultured in DMEM supplemented with 10% fetal bovine serum at 37°C in an atmosphere containing 5% CO₂.

Viability assay

Viability/cytotoxicity caused by sorafenib and FTY720 was determined using the cell proliferation kit II

((2,3-Bis-(2-Methoxy-4-Nitro-5-Sulphophenyl)-2H-Tetrazolium-5-Carboxanilide) XTT) from Roche Diagnostics (Basel, Switzerland) according to the manufacturer's instructions. The cells were seeded at a density of 10×10^3 , 6×10^3 or 5×10^3 cells per well for Huh7 cell line and 15×10^3 , 10×10^3 or 8×10^3 cells per well for HepG2 cell line in 96-well plates depending on the treatment time and allowed to adhere overnight. Subsequently the cells were treated for 24, 48 and 72 h with different doses of sorafenib alone or in combination with FTY720 or vehicle (0.04% DMSO). In the final step they were incubated with the XTT solution for two hours. The optical density reflecting viable cells was obtained by measuring the absorbance at 470 nm and subtracting the background absorbance at 750 nm. Dose-response curves were constructed for treatment with FTY720. The least cytotoxic dose, which had a marginal effect on the viability of cells when used as a single agent, was determined from two individual experiments by comparing the top and bottom plateaus of the dose-response curve. In order to study sensitization of HCC cells to treatment with sorafenib, this dose was used in the subsequent experiments.

Cell death analysis by flow cytometry

The Annexin-V-FLUOS Staining Kit, ROCHE Diagnostics GmbH (Mannheim, Germany) was used to distinguish between viable and dead cells following treatment with sorafenib in the presence or absence of FTY720. Huh7 cells were seeded at a density of 8.0×10^5 in 25 cm² flasks and allowed to adhere overnight. Thereafter, the cells were treated for 6, 12, or 24 h with different doses of sorafenib/sorafenib and FTY720 or DMSO as indicated in the Results section. Cells were harvested and labeled with annexin-V and propidium iodide (PI) according to the manufacturer's instruction and analyzed by flow cytometry in BD FACS caliber (BD Bioscience).

Cell cycle analysis by flow cytometry

The BD Cycletest Plus reagent kit, BD Biosciences was used to determine the cell cycle distribution and sub G1 fractions. Huh7 cells were seeded at the densities of 4.0×10^5 , 4.8×10^5 or 8.0×10^5 cells in 25 cm² flasks depending on the treatment time and allowed to adhere overnight. Subsequently the cells were treated for 24 and 48 h with different doses of sorafenib alone or in combination with FTY720 or vehicle (0.04% DMSO). The cells were harvested and stained with PI according to the manufacturer's instruction. Cell cycle distribution was analyzed using BD LSRIL (BD Bioscience) and the data were analyzed using the ModFit LT v3.3, (BD Bioscience).

Western blot analysis

Whole cell protein was extracted from the Huh7 cells, using NP-40 lysis buffer (Invitrogen) containing 50 mmol/L Tris, 250 mmol/L NaCl, 5 mmol/L Ethylenediaminetetraacetic acid (EDTA), 50 mmol/L NaF, 1 mmol/L Na₃VO₄, 1% NP-40 and 0.02% NaN₃, to which protease inhibitor cocktail p8340 had been added. Protein concentration was measured using Biorad DC protein concentration determination (Bio-Rad Laboratories Inc., Berkeley, California, USA) and 100 µg of total protein was separated with Nu-PAGE (Invitrogen) using gels of different concentrations depending on the size of the protein of interest. Proteins were transferred to either PVDF (Immobilon[®]-P; Millipore, Billerica, MA) or to nitrocellulose membranes (Thermo Scientific, Waltham, Massachusetts, USA) at 33 mA for 1 h using a semidry blotter Hoefer[™] TE 70 (Amersham Biosciences Corp. New Jersey, USA). The membranes were blocked in 5% non-fat milk solution in Phosphate-buffered saline (PBS)-T (0, 1% Tween 20) at room temperature for 1 h before incubation with individual primary antibodies at 4°C over-night. The membranes were washed for 6 × 5 min with PBS-T followed by 1 h incubation with HRP/Fluorescence-conjugated secondary antibodies at room temperature. HRP-conjugated antibodies were detected using Supersignal west Femto Maximum Sensitivity Substrate (Thermo Scientific) whereas Fluorescence conjugated antibodies were imaged directly using LICOR imaging system (LI-COR Biosciences, Ltd., Nebraska, USA). Relative density from western blots was calculated using ImageJ 1.48v software, background was subtracted from individual bands.

