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 (CRC), tumor resistance is a frequent cause of chemotherapy failure. Therefore, new treatment options are needed to improve survival of patients with irinotecan-refractory CRCs, 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 antitumoral 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 CRC, particularly in KRAS-mutated tumors. Mol Cancer Ther; 12(10); 2121–34. ©2013 AACR.

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-fluorouracil (5-FU)/LV, capecitabine, irinotecan, oxaliplatin, bevacizumab, cetuximab, and panitumumab] either in combination or as single agents (2). Owing 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 irinotecan. 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 multitargeted tyrosine kinase inhibitor (TKI) with antiangiogenic properties mainly by blocking VEGF receptor (VEGFR) and platelet-derived growth factor receptor (PDGFR) activation (16) and has proven its efficiency for the treatment of metastatic renal cell carcinoma and advanced hepatocellular carcinoma (17, 18). In addition, it 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.

Materials and Methods

Drugs

Sorafenib (Nexavar, Bayer Schering) 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 to 50% concentration with saline. SN-38, the active metabolite of irinotecan, was kindly provided by Sanofi-Aventis. Irinotecan (Campto) was from Pfizer (20 mg/mL stock).

Cell lines

The HCT116, SW48, SW620, and HT29 colon adenocarcinoma cell lines from the American Type Culture Collection (ATCC, Manassas, Virginia) were grown in RPMI-1640 supplemented with 10% fetal calf serum (FCS) and 2 mmol/L 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, although no authentication was done by the authors. The SN-38-resistant HCT116 and SW48 cell clones were obtained as previously described (7, 9, 12). Briefly, the reference SN-38-sensitive HCT116 cell clone (HCT116-s) was exposed to 10 nmol/L SN-38 and cloned to obtain the HCT116-SN6 and HCT116-A2 clones. In addition, HCT116-s cells were exposed to 15 nmol/L 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 to 150 nmol/L during eight 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 five days before 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 (1,000 cells/well) in RPMI-1640 supplemented with 10% FCS. After 24 hours, 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). The sulforhodamine B fixed to the cells was dissolved in 10 mmol/L Tris–HCl and absorbance at 540 nm was read using an MRX plate reader (Dynex, Inc.). Each IC50 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 by the authors. The SN-38-resistant HCT116 and SW48 cell clones were obtained as previously described. The SN-38–resistant HCT116 and SW48 cells were continuously exposed to SN-38 with a stepwise increase in concentration from 4 to 150 nmol/L during eight months. SN-38–resistant cells were then 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 to 150 nmol/L during eight 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 five days before any experiment.

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Short hairpin RNA constructs

HCT116-A2 and HCT116-SN50 cells that express short hairpin RNAs (shRNA) 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 conducted using the Annexin V-FITC /7-AAD Kit (Beckman Coulter). Cells were seeded in 25 cm2 flasks at 20,000 cells/flask. After a 24-hour rest, cells were treated for 96 hours with either 0.5 or 3 μmol/L sorafenib, or 0.5 μmol/L sorafenib + 0.7 μmol/L SN-38. The cells (1 × 106) 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–fluorescein isothiocyanate (FITC)-positive cells were quantified using Flow Jo analysis software (Treestar Inc).

Cell-cycle analysis

The cells were seeded in 25 cm2 flasks (2 × 105 cells/flask). After a 24-hour rest, the cells were treated for 96 hours with either 0.5 or 3 μmol/L sorafenib, or 0.5 μmol/L sorafenib + 0.7 μmol/L 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).
Intracellular drug accumulation

The relative intracellular drug content was measured using a Cell Lab Quanta flow cytometer (Beckman Coulter) 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 μmol/L SN-38, 50 μmol/L sorafenib, or 25 μmol/L SN-38 + 50 μmol/L sorafenib for two hours. Intracellular drug accumulation was stopped by cooling on ice and cells were washed in ice-cold PBS before fluorescence-activated cell sorting (FACS) analysis. Assays were conducted at least three times. Data acquisition and analysis were carried out using the FlowJo software (Becton Dickinson).

Tumor xenografts in nude mice

All in vivo experiments were conducted 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 × 10⁶ cells. When tumors of approximately 0.1 cm³ 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/d (except during weekends) every two weeks for three months (except during weekends) every two weeks for three weeks in total. SN-38 was administered by gavage at 25 mg/kg/d (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 weighed every day to adjust the drug doses and follow the drug toxicity. Tumor size was measured with a caliper in mice weighed every day to adjust the drug doses and follow the drug toxicity.

