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Cancer Therapeutics Insights

Sorafenib overcomes irinotecan resistance in colorectal cancer by inhibiting the ABCG2 drug-efflux pump.

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Abstract

Despite recent advances in the treatment of colorectal cancer, tumor resistance is a frequent cause of chemotherapy failure. Thus, new treatment options are needed to improve survival of patients with irinotecan-refractory colorectal cancers, particularly those bearing *KRAS* mutations that preclude the use of anti-EGFR therapies. In this study, we investigated whether sorafenib could reverse irinotecan resistance, thereby enhancing the therapeutic efficacy of routinely used irinotecan-based chemotherapy. We used both *in vitro* (the HCT116, SW48, SW620 and HT29 colon adenocarcinoma cell lines and four SN-38 resistant HCT-116 and SW48 clones) and *in vivo* models (nude mice xenografted with SN-38 resistant HCT116 cells) to test the efficacy of sorafenib alone or in combination with irinotecan, or its active metabolite SN-38. We have shown that sorafenib improved the anti-tumoral activity of irinotecan *in vitro*, in both parental and SN-38 resistant colon adenocarcinoma cell lines independently of their *KRAS* status, as well as *in vivo*, in xenografted mice. By inhibiting the drug-efflux pump ABCG2, sorafenib favors irinotecan intracellular accumulation and enhances its toxicity. Moreover, we found that sorafenib improved the efficacy of irinotecan by inhibiting the irinotecan-mediated p38 and ERK activation. In conclusion, our results show that sorafenib can suppress resistance to irinotecan and suggest that sorafenib could be used to overcome resistance to irinotecan-based chemotherapies in colorectal cancer, particularly in *KRAS* mutated tumors.

Introduction

Colorectal cancer (CRC) is the fourth most common cancer worldwide. In 2008, the estimated CRC incidence was of 436.000 cases in Europe and CRC caused more than 212.000 deaths (1). Moreover, despite the significant progress made in the screening and early management of the disease, 30% of patients present synchronous metastases and 50 to 60% will develop metastases that will require chemotherapy. The current management is based on various active drugs (5-FU/LV, capecitabine, irinotecan, oxaliplatin, bevacizumab cetuximab and panitumumab) either in combination or as single agents (2). Thanks to these treatments, the patients' overall survival has been significantly improved, but tumor resistance is still a frequent cause of chemotherapy failure.

Irinotecan (CPT-11) is a derivative of camptothecin and its active metabolite is SN-38 (3). Like other camptothecin derivatives, SN-38 is an inhibitor of topoisomerase I, a nuclear enzyme needed for replication and transcription through relaxation of supercoiled DNA (4,5). Cellular mechanisms causing irinotecan/SN-38 resistance have been reported for each step of the CPT-11 pathway (6–9). Among them, it has been shown that cultured cells that are resistant to camptothecin derivatives have reduced intracellular drug accumulation, mediated by the ATP-binding cassette (ABC) transporter ABCG2 (10), especially in colon cancer cells (11,12). In metastatic CRC, several studies have reported that cetuximab, an anti-EGFR monoclonal antibody, can overcome acquired resistance to irinotecan chemotherapy (13). The underlying mechanism was not clearly identified, but these results suggest a crucial role for the Ras/Raf/MEK/ERK cell signaling pathway in sensitivity to cetuximab. However, patients with *KRAS* mutated CRC do not benefit from anti-EGFR antibody treatment (14,15) and new therapeutic options are therefore needed to improve their survival.

Sorafenib is a multi-targeted tyrosine kinase inhibitor (TKI) with anti-angiogenic properties mainly by blocking VEGFR and PDGFR activation (16) and has proven its efficiency for the treatment of metastatic renal cell carcinoma and advanced hepatocellular carcinoma (17,18). It also regulates the Raf/MEK/ERK pathway by inhibiting C- and B-Raf and therefore can affect tumor cell proliferation even in *KRAS* mutated cancers (19). Moreover, sorafenib, like other TKIs, inhibits members of the ABC transporter superfamily, particularly ABCG2 (20).

Based on these findings, in this preclinical study we evaluated whether sorafenib could reverse irinotecan-resistance in cultured CRC cells and xenografts.

Material and Methods

Drugs

Sorafenib (Nexavar, Bayer Schering, Berlin, Germany) was dissolved in 50:50 Cremophor EL (Sigma): 95% ethanol at a final concentration of 7 mg/ml, filtered through 0.2 μm filters and stored at 4°C until use. Just before use, the sorafenib stock was diluted 1/2 with saline. SN-38, the active metabolite of irinotecan, was kindly provided by Sanofi-Aventis (Vitry-Sur-Seine, France). Irinotecan (Campto) was from Pfizer (20mg/ml stock).

Cell lines

The HCT116, SW48, SW620 and HT29 colon adenocarcinoma cell lines from ATCC (Manassas, VA, USA) were grown in RPMI 1640 supplemented with 10% fetal calf serum (FCS) and 2 mM L-glutamine at 37°C under a humidified atmosphere with 5% CO₂. These cell lines have been obtained in 2000, were amplified and frozen, and one aliquot of each was thawed for this project, though no authentication was done by the authors. The SN-38-

resistant HCT116 and SW48 cell clones were obtained as previously describe (7,9,12). Briefly, the reference SN-38 sensitive HCT116 cell clone (HCT116-s) was exposed to 10 nM SN-38 and cloned to obtain the HCT116-SN6 and HCT116-A2 clones. HCT116-s cells were also exposed to 15 nM SN-38 and cloned to obtain the HCT116-SN50 and HCT116-G7 clones. Parental SW48 cells were cloned to obtain a reference SN-38 sensitive clone (SW48-s). SW48-s cells were continuously exposed to SN-38 with a stepwise increase in concentration from 4 nM to 150 nM during 8 months. SN-38 resistant cells were then cloned to obtain the SW48-SN2, SW48-SN3 and SW48-SN4 clones. Drug-selected clones were maintained in the appropriate concentration of SN-38. All the cell lines were cultured in drug-free medium at least 5 days prior to any experiment.

Drug sensitivity assay

Cell growth inhibition and cell viability after SN-38 treatment were assessed using the sulforhodamine B (SRB) assay (9). Exponentially growing cells were seeded in 96-well plates (1000 cells/well) in RPMI 1640 supplemented with 10% FCS. After 24 h, drugs were added in serial dilutions, each concentration in triplicate wells. After 96 hours, cells were fixed with 10% trichloroacetic acid and stained with 0.4% SRB in 1% acetic acid (Sigma Aldrich, St. Louis, MO, USA). The sulforhodamine B fixed to the cells was dissolved in 10 mM Tris-HCl and absorbance at 540 nm was read using an MRX plate reader (Dynex, Inc., Vienna, VA, USA). Each IC_{50} was determined graphically from the cytotoxicity curves. Combination Index (CI) was calculated using the Chou-Talalay method (21,22).