Confocal microscopy

Huh7 cells were grown on sterile coverslips in six-well plates at the density of 3.0×10^5 cells overnight. Cells were then treated by different doses of sorafenib in the presence or absence of FTY720 for 24 h. Thereafter, the cells were fixed with ice-cold methanol, permeabilized with 3:1 ethanol: acetic acid solution. Cells were blocked with 5% BSA in PBS for 1 h at room temperature. Preparations were mounted on slides using mounting medium containing 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Inc. Burlingame, CA) and examined with a confocal microscope (Nikon Confocal Microscope; Nikon Corporation, Tokyo, Japan).

Cytosolic and mitochondrial fraction

Harvested Huh7 cells (2×10^6) were washed in ice-cold PBS and incubated for 25 min on ice with 300 µL of ice cold buffer A (250 mmol/L Sucrose, 20 mmol/L (4-(2-hydroxyethyl)-1-piperazineethanesulfonic

acid) HEPES-KOH (pH 7.4), 10 mmol/L KCl, 1 mmol/L Na-EGTA, 1 mmol/L Na-EDTA, 1.5 mmol/L MgCl₂, 1 mmol/L dithiothreitol (DTT), 0.1 mmol/L phenylmethylsulfonyl fluoride (PMSF), and cocktail of protease inhibitors [Sigma-Aldrich Co. LLC.]. Cells were then lysed with 30 strokes using a syringe fitted with a needle (22 gauges and 30 mm) and centrifuged at 4°C (600 g). The supernatant was centrifuged at 21,000g for 10 min. The supernatant (cytosol) was saved at -20°C and the pellet (mitochondria) was incubated for 20 min on ice in 100 μL of buffer B (50 mmol/L HEPES (pH 7.4), 1% Nonidet P-40 (NP-40), 10% (v/v) glycerol, 1 mmol/L Na-EDTA, 2 mmol/L DTT, 0.1 mmol/L PMSF, and cocktail of protease inhibitors [Sigma-Aldrich Co. LLC.]). Samples were centrifuged at 21,000g for 15 min, the supernatant containing mitochondrial proteins was stored at -20°C. 40 μg protein of each fraction were electrophoresed in 4–10% precasted SDS-PAGE gel (Thermo Scientific) prior to immunoblotting (see previous section).

Statistical analyses

Paired *T*-test was performed to assess the difference in viability between cells treated with sorafenib alone and with sorafenib and 8 μmol/L or 2 μmol/L of FTY720. Differences in cell death and cell cycle distribution between groups were analyzed by analysis of variance, with the least significance difference (LSD) as *post hoc*. **P* < 0.05, ***P* < 0.005, ****P* < 0.0005.

Results

Increased cytotoxicity after combined treatment with sorafenib and FTY720

FTY720 has earlier shown cytotoxicity toward HCC cells (Lee *et al.* 2004; Ushitora *et al.* 2009; Li *et al.* 2012). In order to investigate whether FTY720 could sensitize HCC cells to treatment with sorafenib, we initially titrated the