Immunohistochemistry

One third of each tumor xenograft was formalin-fixed, paraffin-embedded, and mounted on Superfrost-plus slides. Before immunodetection, specimens were deparaffinized and rehydrated. Antigen retrieval was done by heat treatment in 10 mmol/L citrate buffer at pH 6. Endogenous peroxidase and biotin activities were blocked with 8% H₂O₂ and 10% normal goat serum, respectively. Slides 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 one hour. Both antibodies were diluted at 1:400 in PBS/3% BSA. Antibody binding was revealed using the Strept a ABCComplex/HRP Duet kit (streptavidin–biotin–peroxidase complex method; DakoCytomation). 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 selected 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) 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 mmol/L, Tris pH 7.4 10 mmol/L, EDTA 1 mmol/L, Triton X100 1%, NP40 0.5%, phenylmethylsulfonylfluoride (PMFSD) 2 mmol/L, NaF 100 mmol/L, Orthovanadate 10 mmol/L, one 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 preincubated in 1 mL Array Buffer 1 for one 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 1× Array Buffer 1), washing, and incubating in a Streptavidin–HRP solution (1:2,000). After washing again, proteins were detected by enhanced chemoluminescence (ECL) by using the ECL detection system from GE Healthcare Life Sciences and recorded using the G/BOX iChem 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 postgraft 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 data until the event of interest (i.e., a volume of 1,000 mm³) with the Kaplan–Meier method. The log-rank test was used to compare survival curves between groups. Statistical analysis was conducted using the STATA 10.0 software (StataCorp).

Results

Sorafenib enhances SN-38 cytotoxic effect in vitro

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 (IC_{50} = 3 μmol/L ± 0.5, P value = 1.5 × 10^{-7}), whereas it was comparable in the other cell lines (IC_{50} = 5.4 μmol/L ± 0.7 for SW48 cells; IC_{50} = 6 μmol/L ± 0.2 for SW620 cells; IC_{50} = 5.1 μmol/L ± 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 (0.5 μmol/L; Fig. 1C). The sorafenib plus SN-38 combination decreased the IC_{50} of SN-38 in all four cell lines in comparison with 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 is also independent of KRAS, BRAF, p53, and PIK3CA mutations.

**Sorafenib associated with SN-38 reverses chemoresistance 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 2.2 μmol/L (HCT116-SN6 clone) and 3.45 μmol/L (HCT116-G7 clone; Fig. 1D) and, thus, comparable with 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 with 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 3.9 to 3.2 μmol/L, whereas the SN-38–sensitive SW48-s clone had an IC₅₀ of 5.4 μmol/L.

We then tested the effect of suboptimal 0.5 μmol/L sorafenib in combination with SN-38 in the SN-38–resistant clones (Fig. 1E and G). 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 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 (Table 3; ref. 22). This calculation method provides a quantitative definition for drug combination called the CI. The CI define additive effect (CI = 1), synergistic effect (CI < 1), or antagonism effect (CI > 1). 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 (range of CI values, 0.095–0.381).

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 μmol/L (suboptimal dose used in combination with SN-38) and 3 μmol/L (IC₅₀) during 96 hours (Fig. 2A, B and C). Although sorafenib at a suboptimal dose (0.5 μmol/L) had no effect on cell proliferation and cell death, the IC₅₀ dose (3 μmol/L) inhibited cell proliferation (Fig. 2A) and increased cell death (Fig. 2B). However, the drug has no effect on cell cycle, except on the sub-G₁ phase (Fig. 2C), representative of late apoptosis, confirming the results of Fig 1B. The combination of sorafenib (0.5 μmol/L) and SN-38 (0.7 nmol/L) 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 chemoresistance 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 a volume of approximately 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 with 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 with that by irinotecan; conversely, it was significantly higher when irinotecan and sorafenib were associated (P = 0.045 vs. irinotecan alone and P = 0.005 vs. sorafenib alone). This result indicates that sorafenib improves irinotecan efficiency in vivo as well.

Moreover, the modified Kaplan–Meier plot showed that a significantly longer time was required for 50% mice to reach a 1,000 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).

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 and 3 μmol/L. Data are the mean of three independent experiments. B, apoptosis of HCT116 cells following exposure to sorafenib (0.5 and 3 μmol/L) for 96 hours. Apoptosis was determined by 7-AAD and Annexin V-FLUOS staining with a FACSScan. C, HCT116 cell-cycle distribution after sorafenib treatment was measured by propidium iodide staining with a FACSScan 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 sub-G1 phase (gray). Cells in sub-G1 represent cells in late apoptosis. D, number of viable HCT116 cells determined by counting after 96 hours treatment with sorafenib 0.5 μmol/L + SN-38 0.7 nmol/L. Data are the mean of three independent experiments. E, apoptosis of HCT116 cells following exposure to sorafenib 0.5 μmol/L + SN-38 0.7 nmol/L for 96 hours. Apoptosis was determined by 7-AAD and Annexin V-FLUOS staining with a FACSScan. F, HCT116 cell-cycle distribution after sorafenib 0.5 μmol/L + SN-38 0.7 nmol/L was measured by propidium iodide staining with a FACSScan 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 sub-G1 phase (gray). Cells in sub-G1 represent cells in late apoptosis.