Evaluation of protein expression by western blotting

After counting, cells were lysed in SDS buffer (bromophenol blue, 5% β -mercaptoethanol, 2% SDS, 10% glycerol, 62.5 mM Tris-HCl pH 6.8). Extracts were treated with benzonase,

boiled for 5 min and separated by SDS-PAGE. Proteins were electro-transferred to nitrocellulose membranes (Amersham Pharmacia Biotech, Uppsala, AB, Sweden). Primary antibodies were against ABCG2 (BXP-21, Millipore, Billerica, MA, USA) and GAPDH (6C5, Millipore, Billerica, MA, USA). Secondary antibodies were horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgGs (Sigma Aldrich, St. Louis, MO, USA). Proteins were detected by enhanced chemoluminescence (ECL) by using the ECL detection system from GE Healthcare Life Sciences (Buckinghamshire, UK) and recorded using the G/BOX iChemi imaging system (Syngene).

ShRNA constructs

HCT116-A2 and HCT116-SN50 cells that express ShRNAs targeting *luciferase* (ShLuc) or three different regions of the *ABCG2* mRNA sequence (ShABCG2-1, -2 and -3) were obtained by retroviral gene transduction of the ShRNA constructs in the pSIREN vector. Cells were selected with 2 µg/mL puromycin and stable clones were pooled.

Cell death analysis

Cell death analysis was performed using the Annexin V-FITC/7-AAD Kit (Beckman Coulter, Indianapolis, USA). Cells were seeded in 25 cm² flasks at 20 000 cells/flask. After a 24-hour rest, cells were treated 96 hours with either 0.5 or 3 µM sorafenib, or 0.5 µM sorafenib + 0.7µM SN-38. The cells (1×10^6) and corresponding supernatants were labeled using the Annexin V-FITC/7-AAD Kit. Analyses were done on a FC500 Beckman Coulter Flow Cytometer. Annexin V-FITC positive cells were quantified using Flow Jo analysis software (Treestar Inc, Ashland, Oregon , USA).

Cell cycle analysis

The cells were seeded in 25cm² flasks (2 x 10⁴ cells/flask). After a 24-hour rest, the cells were treated for 96 hours with either 0.5 or 3 μM sorafenib, or 0.5 μM sorafenib + 0.7μM SN-38. One million cells were pelleted, washed with PBS, fixed in 75% ethanol, treated with 100 μg/mL of RNase (QIAGEN) and stained with 40 μg/mL of propidium iodide. Analyses were done on a FC500 Beckman Coulter Flow Cytometer. Cell cycle distribution was then determined with a FC500 Beckman Coulter Flow Cytometer using the FL-3 channel. Cells were gated on a dot plot that displayed DNA pulse-peak versus DNA-pulse area to exclude doublets. Cell cycle distributions were illustrated using Flow Jo analysis software (Treestar Inc, Ashland, Oregon , USA).

Intracellular drug accumulation

The relative intracellular drug content was measured using a Cell Lab Quanta flow cytometer (Beckman Coulter, Brea, CA, USA) with excitation at 345 nm (ultraviolet laser) and a 540 nm band-pass filter to detect emission for SN-38 (12). Subconfluent cells were harvested and 500,000 cells were incubated at 37°C in complete medium supplemented with diluent only, 25 μM SN-38, 50 μM sorafenib, or 25μM SN-38 + 50μM sorafenib for 2 hr. Intracellular drug accumulation was stopped by cooling on ice and cells were washed in ice-cold PBS before FACS analysis. Assays were performed at least three times. Data acquisition and analysis were performed using the FlowJo software (Becton Dickinson).

Tumor xenografts in nude mice

All *in vivo* experiments were performed by accredited researchers (Dr. B. Robert, N°34-156, Dr. Céline Gongora, N°34-142) in compliance with the French regulations and ethical guidelines for experimental animal studies. Six-week/old female athymic mice (Harlan),

which were maintained in a specific pathogen-free facility in an accredited establishment (Agreement No. C34-172-27), were xenografted subcutaneously in the right flank with 1.5×10^6 cells. When tumors of approximately 0.1 cm^3 in diameter were detected, mice were randomly distributed in four groups (n=6) and treated with vehicle alone (control), sorafenib alone, SN-38 alone or sorafenib plus SN-38. Sorafenib was administered by gavage at 25 mg/kg/day (except during weekends) every two weeks for three weeks in total. SN-38 was injected intraperitoneally (i.p.) at 40 mg/kg twice a week during five weeks. Mice were weighted every day to adjust the drug doses and follow the drug toxicity. Tumor size was measured with a caliper in two perpendicular dimensions. Tumor volumes were calculated using the formula $(r^2 \times R)/2$, where r is the smaller dimension. Mice were euthanized when tumor volume reached 2000 mm^3 and explanted tumor xenografts were fixed for immunohistochemistry.

Immunohistochemistry (IHC)

One third of each tumor xenograft was formalin-fixed, paraffin-embedded and mounted on Superfrost-plus slides. Prior to immunodetection, specimens were deparaffinized and rehydrated. Antigen retrieval was performed by heat treatment in 10 mM citrate buffer at pH 6. Endogenous peroxidase and biotin activities were blocked with specific reagents. Sections were incubated at room temperature with anti-cleaved Caspase 3 (5AE1, Cell Signaling Technology) for 30 minutes or with anti-CD34 (MEC14-7, AbD Serotec) antibodies for 1 hour. Both antibodies were diluted at 1:400 in PBS/3% BSA. Antibody binding was revealed using the Strepta ABCComplex/HRP Duet kit (streptavidin-biotin-peroxidase complex method) (DakoCytomation, Trappes, France). Sections were counterstained with hematoxylin and mounted with mounting medium. Immunostaining was analyzed with NDPViewer after

digitalization with the NanoZoomer slide scanner (Hamamatsu). The most representative images were chosen for presentation.

Proteome profiler analysis

The activity of a panel of 46 intracellular kinases was assessed through the determination of their relative levels of phosphorylation using the Proteome Profiler Array (R&D Systems, Minneapolis, USA) according to the manufacturer's instructions. Briefly, xenografts from untreated mice (NT) or from mice treated with sorafenib alone, irinotecan alone or sorafenib + irinotecan were lysed in lysis buffer (NaCl 150 mM, Tris pH 7.4 10 mM, EDTA 1mM, Triton X100 1%, NP40 0.5%, PMSF 2 mM, NaF 100 mM, Orthovanadate 10 mM, 1 tablet of protease inhibitor). After centrifugation at 15,000 RPM for 20 minutes, the protein concentration of the supernatant was determined, and the volume of each sample equivalent to 200 µg of protein was diluted in Array Buffer 1 to yield a final volume of 2 ml. Arrays were pre-incubated in 1 ml Array Buffer 1 for 1 hour before incubating the array strips in the diluted sample at 4°C overnight, washing 3 × 10 minutes in 20 ml wash buffer, incubating in the detection antibody cocktail (1:100 in 1X Array Buffer 1), washing, and incubating in a Streptavidin-HRP solution (1:2000). After washing again, proteins were detected by enhanced chemoluminescence (ECL) by using the ECL detection system from GE Healthcare Life Sciences (Buckinghamshire, UK) and recorded using the G/BOX iChemi imaging system (Syngene).