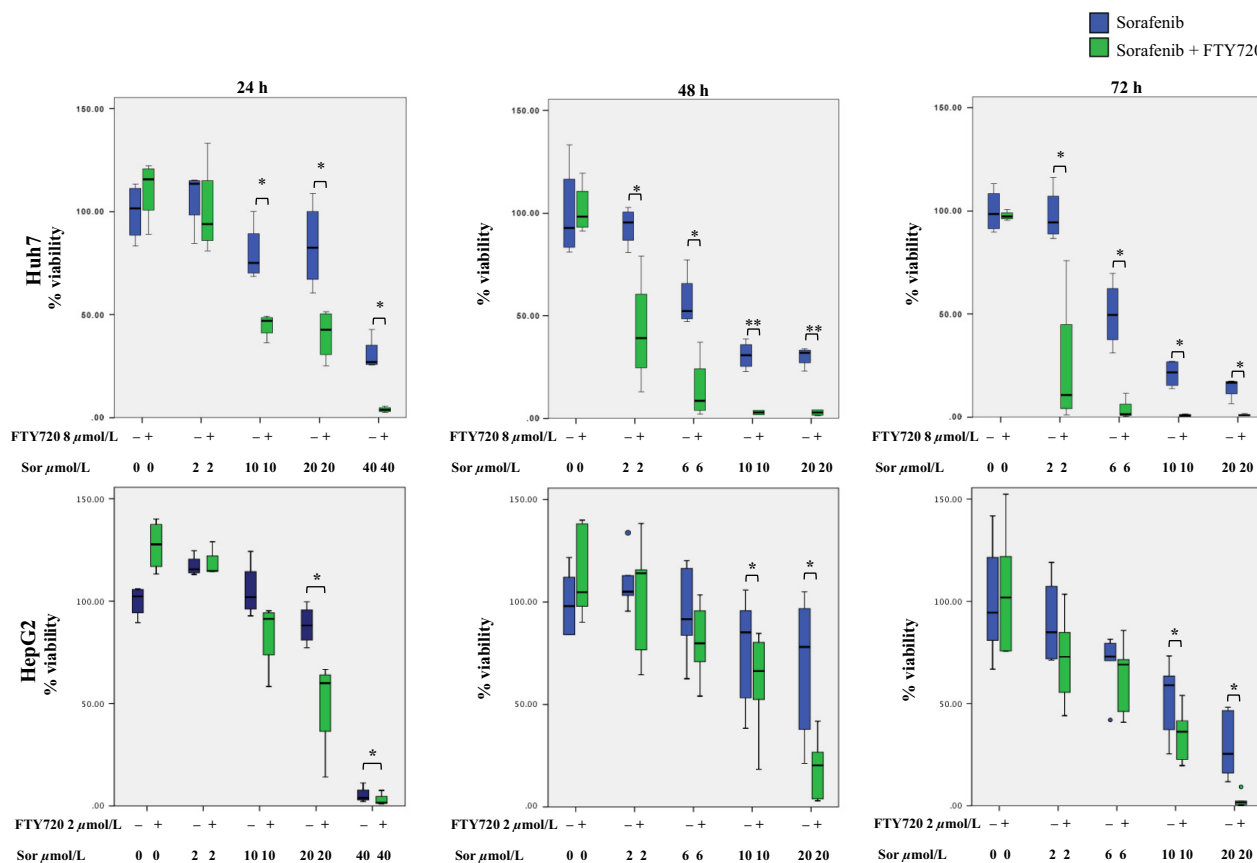


Figure 1. Cell viability following combined treatment with sorafenib and FTY720. Huh7 and HepG2 cells were treated with increasing doses of sorafenib in the presence or absence of 8 or 2 μmol/L of FTY720, respectively for 24, 48 or 72 h, where after XTT assay was performed. The graphs are based on at least four individual experiments. The boxes represent the interquartile range (IQR) for each treatment, the bands represent the median, while the whiskers represent the highest and lowest values, respectively, that are not considered outliers. Outliers that are 1.5× the IQR or more are presented as circles. The paired *T*-test was performed to assess the difference in viability between cells treated with sorafenib alone and with sorafenib and 8 or 2 μmol/L of FTY720. **P* < 0.05, ***P* < 0.005, ****P* < 0.0005.

dose of FTY720 using the XTT assay as an estimation of viability. The least cytotoxic dose for FTY720 was determined to be 8 and 2 $\mu\text{mol/L}$ for Huh7 and HepG2, respectively (see Materials and Methods). The decreased viability induced by treatment of HCC cells with increasing doses of sorafenib for 24, 48 or 72 h was significantly enhanced after cotreatment of Huh7 and HepG2 with 8 $\mu\text{mol/L}$ or 2 $\mu\text{mol/L}$ of FTY720 respectively, Figure 1. The effect increased over time and was more pronounced in Huh7 as compared to HepG2. These results indicate that FTY720 sensitizes HCC cells to cytotoxicity induced by sorafenib.

Effects on cell cycle distribution following combined treatment with sorafenib and FTY720

In order to examine the mechanisms behind the enhanced cytotoxicity following combined treatment, further studies

were performed in the Huh7 cell line, which, similarly to a majority of primary HCC, and in contrast to HepG2, harbor mutated p53 (Bressac et al. 1990). To investigate whether the cell cycle distribution was affected by the combined treatment, Huh7 cells were treated with sorafenib in the presence or absence of FTY720 for 24 or 48 h. After both 24 and 48 h, combined treatment with 2 $\mu\text{mol/L}$ of sorafenib and 8 $\mu\text{mol/L}$ of FTY720 caused a significant accumulation of cells in the G1 phase and a concomitant decrease of the number of cells in the S phase compared to treatment with sorafenib alone, Figure 2. In contrast, at higher doses of sorafenib, cotreatment with 8 $\mu\text{mol/L}$ of FTY720 caused a significant decrease or increase of cells in the G2 phase after 24 or 48 h, respectively. At the latter time point, FTY720 also significantly increased the sub G1 fraction, representing dead cells, observed after treatment with 6 $\mu\text{mol/L}$ or 10 $\mu\text{mol/L}$ doses of sorafenib alone, Figure 2.

