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, capcitabine, 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 incubating with 0.5 or 3 mmol/L sorafenib, or 0.5 mmol/L SN-38. One million cells were harvested, washed with PBS, fixed in 40 g/mL of RNAse (Qiagen), and stained with 40 g/mL of propidium iodide. Analyses were done on a FC500 Beckman Coulter Flow Cytometer. Annexin V–fluorescein isothiocyanate (FITC)-positive cells were quantified using FlowJo analysis software (Treestar Inc).

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 mmol/L sorafenib, or 0.5 mmol/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 FlowJo analysis software (Treestar Inc).

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-cycle analysis
The cells were seeded in 25 cm² flasks (2 × 10⁴ cells/flask). After a 24-hour rest, the cells were treated for 96 hours with either 0.5 or 3 mmol/L sorafenib, or 0.5 mmol/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 FlowJo 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 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 administered by gavage at 25 mg/kg/d (except during weekends) every two weeks for three weeks in total. Sorafenib was administered by gavage at 25 mg/kg/d (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. Sorafenib was administered by gavage at 25 mg/kg/d (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 administered by gavage at 25 mg/kg/d (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. Sorafenib was administered by gavage at 25 mg/kg/d (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. Sorafenib was administered by gavage at 25 mg/kg/d (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. Sorafenib was administered by gavage at 25 mg/kg/d (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. Sorafenib was administered by gavage at 25 mg/kg/d (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. Sorafenib was administered by gavage at 25 mg/kg/d (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. Sorafenib was administered by gavage at 25 mg/kg/d (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. Sorafenib was administered by gavage at 25 mg/kg/d (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. Sorafenib was administered by gavage at 25 mg/kg/d (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. Sorafenib was administered by gavage at 25 mg/kg/d (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. Sorafenib was administered by gavage at 25 mg/kg/d (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. Sorafenib was administered by gavage at 25 mg/kg/d (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.

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 specific reagents. Sections were incubated at room temperature with anti-cleaved Caspase 3 (SAEI, Cell Signaling Technology) for 30 minutes or with anti-CD54 (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 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 (PMSD) 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 from the xenograft date 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 IC$_{50}$ 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 IC$_{50}$ in SN-38–sensitive HCT116-s cells (Fig. 1B). Only the HCT116-SN6 clone showed a small, but significant decrease in IC$_{50}$ 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 IC$_{50}$ ranged from 3.9 to 3.2 μmol/L, whereas the SN-38–sensitive SW48-s clone had an IC$_{50}$ 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 IC$_{50}$ 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 (IC$_{50}$) 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 IC$_{50}$ 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-G$_1$ 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 mM/L) at suboptimal dose, display the same effect on cell proliferation, cell death, and cell cycle (Fig. 2D, E and F) as sorafenib at IC$_{50}$ 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 mm$^3$, 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 mm$^3$ 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 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
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 intracellular accumulation of SN-38 not only 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 overexpress ABCG2 (Fig. 4E). These data suggest that sorafenib regulates SN-38 accumulation mainly, but not exclusively, via inhibition of the efflux pump ABCG2.

In addition, increased SN-38 accumulation was 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 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 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 downregulated (HCT116-A2-ShABCG2 and HCT116-SN50-ShABCG2 cells) in comparison with 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 and 15-fold in HCT116-SN50-ShLuc cells in comparison with cells treated with SN-38 alone). Conversely, in cells in which ABCG2 was downregulated, sorafenib increased SN-38 intracellular accumulation only weakly (7-fold increase in HCT116-A2-ShABCG2-3 and 5-fold in HCT116-SN50-ShABCG2-3 in comparison with cells treated with SN-38 alone), indicating that the sorafenib effect on intracellular drug accumulation is impaired when ABCG2 is knocked down. This result indicates that sorafenib is mainly inhibiting ABCG2 activity because it does not impair its expression (Fig. 4G and 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 conducted cytotoxic assays in control cells and in cells in which ABCG2 was downregulated. Sorafenib significantly sensitized HCT116-A2-ShLuc and HCT116-SN50-ShLuc control cells to SN-38 as indicated by the 7.6- and 10-fold reduction of IC₅₀, respectively, and similarly to what is observed in the parental HCT116-A2 and HCT116-SN50 cell lines. In addition, sorafenib increased SN-38 cytotoxicity in cells in which ABCG2 was knocked down, but to a lesser extent than in control cells (3.4-fold reduction of IC₅₀ in HCT116-A2-ShABCG2 cells and 2.8-fold in HCT116-SN50-ShABCG2 cells). Taken together, 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 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 inhibits 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. Jayanthan 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 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 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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