Statistical analysis

A linear mixed-regression model, containing both fixed and random effects, was used to determine the relationship between tumor growth and number of days after grafting. BLI data were first transformed using the natural log scale to better fit the assumptions of the linear

mixed model. The fixed part of the model included variables corresponding to the number of post-graft days and the different treatments. Interaction terms were built into the model; random intercepts and random slopes were included to take time into account. The coefficients of the model were estimated by maximum likelihood and considered significant at the 0.05 level. Survival rates were estimated from the xenograft date until the event of interest (i.e., a volume of 1000 mm³) with the Kaplan-Meier method. The log-rank test was used to compare survival curves between groups. Statistical analysis was performed using the STATA 10.0 software (StataCorp).

Results

Sorafenib enhances SN-38 cytotoxic effect *in vitro*

In order to study the combined effect of sorafenib and SN-38 (Fig. 1A) in CRC cells with different genetic features, we used four CRC cell lines (HCT116, SW48, SW620 and HT29 cells) that harbor various mutations in the *KRAS*, *BRAF*, *PIK3CA* and *p53* genes (Table 1). Sensitivity to sorafenib (Fig. 1B), evaluated using the SBR assay, was highest in HCT116 cells (IC₅₀: 3 μM ± 0.5, p-value = 1.5 10⁻⁷), while it was comparable in the other cell lines (IC₅₀: 5.4 μM ± 0.7 for SW48 cells; IC₅₀: 6 μM ± 0.2 for SW620 cells; IC₅₀: 5.1 μM ± 0.5 for HT29 cells). These results indicate that CRC cell lines are sensitive to sorafenib and that its toxicity does not seem to be correlated with *KRAS*, *BRAF*, *PIK3CA* and *p53* mutations. We then assessed the effect of sorafenib on the sensitivity of cells to SN-38 by using a suboptimal dose of sorafenib (0.5μM) (Fig. 1C). The sorafenib plus SN-38 combination decreased the IC₅₀ of SN-38 in all four cell lines in comparison to SN-38 alone (from 1.6-fold reduction in HCT116 cells to 4.1-fold in SW48 cells). These results indicate that the sorafenib-mediated increase of SN-38 cytotoxicity also is independent of *KRAS*, *BRAF*, *p53* and *PIK3CA* mutations.

Sorafenib associated with SN-38 reverses chemo-resistance *in vitro*

We then asked whether sorafenib could reverse the resistance of CRC cells to SN-38. To this aim, we used SN-38 resistant HCT116 (Table 2) and SW48 clones (9). The sorafenib IC₅₀ in SN-38 resistant HCT116 clones were between 2.2μM (HCT116-SN6 clone) and 3.45 μM (HCT116-G7 clone) (Fig. 1D) and thus comparable to the IC₅₀ in SN-38 sensitive HCT116-s cells (Fig. 1B). Only, the HCT116-SN6 clone showed a small, but significant decrease in IC₅₀ compared to the other cell lines (Fig. 1D). The SN-38 resistant SW48 clones were slightly, but significantly more sensitive to sorafenib than the parental cell line (Fig. 1F). Indeed, their IC₅₀ ranged from 3.9μM to 3.2μM, whereas the SN-38-sensitive SW48-s clone had an IC₅₀ of 5.4μM.

We then tested the effect of suboptimal 0.5μM sorafenib in combination with SN-38 in the SN-38 resistant clones (Fig. 1E and 1G). SN-38 toxicity was increased in all SN-38 resistant HCT116 and SW48 clones when associated with sorafenib. Specifically, the IC₅₀ was 1.5- (HCT116-G7 cells) to 5-fold (HCT116-A2 cells) reduced in SN-38 resistant HCT116 clones, and 2.3- (SW48-SN2 cells) to 5-fold (SW48-SN4 cells) decreased in SN-38 resistant SW48 clones. These findings suggest that sorafenib enhances also SN-38 toxicity in SN-38 resistant CRC cells.

Sorafenib associated with SN-38 inhibits cell proliferation and induces cell death.

The combined effects of Sorafenib and SN-38 on cell survival were then analyzed using the *Chou-Talalay method* (22) (Table 3). This calculation method provides a quantitative definition for drug combination called Combination Index (CI). The CI define additive effect (CI=1), synergistic effect (CI<1) or antagonism effect (CI>1). Combination index (CI) values

were calculated for all the 14 cell lines tested in this study, and we found that treatment with sorafenib and SN-38 was always synergistic (CI values ranged from 0.095 to 0.381).

We then determined if the synergistic effect involves cell death or inhibition of cell proliferation. We first studied the effect of sorafenib alone on HCT116 at 0.5 μ M (suboptimal dose used in combination with SN-38) and 3 μ M (IC₅₀) during 96 hours (Fig. 2A, B, C). While Sorafenib at suboptimal dose (0.5 μ M) had no effect on cell proliferation and cell death, the IC₅₀ dose (3 μ M) inhibit cell proliferation (Fig. 2A) and increase cell death (Fig. 2B). However, the drug has no effect on cell cycle, except on the subG1 phase (Fig. 2C), representative of late apoptosis, confirming the results of Fig 1B. The combination of sorafenib (0.5 μ M) and SN-38 (0.7 nM), at suboptimal dose, display the same effect on cell proliferation, cell death and cell cycle (Fig. 2D, E and F) as sorafenib at IC₅₀ dose.

Sorafenib associated with irinotecan reverses chemo-resistance *in vivo*

To determine whether sorafenib could reverse resistance to SN-38 *in vivo*, we xenografted SN-38 resistant HCT116-SN50 cells in nude mice. When tumors reached approximately a volume of 100 mm³, mice were assigned to four groups that received vehicle (control, NT), sorafenib alone (by gavage), irinotecan alone (i.p.), or irinotecan plus sorafenib (Fig. 3A). Tumor growth was slightly inhibited by irinotecan alone in comparison to controls, as expected. Indeed, we previously showed that HCT116-SN50 cells are less sensitive to irinotecan than HCT116-s cells (9). Inhibition of tumor growth by sorafenib alone was comparable to that by irinotecan, conversely it was significantly higher when irinotecan and sorafenib were associated (p=0.045 versus irinotecan alone and p=0.005 versus sorafenib alone). This result indicates that sorafenib improves irinotecan efficiency also *in vivo*.

Moreover, the modified Kaplan-Meier plot showed that a significantly longer time was required for 50% mice to reach a 1000 mm³ tumor volume in the group treated with irinotecan

plus sorafenib (median survival, MS, not reached, $p = 0.0081$) or sorafenib alone (MS = 33 days, $p = 0.015$) than in control mice (MS = 28 days) (Fig. 3B). These data indicate that the irinotecan plus sorafenib combination is significantly more effective than sorafenib or irinotecan alone in inhibiting growth of SN-38 resistant HCT116 CRC xenografts.