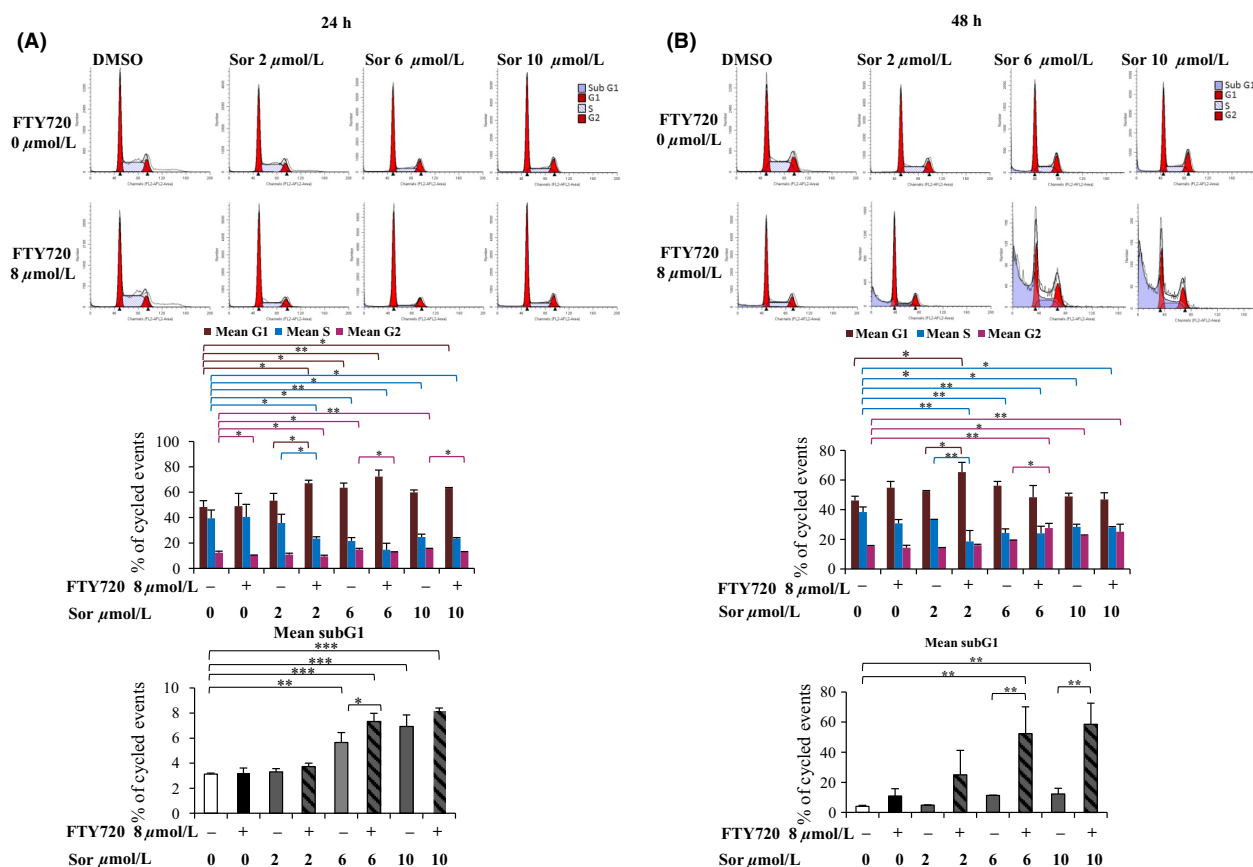


Figure 2. Cell cycle distribution following combined treatment with sorafenib and FTY720. Huh7 cells were treated with sorafenib in the absence or presence of 8 $\mu\text{mol/L}$ FTY720 and stained with propidium iodide prior to analysis by flow cytometry for (A) 24 h or (B) 48 h. Cytoplots show one representative experiment out of two (upper part). The columns in the histograms (lower part) show mean of two individual experiments, error bars represent standard deviation. Differences between groups were analyzed by analysis of variance, with the least significance difference as *post hoc*. * $P < 0.05$, ** $P < 0.005$, *** $P < 0.0005$.

Together, the cell cycle analysis shows synergistic effects of combined treatment with FTY720 and sorafenib on both cell cycle progression and cell death.