**P < 0.01.**
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 and 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 IC₅₀ reduction upon combined exposure to sorafenib and SN-38 (Fig. 1E) also overexpress ABCG2, we investigated whether sorafenib could affect SN-38 intracellular accumulation.

Western blot analysis confirmed that ABCG2 was overexpressed 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 analyzed the proportion of cells containing SN-38 using flow cytometry assay, because SN-38 is naturally fluorescent (12). We found that ABCG2 overexpression correlated with lower intracellular accumulation of SN-38 in these clones (8% of cells containing intracellular SN-38) in comparison with SN-38-sensitive HCT116-s cells (67%) and the

![Figure 4](https://example.com/figure4.png)

**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 cytometric 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 cytometric 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) to compare the fold-change between conditions. F, flow cytometric 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 μmol/L 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 μmol/L), SN-38 (20 nmol/L), or sorafenib (0.5 μmol/L) + SN-38 (20 nmol/L) during 72 hours. Protein loading is shown by tubulin. *P < 0.05; **P < 0.01; ***P < 0.001.
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 HCT116-SN50 (Fig. 4G and H). The higher SN-38 intracellular accumulation in HT29 cells correlated with ABCG2 expression (Fig. 4B).

To determine whether the sorafenib effect on ABCG2 function is related to a decrease of ABCG2 expression, we conducted 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 does not affect ABCG2 expression in HCT116 cells resistant to SN-38.

**Sorafenib inhibits the phosphorylations of p38 and ERK induced by irinotecan**

Next, we assessed differences in the cell signaling responses to sorafenib alone, irinotecan alone, and both drugs together in xenografts by using proteome profiler (Fig. 6A). We observed that irinotecan alone activates 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 with 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 signaling pathways, p38 and ERK.

To confirm the sorafenib effect on p38 and ERK phosphorylation, we conducted Western blot analysis on five xenografts per treatment (NT, sorafenib, irinotecan, sorafenib + irinotecan). As shown in Fig. 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. In addition, sorafenib, by inhibiting p38 and ERK signaling 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 antitumor activity of irinotecan and SN-38 was more pronounced when they were used in combination with sorafenib both in vitro and in vivo. In addition, this sorafenib effect was observed in SN-38–resistant CRC cell clones, indicating that sorafenib can restore irinotecan sensitivity. Sorafenib clearly improves inhibition of cell proliferation and enhances apoptosis. Furthermore, in vivo, angiogenesis was reduced and sorafenib could promote the effects of irinotecan 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 overexpress 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 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 and colleagues reported that sorafenib decreases the expression level of ABCC2 in some breast cancer cell

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 five 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 five different mice for each treatment. *, $P < 0.05$; **, $P < 0.01$. 
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 and colleagues proposed that sorafenib induces ABCG2 degradation via the lysosome (20). Taken together, 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 suggest that sorafenib acts on ABCG2 function and not on ABCG2 expression.

Increased ABCG2 expression has also been reported in cancer stem cells (CSC) and ABCG2 may serve as a CSC marker (34). Spheroids derived from tumor biopsies of patients with CRC display CSC properties, such as enhanced self-renewal capacity, slow division, drug resistance, and expression of ABCG2, CD133, and CD44 (35). As the CSC 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 overexpress 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 also more efficient than irinotecan alone in CRC cells in which ABCG2 was downregulated 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 also be related to activation of NF-κB and inhibition of the apoptotic cascade. Jayanth and colleagues (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-κB activation by retaining NF-κB in the cytoplasm and, therefore, preventing induction of antiapoptotic genes. Another hypothesis is that sorafenib could modify metabolism of irinotecan. 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 can 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 shown by Namboodiri and Grossi, respectively, in vitro and in cellulo (39, 40). Therefore, sorafenib, by inhibiting the MAPK p38 pathway, makes 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 where 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, the ERK pathway as well 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.

Disclosure of Potential Conflicts of Interest
E. Assenat is a consultant/advisory board member for Novartis, Roche, Ipsen, and Bayer. M. Ychou is a consultant/advisory board member for Bayer. No potential conflicts of interest were disclosed by the other authors.

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