Sorafenib associated with irinotecan induces apoptosis *in vivo*

At the end of the *in vivo* experiment, the number of mitoses and the expression of CD34 (angiogenesis marker) and cleaved Caspase 3 (apoptosis marker) were analyzed in contiguous sections of the explanted HCT116-SN50 xenografts. In samples from animals treated with sorafenib plus irinotecan significantly fewer mitoses and CD34-positive endothelial cells and more cleaved Caspase 3-positive cells were observed than in xenografts from controls or from mice treated with sorafenib or irinotecan alone (Fig. 3C, D). These results indicate that, in SN-38 resistant HCT116-SN50 xenografts, the combination of sorafenib plus irinotecan inhibits tumor cell proliferation and angiogenesis and enhances apoptosis more efficiently than when the two drugs are used alone as observed *in vitro*.

Sorafenib enhances SN-38 cell accumulation

We next investigated whether sorafenib overcame irinotecan resistance by bypassing some mechanism(s) of drug resistance. As the drug-efflux pump ABCG2 is involved in acquired resistance to SN-38 (12), and because the SN-38 resistant HCT116-SN50 and -A2 clones that showed the most significant IC_{50} reduction upon combined exposure to sorafenib and SN-38 (Fig. 1E) also over-express ABCG2, we investigated whether sorafenib could affect SN-38 intracellular accumulation.

Western blot analysis confirmed that ABCG2 was over-expressed only in HCT116-SN50 and -A2 cells, but not in the parental cell line or in the other SN-38 resistant clones (Fig. 4A). Then, we have analyzed the proportion of cells containing SN-38 using flow cytometry assay,

since SN-38 is naturally fluorescent (12). We found that ABCG2 over-expression correlated with lower intracellular accumulation of SN-38 in these clones (8% of cells containing intracellular SN-38) in comparison to SN-38 sensitive HCT116-s cells (67%) and the other SN-38 resistant clones that do not express ABCG2 (HCT116-SN6 cells: 60 %, and HCT116-G7 cells: 47 %) (Fig. 4C). We then exposed cells to sorafenib and SN-38. Sorafenib significantly increased SN-38 intracellular accumulation in HCT116-SN50 and -A2 cells (10-fold), but also, albeit less strongly (1.4 to 2.4 fold increase), in HCT116-s cells and in the other SN-38 resistant clones that do not over-express ABCG2 (Fig. 4E). These data suggest that sorafenib regulates SN-38 accumulation mainly, but not exclusively, via inhibition of the efflux pump ABCG2.

Increased SN-38 accumulation was also observed in parental HCT116 (1.6 fold), SW48 (1.8 fold), SW620 (2.4 fold) and HT29 (4.9 fold) CRC cells treated with sorafenib (Fig. 4D and F). The higher SN-38 intracellular accumulation in HT29 cells correlated with ABCG2 expression (Fig. 4B).

To determine whether sorafenib effect on ABCG2 function is related to a decrease of ABCG2 expression, we performed Western Blot analysis. Dose-response analysis after 72 hours of sorafenib incubation showed no inhibition of ABCG2 expression in both HCT116-A2 and HCT116-SN50 (Fig. 4G). Furthermore, we have tested the sorafenib and SN-38 combination on ABCG2 expression, and obtained again no ABCG2 expression inhibition (Fig. 4H). This result indicates that sorafenib doesn't affect ABCG2 expression in HCT116 cells resistant to SN-38.

Sorafenib overcomes SN-38 resistance mainly by inhibiting ABCG2 drug-efflux activity

To determine if sorafenib enhances SN-38 intracellular accumulation essentially via ABCG2 inhibition, we generated stable HCT116-A2 and -SN50 cell lines in which ABCG2 was

knocked down with retroviruses that express three different anti-*ABCG2* ShRNAs. The ShRNA-*ABCG2*-3 construct efficiently reduced *ABCG2* expression in both HCT116-A2 and -SN50 cells (Fig. 5A) and the stable cell lines generated with this construct were used for all further experiments. First, we showed that SN-38 intracellular accumulation was significantly increased in cells in which *ABCG2* was down-regulated (HCT116-A2-Sh*ABCG2* and HCT116-SN50-Sh*ABCG2* cells) in comparison to control HCT116-A2-ShLuc and HCT116-SN50-ShLuc cells (Fig. 5B). Then, we compared the effect of sorafenib on SN-38 intracellular accumulation in these cell lines (Fig. 5C). As expected, in control cells that express *ABCG2*, combined exposure to sorafenib and SN-38 strongly stimulated SN-38 accumulation (20-fold increase in HCT116-A2-ShLuc cells, 15-fold in HCT116-SN50-ShLuc cells in comparison to cells treated with SN-38 alone). Conversely, in cells in which *ABCG2* was down-regulated, sorafenib increased only weakly SN-38 intracellular accumulation (7-fold increase in HCT116-A2-Sh*ABCG2*-3, 5-fold in HCT116-SN50-Sh*ABCG2*-3 in comparison to cells treated with SN-38 alone), indicating that sorafenib effect on intracellular drug accumulation is impaired when *ABCG2* is knocked down. This result indicates that sorafenib is mainly inhibiting *ABCG2* activity since it doesn't impair its expression (Fig. 4G-H).

Finally, to determine whether inhibition of *ABCG2* activity by sorafenib was responsible for the higher SN-38 cytotoxicity when the two drugs are used in combination (Fig. 5D), we performed cytotoxic assays in control cells and in cells in which *ABCG2* was down-regulated. Sorafenib significantly sensitized HCT116-A2-ShLuc and HCT116-SN50-ShLuc control cells to SN-38 as indicated by the 7.6-fold and 10-fold reduction of IC_{50} , respectively, and similarly to what observed in the parental HCT116-A2 and HCT116-SN50 cell lines. Sorafenib increased SN-38 cytotoxicity also in cells in which *ABCG2* was knocked down, but to a lesser extent than in control cells (3.4-fold reduction of IC_{50} in HCT116-A2-Sh*ABCG2* cells and

2.8-fold in HCT116-SN50-ShABCG2 cells). Altogether, these results indicate that sorafenib potentiates SN-38 cytotoxicity mainly via inhibition of ABCG2 drug-efflux activity and that as a consequence sorafenib can efficiently overcome resistance to irinotecan-related drugs.

Sorafenib inhibits the p38 and ERK's phosphorylations induced by irinotecan

Next, we assessed differences in the cell signalling responses to sorafenib alone, irinotecan alone and both drugs together in xenografts by using proteome profiler (Fig. 6A). We observed that irinotecan alone activate the phosphorylation of the MAPK p38 and ERK. These two kinases were either unaffected or slightly induced by sorafenib alone. Moreover, the presence of sorafenib in combination to irinotecan is able to reduce the irinotecan effect on p38 and ERK. These results indicate that sorafenib can overcome irinotecan activation of the MAPK kinase signalling pathways, p38 and ERK.