Induction of apoptosis after combined treatment with sorafenib and FTY720

To further investigate the mechanisms behind the cytotoxic effect of combined treatment with sorafenib and FTY720, early signs of apoptosis were assessed by flow cytometry analysis of cells labeled with Annexin-V-Fluos and PI. A significant increase in presumably apoptotic, secondary necrotic and necrotic cells in the lower right, upper right and upper left quadrants, respectively, was observed after combined treatment with 10 $\mu\text{mol/L}$ of sorafenib and 8 $\mu\text{mol/L}$ of FTY720 as compared to treatment with sorafenib alone for 6 hours, Figure 3. Condensation of nuclei was observed after treatment with 10 and 20 $\mu\text{mol/L}$ of sorafenib for 24 h, and was further enhanced after cotreatment with 8 $\mu\text{mol/L}$ of FTY, Figure 4A. In order to investigate different apoptotic pathways, cleaved PARP and cytosolic AIF, markers for caspase-dependent and -independent apoptosis, respectively, were analyzed by western blotting. Treatment with a high dose of sorafenib (20 $\mu\text{mol/L}$) for 24 h caused cleavage of PARP in the absence of FTY720, whereas lower doses did not, Figure 4B. The ratio of cleaved

PARP increased when the cells were treated with 20 $\mu\text{mol/L}$ of sorafenib together with 8 $\mu\text{mol/L}$ of FTY720. Furthermore, a weak band representing cleaved PARP was observed when 10 $\mu\text{mol/L}$ of sorafenib was combined with 8 $\mu\text{mol/L}$ of FTY720. Treatment with either 2 or 10 $\mu\text{mol/L}$ of sorafenib or 8 $\mu\text{mol/L}$ of FTY720 alone for 24 h induced a slight release of AIF into the cytosol, which was further increased after combination treatment, Figure 4C. The purity of the mitochondrial and cytosolic fractions was confirmed using the mitochondrial and cytoskeletal markers COX IV and β -actin, respectively. The expression of COX IV, declined after treatment with 10 $\mu\text{mol/L}$ sorafenib \pm 8 $\mu\text{mol/L}$ FTY720. The role of mitochondria-mediated apoptosis was supported by release of Cyt *c* into the cytosol. Taken together, these results indicate that the induction of cell death by combined treatment with sorafenib and FTY720 could partly be attributed to mitochondria-mediated apoptosis in both caspase-independent and, at higher doses of sorafenib, caspase-dependent manners.

Effect of combined treatment with sorafenib and FTY720 on proteins involved in autophagy

The earlier observed opposing effects of sorafenib and FTY720 on autophagy directed us to investigate hallmarks