To confirm the sorafenib effect on p38 and ERK phosphorylation, we performed western blot analysis on 5 xenografts per treatment (NT, sorafenib, Irinotecan, sorafenib+irinotecan). As shown in Figure 6B, we found that irinotecan induces p38 and ERK phosphorylation *in vivo*. Then, the addition of sorafenib to irinotecan is able to inhibit this induction. Those results are essential in the understanding of irinotecan resistance and way to circumvent it. Indeed, this is the first time, to our knowledge, that p38 and ERK are shown to be activated by irinotecan *in vivo*. And sorafenib, by inhibiting p38 and ERK signalling pathways, enhance the cytotoxic activity of irinotecan.

Discussion

The findings of the present study indicate that sorafenib might represent a new treatment option for the management of metastatic CRC. Specifically, this multi-TKI exhibited cytotoxic effects in different CRC cell lines regardless of their *KRAS* status, as

described in other preclinical studies (23,24) and suggested by several phase I studies, involving a large number of patients with CRC (25–28).

Moreover, sorafenib enhances irinotecan activity. The anti-tumor activity of irinotecan and SN-38 was more pronounced when they were used in combination with sorafenib both *in vitro* and *in vivo*. This sorafenib effect was observed also in SN-38 resistant CRC cell clones, indicating that sorafenib can restore irinotecan sensitivity. Sorafenib clearly improve cell proliferation inhibition and enhance apoptosis. Furthermore, *in vivo*, angiogenesis was reduced and sorafenib could promote the irinotecan effects on angiogenesis. This hypothesis was also discussed in previous early phase I clinical studies (29,30), in which several patients with advanced CRC who did not respond to irinotecan-based regimens showed partial response or prolonged stable disease following treatment with sorafenib and irinotecan. Sorafenib could thus be used to overcome resistance to irinotecan-based chemotherapies in CRC, particularly in *KRAS* mutated tumors for which the therapeutic options are extremely limited.

We then show that sorafenib overcomes resistance to irinotecan/SN-38 mainly by inhibiting the drug-efflux pump ABCG2, leading to increased SN-38 intracellular accumulation and consequently higher toxicity. These effects were stronger in the SN-38 resistant CRC cell lines that over-express this ABC transporter. Indeed, the ABCG2 efflux pump mediates transport of irinotecan and SN-38 out of the cell and is directly involved in drug resistance. The molecular mechanism underlying the sorafenib effect on ABCG2 efflux function is still under investigation. In addition to ABCG2, sorafenib also blocks the function of other ABC transporters, including ABCB1, ABCC2 and ABCC4 (20,31). Hu and colleagues showed that sorafenib inhibits the ATPase activity of ABCC2 by directly interacting with this ABC transporter (31). Carloni et al reported that sorafenib decreases the expression level of ABCC2 in some breast cancer cell lines (32). Moreover, several authors

(33) reported that the AKT and Ras/Raf/MEK/ERK signaling pathways, which are frequently dysregulated in cancer, regulate the expression of the ABC transporters, specifically ABCG2, at the plasma membrane. Sorafenib may thus decrease ABCG2 membrane expression by inhibiting these signaling pathways. Finally, Wei et al, proposed that sorafenib induces ABCG2 degradation via the lysosome (20). Altogether these works suggest that sorafenib might inhibit both the function and the cell surface expression of ABCG2, ultimately leading to increased irinotecan cell concentration. Our results rather suggest sorafenib acts on ABCG2 function and not on ABCG2 expression.

Increased ABCG2 expression has also been reported in cancer stem cells (CSCs) and ABCG2 may serve as a CSC marker (34). Spheroids derived from tumor biopsies of patients with CRC display cancer stem-cell properties, such as enhanced self-renewal capacity, slow division, drug resistance and expression of ABCG2, CD133 and CD44 (35). As the cancer stem cell hypothesis suggests that tumor formation and growth are driven by drug-resistant CSCs, it is tempting to hypothesize that sorafenib, as an ABCG2 inhibitor, could both facilitate the elimination of CSCs and enhance the therapeutic efficacy of irinotecan.

Furthermore, we think that ABCG2 expression may become a predictive marker for sorafenib response in irinotecan-resistant tumors. Indeed, as we previously showed that ABCG2 expression is increased in human hepatic metastases treated with irinotecan (12), patients with cancers that over-express ABCG2 could be good candidates for combined treatment with sorafenib and irinotecan.

However, ABCG2 inhibition is clearly not the only mechanism involved in the synergy between sorafenib and irinotecan. Indeed, the sorafenib-irinotecan combination was more efficient than irinotecan alone also in CRC cells in which *ABCG2* was down-regulated by ShRNA. Sorafenib seems to have little effect on two other major ABC transporters

(ABCB1 and ABCC1) involved in irinotecan efflux (20). On the other hand, irinotecan failure can be related also to activation of Nuclear Factor kappa B (NF- κ B) and inhibition of the apoptotic cascade. Jayanthan et al (36), have shown that in cell lines derived from atypical teratoid rhabdoid tumors of the central nervous system, sorafenib could interfere with irinotecan-mediated NF- κ B activation by retaining NF- κ B in the cytoplasm and therefore preventing induction of anti-apoptotic genes. Another hypothesis is that sorafenib could modify irinotecan's metabolism. Indeed, the active metabolite SN-38, is converted to inactive SN-38 glucuronide (SN-38-G) by UGT1A1 but the literature (29,37) also suggests that UGT1A1 can be inhibited by sorafenib which can consequently decrease the level of inactive SN-38-G into the cancer cells. The HCT116 cells, which we have used to describe the molecular mechanism of the synergism between sorafenib and SN-38, lack glucuronidation activity (38). In conclusion, even if we cannot exclude that a part of the sorafenib mechanism in overcoming irinotecan resistance is based on UGT1A1 inhibition, our data indicate that another mechanism is involved. We have recently published that p38 MAPK is induced by SN-38 and is responsible for the development of resistance to SN-38 and that the use of p38 inhibitor enhance the cytotoxic activity of SN-38 (9). Interestingly, we have found that sorafenib can inhibit the p38 activation mediated by irinotecan *in vivo*, confirming its inhibitory effect demonstrated by Namboodiri and Grossi, respectively *in vitro* and *in cellulo* (39,40). So, sorafenib, by inhibiting the MAPK p38 pathway make the cells more sensitive to irinotecan. Moreover, it has been published that cytotoxic therapies like Taxol (41) may inappropriately induce the expression of cytoprotective signaling cascades like ERK/MEK pathway whose many of the substrates play a role in promoting cell proliferation and survival and can in fact mediate irinotecan resistance. Here, we are the first to show that irinotecan can activate, *in vivo*, ERK pathway too and that sorafenib can block this ERK phosphorylation, providing another explanation for the efficiency of the combination in colon cancer cells.