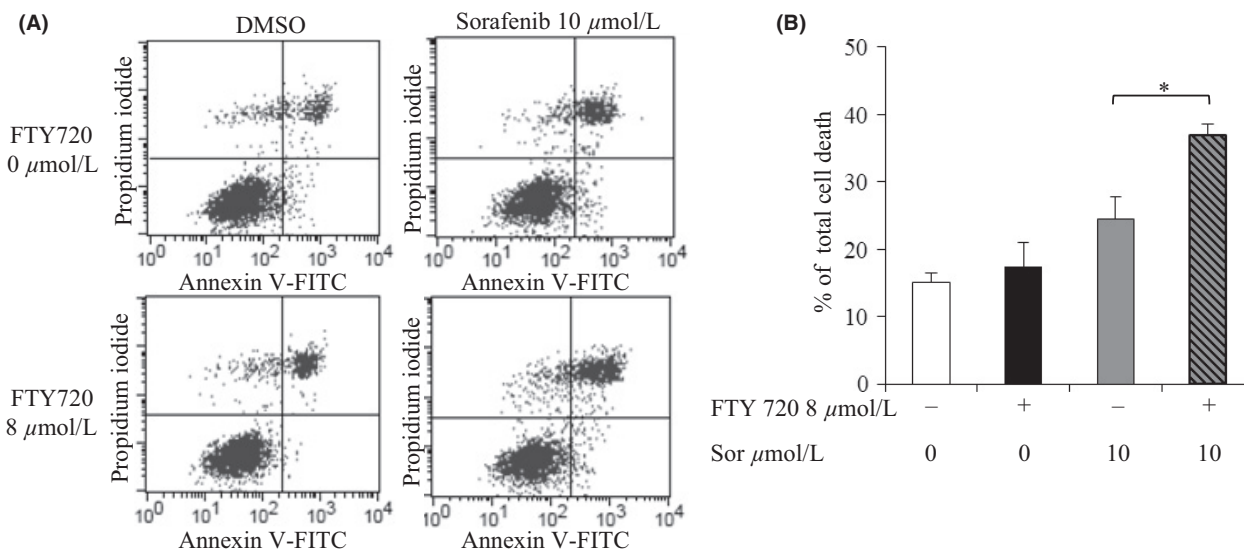


Figure 3. Induction of cell death following treatment with sorafenib and FTY720. Huh7 cells were exposed to 10 $\mu\text{mol/L}$ of sorafenib and/or 8 $\mu\text{mol/L}$ of FTY720 for 6 h and stained with FITC-Annexin V and propidium iodide (PI) prior to analysis by flow cytometry. (A) Cytograms show one representative experiment out of two, the percentages show unaffected cells which are negative for both stains (lower left quadrant), early apoptotic cells stained with Annexin V (lower right quadrant), late apoptotic cells stained with both Annexin V and PI (upper right quadrant) and necrotic cells stained with only PI (upper left quadrant). (B) Histogram showing total percentage of apoptotic and necrotic cells which were stained with Annexin V, PI or both, mean of two individual experiments. Error bars represent standard deviation. Differences between groups were analyzed by analysis of variance, with the least significance difference as *post hoc*. * $P < 0.05$.