In conclusion, sorafenib is a promising option for the treatment of irinotecan-resistant CRCs as suggested by this study and the feedback on its clinical use in other cancer types. The sorafenib-irinotecan combination is not toxic in xenografted mice and some clinical trials have reported that they can be associated for the treatment of *KRAS* mutated tumors (29,36). Moreover, a not yet published phase II trial (42), carried out in patients with irinotecan-resistant and *KRAS* mutated CRCs, shows hopeful results. Therefore, it is worth to continue investigating the clinical effects of the sorafenib-irinotecan combination in CRC.

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References

1. Ferlay J, Shin H-R, Bray F, Forman D, Mathers C, Parkin DM. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *Int J Cancer* 2010;127:2893-2917.

2. Van Cutsem E, Nordlinger B, Cervantes A. Advanced colorectal cancer: ESMO Clinical Practice Guidelines for treatment. *Ann Oncol* 2010;21 Suppl 5:v93-97.
3. Pommier Y. Camptothecins and topoisomerase I: a foot in the door. Targeting the genome beyond topoisomerase I with camptothecins and novel anticancer drugs: importance of DNA replication, repair and cell cycle checkpoints. *Curr Med Chem Anticancer Agents* 2004;4:429-434.
4. Pommier Y, Leo E, Zhang H, Marchand C. DNA topoisomerases and their poisoning by anticancer and antibacterial drugs. *Chem Biol* 2010;17:421-433.
5. Pommier Y. DNA topoisomerase I inhibitors: chemistry, biology, and interfacial inhibition. *Chem Rev* 2009;109:2894-2902.
6. Pommier Y, Pourquier P, Urasaki Y, Wu J, Laco GS. Topoisomerase I inhibitors: selectivity and cellular resistance. *Drug Resist Updat* 1999;2:307-318.
7. Gongora C, Vezzio-Vie N, Tuduri S, Denis V, Causse A, Auzanneau C, et al. New Topoisomerase I mutations are associated with resistance to camptothecin. *Mol Cancer* 2011;10:64-77.
8. Xu Y, Villalona-Calero MA. Irinotecan: mechanisms of tumor resistance and novel strategies for modulating its activity. *Ann Oncol* 2002;13:1841-1851.
9. Paillas S, Boissière F, Bibeau F, Denouel A, Mollevi C, Causse A, et al. Targeting the p38 MAPK pathway inhibits irinotecan resistance in colon adenocarcinoma. *Cancer Res* 2011;71:1041-1049.

10. Maliepaard M, Van Gastelen MA, De Jong LA, Pluim D, Van Waardenburg RC, Ruevekamp-Helmers MC, et al. Overexpression of the BCRP/MXR/ABCP gene in a topotecan-selected ovarian tumor cell line. *Cancer Res* 1999;59:4559-4563.
11. Brangi M, Litman T, Ciotti M, Nishiyama K, Kohlhagen G, Takimoto C, et al. Camptothecin resistance: role of the ATP-binding cassette (ABC), mitoxantrone-resistance half-transporter (MXR), and potential for glucuronidation in MXR-expressing cells. *Cancer Res* 1999;59:5938-5946.
12. Candeil L, Gourdier I, Peyron D, Vezzio N, Copois V, Bibeau F, et al. ABCG2 overexpression in colon cancer cells resistant to SN38 and in irinotecan-treated metastases. *Int J Cancer* 2004;109:848-854.
13. Cunningham D, Humblet Y, Siena S, Khayat D, Bleiberg H, Santoro A, et al. Cetuximab monotherapy and cetuximab plus irinotecan in irinotecan-refractory metastatic colorectal cancer. *N Engl J Med* 2004;351:337-345.
14. Amado RG, Wolf M, Peeters M, Van Cutsem E, Siena S, Freeman DJ, et al. Wild-type KRAS is required for panitumumab efficacy in patients with metastatic colorectal cancer. *J Clin Oncol* 2008;26:1626-1634.
15. Karapetis CS, Khambata-Ford S, Jonker DJ, O'Callaghan CJ, Tu D, Tebbutt NC, et al. K-ras mutations and benefit from cetuximab in advanced colorectal cancer. *N Engl J Med* 2008;359:1757-1765.
16. Wilhelm SM, Adnane L, Newell P, Villanueva A, Llovet JM, Lynch M. Preclinical overview of sorafenib, a multikinase inhibitor that targets both Raf and VEGF and PDGF receptor tyrosine kinase signaling. *Mol Cancer Ther* 2008;7:3129-3140.

17. Escudier B, Eisen T, Stadler WM, Szczylik C, Oudard S, Siebels M, et al. Sorafenib in advanced clear-cell renal-cell carcinoma. *N Engl J Med* 2007;356:125-134.
18. Llovet JM, Ricci S, Mazzaferro V, Hilgard P, Gane E, Blanc J-F, et al. Sorafenib in advanced hepatocellular carcinoma. *N Engl J Med* 2008;359:378-390.
19. Beeram M, Patnaik A, Rowinsky EK. Raf: a strategic target for therapeutic development against cancer. *J Clin Oncol* 2005;23:6771-6790.
20. Wei Y, Ma Y, Zhao Q, Ren Z, Li Y, Hou T, et al. New Use for an Old Drug: Inhibiting ABCG2 with Sorafenib. *Mol Cancer Ther* 2012;11:1693-1702.
21. Chou T-C. Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies. *Pharmacol Rev* 2006;58:621-681.
22. Chou T-C. Drug combination studies and their synergy quantification using the Chou-Talalay method. *Cancer Res* 2010;70:440-446.
23. Wilhelm SM, Carter C, Tang L, Wilkie D, McNabola A, Rong H, et al. BAY 43-9006 exhibits broad spectrum oral antitumor activity and targets the RAF/MEK/ERK pathway and receptor tyrosine kinases involved in tumor progression and angiogenesis. *Cancer Res* 2004;64:7099-7109.
24. Martinelli E, Troiani T, Morgillo F, Rodolico G, Vitagliano D, Morelli MP, et al. Synergistic antitumor activity of sorafenib in combination with epidermal growth factor receptor inhibitors in colorectal and lung cancer cells. *Clin Cancer Res* 2010;16:4990-5001.

25. Awada A, Hendlisz A, Gil T, Bartholomeus S, Mano M, De Valeriola D, et al. Phase I safety and pharmacokinetics of BAY 43-9006 administered for 21 days on/7 days off in patients with advanced, refractory solid tumours. *Br J Cancer* 2005;92:1855-1861.
26. Clark JW, Eder JP, Ryan D, Lathia C, Lenz H-J. Safety and pharmacokinetics of the dual action Raf kinase and vascular endothelial growth factor receptor inhibitor, BAY 43-9006, in patients with advanced, refractory solid tumors. *Clin Cancer Res* 2005;11:5472-5480.
27. Moore M, Hirte HW, Siu L, Oza A, Hotte SJ, Petrenciuc O, et al. Phase I study to determine the safety and pharmacokinetics of the novel Raf kinase and VEGFR inhibitor BAY 43-9006, administered for 28 days on/7 days off in patients with advanced, refractory solid tumors. *Ann Oncol* 2005;16:1688-1694.
28. Strumberg D, Richly H, Hilger RA, Schleucher N, Korfee S, Tewes M, et al. Phase I clinical and pharmacokinetic study of the Novel Raf kinase and vascular endothelial growth factor receptor inhibitor BAY 43-9006 in patients with advanced refractory solid tumors. *J Clin Oncol* 2005;23:965-972.
29. Mross K, Steinbild S, Baas F, Gmehling D, Radtke M, Voliotis D, et al. Results from an in vitro and a clinical/pharmacological phase I study with the combination irinotecan and sorafenib. *Eur J Cancer* 2007;43:55-63.
30. Azad N, Dasari A, Arcaroli J, Taylor GE, Laheru DA, Carducci MA, et al. Phase I pharmacokinetic and pharmacodynamic study of cetuximab, irinotecan and sorafenib in advanced colorectal cancer. *Investigational new drugs* 2012; 31:345-54.