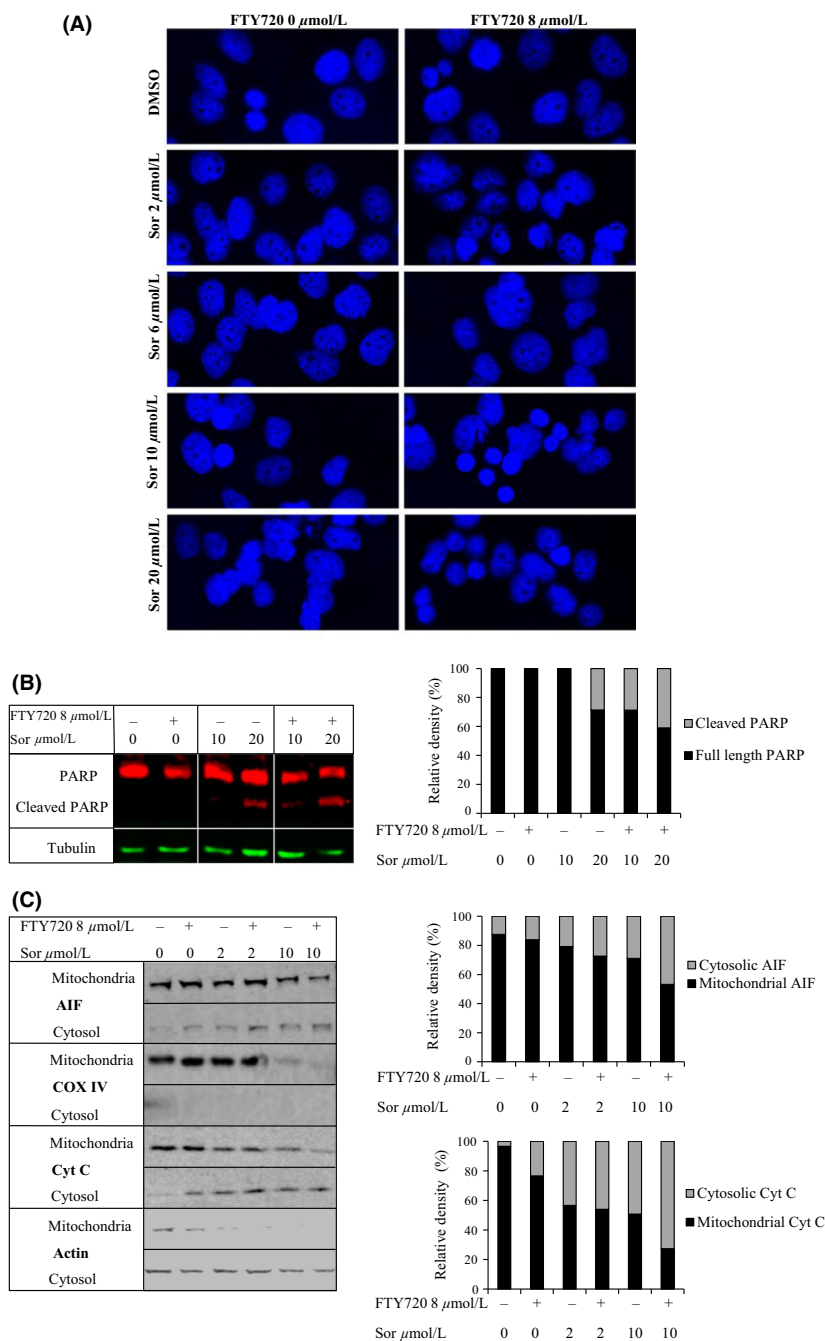


Figure 4. Apoptosis following combined treatment with sorafenib and FTY720. (A) Huh7 cells were treated with increasing doses of sorafenib in the presence or absence of 8 $\mu\text{mol/L}$ FTY720 for 24 h, fixed with ice-cold methanol and stained with 4',6-diamidino-2-phenylindole (DAPI) prior to visualization of nuclei (in blue) by confocal microscopy. (B) Western blot showing poly-ADP ribose polymerase (PARP) cleavage as an indicator of caspase-mediated apoptosis following treatment with 10 or 20 $\mu\text{mol/L}$ of sorafenib in the presence or absence of 8 $\mu\text{mol/L}$ FTY720. Tubulin was used as a loading control. In the histogram relative density was used to calculate the percentage of cleaved PARP compared to total PARP. (C) Western blots showing translocation of apoptosis-inducing factor (AIF) from mitochondria into the cytosol as an indicator of caspase-independent apoptosis. Huh7 cells were treated with 2 or 10 $\mu\text{mol/L}$ of sorafenib in the presence or absence of 8 $\mu\text{mol/L}$ FTY720 for 24 h, fractionated into mitochondrial and cytosolic compartments and analyzed for the protein levels of AIF and cytochrome c. In the histogram, relative density was used to calculate the percentages of cytosolic and mitochondrial fractions for both proteins. β -actin was used as cytoskeletal and COX IV as mitochondrial control. In all cases, one of two individual experiments is shown.

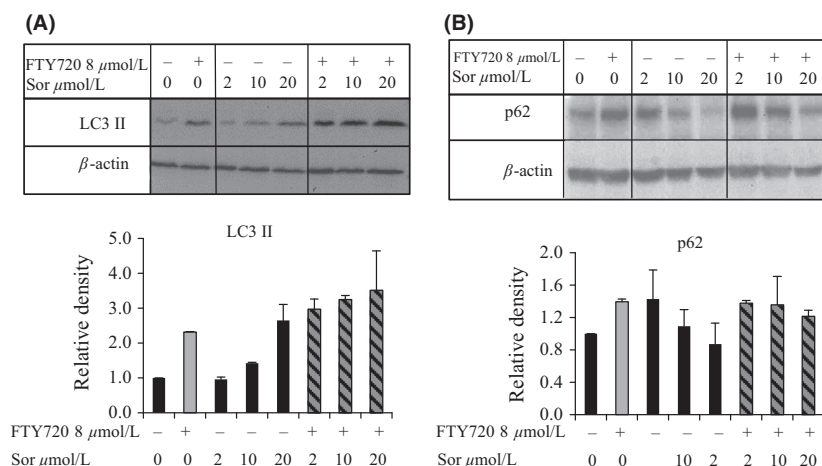


Figure 5. Western blots showing the levels of autophagic proteins following cotreatment with sorafenib and FTY720. Huh7 cells were treated with increasing doses of sorafenib with or without 8 $\mu\text{mol/L}$ FTY720 for 24 h. Protein levels of (A) LC3-II and (B) p62 are shown in the blot and in the histograms. Relative densities from two different experiments are presented in the histograms. Values are normalized against vehicle control as well as β -actin. Error bars represent standard deviation.

of this cellular process following combined treatment of Huh7 cells with sorafenib and FTY720. Treatment with high doses of sorafenib alone caused increased levels of LC3II protein concurrently with decreased p62 protein levels, Figure 5. In contrast, an increase in both LC3II and p62 protein levels was observed following treatment with FTY720 alone. Cotreatment with FTY720 further increased the induction of LC3II protein observed after treatment with sorafenib alone, and attenuated the sorafenib-induced decrease in p62 protein levels. These results indicate that sorafenib may induce autophagosome formation, whereas FTY720 may inhibit the fusion of autophagosomes with lysosomes.

Discussion

At present, sorafenib is the standard systemic therapy for treatment of patients with advanced HCC. Combination of sorafenib with compounds that target alternate mechanisms of action may lead to new therapeutic options resulting in decreased risk for metastasis and increased survival. Among the combination therapies undergoing clinical trials (Villanueva and Llovet 2011), analogs of bioactive sphingolipids are currently not being tested in combination with sorafenib, despite their suggested potential as anticancer agents (Pyne *et al.* 2011; Delgado *et al.* 2012). In this study, we have investigated the effects of combined treatment of HCC cells with sorafenib and the sphingosine analog FTY720, which is approved for treatment of relapsing multiple sclerosis. We have presently shown that the sphingosine analog FTY720 sensitized HCC cells to treatment with sorafenib. At doses of FTY720 that itself showed

no cytotoxicity, cotreatment with sorafenib significantly increased the effects on viability, cell cycle progression and cell death observed after treatment with sorafenib alone. Furthermore, whereas sorafenib alone has been shown to favor cancer cell survival by inducing autophagy (Shi *et al.* 2011; Shimizu *et al.* 2012), the autophagic process appeared to be blocked by cotreatment with FTY720.