31. Hu S, Chen Z, Franke R, Orwick S, Zhao M, Rudek MA, et al. Interaction of the multikinase inhibitors sorafenib and sunitinib with solute carriers and ATP-binding cassette transporters. *Clin Cancer Res* 2009;15:6062-6069.
32. Carloni S, Fabbri F, Brigliadori G, Ulivi P, Silvestrini R, Amadori D, et al. Tyrosine kinase inhibitors gefitinib, lapatinib and sorafenib induce rapid functional alterations in breast cancer cells. *Curr Cancer Drug Targets* 2010;10:422-431.
33. Mogi M, Yang J, Lambert J-F, Colvin GA, Shiojima I, Skurk C, et al. Akt signaling regulates side population cell phenotype via Bcrp1 translocation. *J Biol Chem* 2003;278:39068-39075.
34. Ding X, Wu J, Jiang C. ABCG2: a potential marker of stem cells and novel target in stem cell and cancer therapy. *Life Sci* 2010;86:631-637.
35. Fang DD, Kim YJ, Lee CN, Aggarwal S, McKinnon K, Mesmer D, et al. Expansion of CD133(+) colon cancer cultures retaining stem cell properties to enable cancer stem cell target discovery. *Br J Cancer* 2010;102:1265-1275.
36. Jayanthan A, Bernoux D, Bose P, Riabowol K, Narendran A. Multi-tyrosine kinase inhibitors in preclinical studies for pediatric CNS AT/RT: Evidence for synergy with Topoisomerase-I inhibition. *Cancer Cell Int* 2011;11:44-55.
37. Peer CJ, Sissung TM, Kim A, Jain L, Woo S, Gardner ER, et al. Sorafenib is an inhibitor of UGT1A1 but is metabolized by UGT1A9: implications of genetic variants on pharmacokinetics and hyperbilirubinemia. *Clin. Cancer Res* 2012;18:2099-2107.

38. Cummings J, Ethell BT, Jardine L, Boyd G, Macpherson JS, Burchell B, et al. Glucuronidation as a mechanism of intrinsic drug resistance in human colon cancer: reversal of resistance by food additives. *Cancer Res* 2003;63:8443-8450.
39. Namboodiri HV, Bukhtiyarova M, Ramcharan J, Karpusas M, Lee Y, Springman EB. Analysis of imatinib and sorafenib binding to p38alpha compared with c-Abl and b-Raf provides structural insights for understanding the selectivity of inhibitors targeting the DFG-out form of protein kinases. *Biochemistry* 2010;49:3611-3618.
40. Grossi V, Liuzzi M, Murzilli S, Martelli N, Napoli A, Ingravallo G, et al. Sorafenib inhibits p38 α activity in colorectal cancer cells and synergizes with the DFG-in inhibitor SB202190 to increase apoptotic response. *Cancer Biol Ther* 2012;13:1471-1481.
41. McDaid HM, Lopez-Barcons L, Grossman A, Lia M, Keller S, Pérez-Soler R, et al. Enhancement of the therapeutic efficacy of taxol by the mitogen-activated protein kinase kinase inhibitor CI-1040 in nude mice bearing human heterotransplants. *Cancer Res* 2005; 65:2854-2860.
42. Ychou M, Bouche O, Thezenas S, Francois E, Adenis A, Bennouna J, et al. Final results of a multicenter phase II trial assessing sorafenib (S) in combination with irinotecan (i) as second- or later-line treatment in metastatic colorectal cancer (mCRC) patients (pts) with KRAS-mutated tumors (mt; NEXIRI). *J Clin Oncol* 29:15s, 2011 (suppl; abstr e14002).

Table 1: Molecular characteristics of the used colon cancer cell lines. Mutations in KRAS, BRAF, p53 and PIK3CA are indicated

Table 2: IC50 values of SN-38 in SN-38 sensitive (HCT116-s) and resistant HCT116 clones.

The resistance factor (RF; between brackets) was determined by dividing the IC50 value of each resistant clone by the IC50 value of the sensitive clone HCT116-s.

Table 3: Evaluation of the inhibitory effects on cytotoxic assay with sorafenib and SN-38. Combination Index (CI) values were calculated according to the Chou and Talalay model for drug interactions. Cancer cells were treated with 8 different concentrations of sorafenib and SN-38 and CI was calculated for each of the 8 combinations (in order to be concise, only one was reported on the table).

	SN38 (nM)	Sorafenib (µM)	CI
HCT116	2	0,5	0,381
SW48	2	0,5	0,263
SW620	2	0,5	0,124
HT29	2	0,5	0,354
HCT116-s	2	0,5	0,381
HCT116-SN6	8	0,5	0,25
HCT116-G7	12,5	0,5	0,222
HCT116-SN50	50	0,5	0,119
HCT116-A2	100	0,5	0,196
SW48-s	2	0,5	0,263
SW48-SN2	12,5	0,5	0,159
SW48-SN3	25	0,5	0,151
SW48-SN4	50	0,5	0,095

Figure legends

Figure 1: Sorafenib enhances the cytotoxic effect of SN-38. **A:** Structures of Sorafenib and SN-38. **B:** Sorafenib cytotoxicity was assessed in the HCT116, SW48, SW620 and HT29 colon cancer cell lines using the Sulforhodamine B (SRB) assay to determine the IC_{50} of sorafenib. **C:** SRB assays to determine the IC_{50} of SN-38 in HCT116, SW48, SW620 and HT29 cells that were incubated with SN-38 alone (white bars) or in combination with sorafenib (black bars). **D:** SRB assays to determine the IC_{50} of sorafenib in SN-38 resistant (HCT116-SN6, HCT116-A2, HCT116-SN50 and HCT116-G7) and SN-38 sensitive (HCT116-s) clones. **E:** SRB assays to determine the IC_{50} of SN-38 in HCT116-s, HCT116-SN6, HCT116-A2, HCT116-SN50 and HCT116-G7 cells that were incubated with SN-38 alone (white bars) or in combination with sorafenib (black bars). **G:** Structures of Sorafenib and SN-38. **F:** Sulforhodamine B (SRB) assays to determine the IC_{50} of sorafenib in SN-38 sensitive SW48-s and SN-38 resistant SW48-SN2, SW48-SN3 and SW48-SN4 cells. **G:** SRB assays to determine the SN-38 IC_{50} in SN-38 sensitive SW48-s and SN-38 resistant SW48-SN2, SW48-SN3 and SW48-SN4 cells that were treated with SN-38 alone (white bars) or in combination with sorafenib (black bars). ★ $p < 0.05$, ★★ $p < 0.01$, ★★★ $p < 0.001$.

Figure 2: Synergistic effect of sorafenib and SN-38. **A:** Number of viable HCT116 cells determined by counting after 96 hours treatment with sorafenib 0.5 μ M and 3 μ M. Data is the mean of three independent experiments. **B:** Apoptosis of HCT116 cells following exposure to sorafenib (0.5 and 3 μ M) for 96 hours. Apoptosis was determined by 7-AAD and Annexin V-FLUOS staining with a FACScan. **C:** HCT116 cell cycle distribution after sorafenib treatment was measured by propidium iodide staining with a FACScan flow cytometer. FACS profiles and the proportion of cells in each phase of the cell cycle are presented: cells in G2/M (black),

S (white and black slashes), G0/G1 (white) and the subG1 phase (grey). Cells in subG1 represent cells in late apoptosis. **D:** Number of viable HCT116 cells determined by counting after 96 hours treatment with sorafenib 0.5 μM + SN-38 0.7 nM. Data is the mean of three independent experiments. **E:** Apoptosis of HCT116 cells following exposure to sorafenib 0.5 μM + SN-38 0.7 nM for 96 hours. Apoptosis was determined by 7-AAD and Annexin V-FLUOS staining with a FACScan. **F:** HCT116 cell cycle distribution after sorafenib 0.5 μM + SN-38 0.7 nM was measured by propidium iodide staining with a FACScan flow cytometer. FACS profiles and the proportion of cells in each phase of the cell cycle are presented: cells in G2/M (black), S (white and black slashes), G0/G1 (white) and the subG1 phase (grey). Cells in subG1 represent cells in late apoptosis. ★ $p < 0.05$, ★★ $p < 0.01$, ★★★ $p < 0.001$.

Figure 3: Sorafenib effect on irinotecan cytotoxicity *in vivo*. **A:** Tumor progression curves. Tumor growth kinetics in mice xenografted with SN-38 resistant HCT116-SN50 cells and treated with vehicle alone (NT), irinotecan, sorafenib, or with the irinotecan + sorafenib combination. **B:** Modified Kaplan-Meier survival plots for the four treatment groups. **C:** Expression of cleaved Caspase 3 and CD34 by IHC in explanted xenografts from the four treatment groups. **D:** Quantification of the number of mitoses, cleaved Caspase 3-positive cells and CD34-positive endothelial cells (average number of positive cells in 10 different fields) in the four treatment groups. ★ $p < 0.05$, ★★ $p < 0.01$, ★★★ $p < 0.001$.

Figure 4: Sorafenib enhances SN-38 intracellular accumulation. **A:** Western blot analysis of ABCG2 expression in the SN-38 sensitive HCT116-s and the SN-38 resistant HCT116-SN6, HCT116-SN50, HCT116-G7 and HCT116-A2 cell lines. Protein loading is shown by GAPDH. **B:** Western blot analysis of ABCG2 expression in HT29, HCT116, SW48 and SW620 cells. Protein loading is shown by GAPDH. **C:** Flow cytometry analysis of SN-38

intracellular accumulation in HCT116-s, HCT116-SN6, HCT116-A2, HCT116-SN50 and HCT116-G7 cells. **D:** Flow cytometric analysis of SN-38 intracellular accumulation in HCT116, SW48, SW620 and HT29 cells. **E:** Flow cytometry analysis of SN-38 intracellular accumulation in HCT116-s, HCT116-SN6, HCT116-A2, HCT116-SN50 and HCT116-G7 cells after exposure to SN-38 alone or in combination with sorafenib. The SN-38+sorafenib values are relative to the SN-38 alone values (set to 1) in order to compare the fold change between conditions. **F:** Flow cytometry analysis of SN-38 intracellular accumulation in HCT116, SW48, SW620 and HT29 cells following exposure to SN-38 alone or in combination with sorafenib. The SN-38+sorafenib values are relative to the SN-38 alone values (set to 1) to compare the fold change between conditions. **G:** Western blot analysis of ABCG2 expression in HCT116-A2 and HCT116 SN50 treated with Sorafenib 0.5, 1 and 2 μ M during 72 hours. Protein loading is shown by tubulin. **H:** Western blot analysis of ABCG2 expression in HCT116-A2 and HCT116 SN50 treated with Sorafenib (2 μ M), SN-38 (20 nM) or Sorafenib (0.5 μ M) + SN-38 (20 nM) during 72 hours. Protein loading is shown by tubulin. ★ $p < 0.05$, ★★ $p < 0.01$, ★★★ $p < 0.001$.

Figure 5: Sorafenib enhances SN-38 cytotoxic effect by inhibiting ABCG2 drug-efflux function. **A:** Western blot analysis of ABCG2 expression in SN-38 resistant HCT116-A2 and HCT116-SN50 cells transfected with three different ShRNAs against *ABCG2* or the control ShRNA ShLuc. Protein loading is shown by GAPDH. **B:** Flow cytometric analysis of SN-38 intracellular accumulation in HCT116-A2 and HCT116-SN50 that express ShLuc or ShABCG2-3. **C:** Flow cytometric analysis of SN-38 intracellular accumulation in HCT116-A2 and HCT116-SN50 that express ShLuc or ShABCG2-3 following exposure to SN-38 alone or in combination with sorafenib. The SN-38+sorafenib values are relative to the SN-38 alone values (set to 1) to compare the fold change between conditions. **D:** Sulforhodamine B

assays to determine the IC_{50} of SN-38 in HCT116-A2 and HCT116-SN50 cells that express ShLuc or ShABCG2-3 following treatment with SN-38 alone or in combination with sorafenib. ★ $p < 0.05$, ★★ $p < 0.01$, ★★★ $p < 0.001$.

Figure 6: Effect of sorafenib + irinotecan on activation of intracellular kinases. **A:** Phosphorylation status of intracellular kinases was compared using proteome profiler array in nuclear extracts from xenografts from untreated (NT) or mice treated with Irinotecan + Sorafenib, Irinotecan alone or Sorafenib alone. Quantitative data were obtained using the G-Box from Syngene and the GenTools software. **B:** The phosphorylation of p38 and ERK was verified by Western Blotting on protein extract from xenografts from 5 different mice. Equal loading is shown by Tubulin. Quantification of phospho-p38 (pp38) and phospho-ERK (pERK) relative to Tubulin. Histograms represent the mean of the 5 different mice for each treatment. ★ $p < 0.05$, ★★ $p < 0.01$, ★★★ $p < 0.001$.