Both FTY720 and sorafenib have earlier been shown to induce arrest in the G1 phase of the cell cycle in human cancer cells of various origins including HCC (Permpongkosol *et al.* 2002; Lee *et al.* 2004; Huether *et al.* 2007; Zheng *et al.* 2010; Marvaso *et al.* 2014). The relatively low dose of FTY720 used in this study had little effect on the cell cycle progression. However, it sensitized the Huh7 cells to treatment with sorafenib, as combined treatment with FTY720 and sorafenib caused significant accumulation of cells in the G1 phase at a dose of sorafenib that alone had no effect. At higher doses of sorafenib, combined treatment also caused a significant increase in the sub G1 fraction, indicating cell death. Earlier studies have shown induction of both apoptosis and necrosis in response to FTY720 treatment (Zhang *et al.* 2010; Pitman *et al.* 2012). We observed a significant synergistic increase in the fraction of apoptotic and necrotic cells after combined treatment with sorafenib and FTY720 for six hours. Similarly to earlier observations (Hung *et al.* 2008), no PARP-cleavage was observed following treatment with 8 $\mu\text{mol/L}$ of FTY720 for 24 h. However, the ratio of cleaved PARP resulting from treatment with sorafenib was increased by cotreatment with FTY720. The relatively high doses of sorafenib required for PARP cleavage are in accordance with earlier observations of sorafenib-induced

apoptosis (Yu *et al.* 2005). This result raised the possibility that caspase-independent apoptosis may have contributed to the cell death observed by detection of Annexin-V and PI. Our results support a modest induction of caspase-independent apoptosis, as a partial release of AIF into the cytosol was observed, whereas a fraction of AIF remained in the mitochondria after treatment of Huh7 cells with sorafenib and FTY720. The observed release of AIF into the cytosol might have triggered the extramitochondrial release of cyt c as demonstrated earlier (Loeffler *et al.* 2001; Liu *et al.* 2004).

Treatment with sorafenib has been shown to induce autophagy in HCC cells (Tai *et al.* 2013), and involvement of autophagy in acquired resistance to sorafenib in HCC cell lines has been demonstrated (Zhai *et al.* 2014). As autophagy counteracts the anti-proliferative effects of sorafenib, we investigated the effects of combined treatment with sorafenib and FTY720. Different studies have reported either induction (Zhang *et al.* 2010; Wallington-Beddoe *et al.* 2011; Liao *et al.* 2012) or blockage of autophagy by treatment of cancer cells with FTY720, the latter leading to enhanced effect of milatuzumab in mantle cell lymphoma (Alinari *et al.* 2012). During cytoprotective autophagy, the p62 protein levels decrease due to degradation in the autolysosome, whereas increased p62 levels may reflect a blockage in autophagy. Our results indicate that treatment with sorafenib alone induced autophagy in Huh7, as increasing doses of sorafenib caused a gradual slight increase in LC3II protein and concomitant decrease in p62 protein levels. In contrast, we observed higher protein levels of both LC3II and p62 following combined treatment with sorafenib and FTY720, indicating that FTY720 protects both proteins from degradation, possibly through blocking the conversion of autophagosomes to autolysosomes. Inhibition of autophagy with chloroquine has earlier been shown to potentiate sorafenib-induced apoptosis *in vitro* and antitumor effects *in vivo* in HCC cell lines and Huh7 xenografts, respectively (Shi *et al.* 2011; Shimizu *et al.* 2012). In line with these observations, inhibition of autophagy by FTY720 may underlie our observed synergistic effects on cell cycle arrest and cell death in response to combined treatment with sorafenib and FTY720.

In conclusion, we have shown that FTY720 enhanced the decrease in viability observed after treatment of Huh7 and HepG2 cells with sorafenib alone. Combined treatment of Huh7 cells with sorafenib and FTY720 generated synergistic effects on cell cycle arrest and apoptosis, possibly through blockage of autophagy. Our results suggest that FTY720 sensitizes HCC cells to sorafenib, and enhances its cytotoxic effects. This encourages further studies aiming at combinatorial use of FTY720 and sora-

fenib, both of which are in clinical use, in order to improve the outcome of HCC treatment.

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Author Contributions

J. Flygare, P.J. de Verdier and D. Ahmed designed the research study, D. Ahmed and O. Lunqe performed the experiments, P. Stål contributed with clinical expertise and experimental design, C. Ryk and D. Ahmed performed statistical data analysis, D. Ahmed and J. Flygare wrote the paper, and P. de Verdier, P. Stål, and C. Ryk critically revised the manuscript.

Disclosures

None to declare.

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