Targeting the p38 MAPK Pathway Inhibits Irinotecan Resistance in Colon Adenocarcinoma

Salomé Paillas¹, Florence Boissière², Frédéric Bibeau², Amélie Denouel², Caroline Mollevi³, Annick Causse¹, Vincent Denis¹, Nadia Vezzio-Vie¹, Laetitia Marzi¹, Cédric Cortijo¹, Imade Ait-Arsa⁴, Nadav Askari⁴, Philippe Pourquier⁵, Pierre Martineau¹, Maguy Del Rio¹, and Céline Gongora¹

Abstract

Despite recent advances in the treatment of colon cancer, tumor resistance is a frequent cause of chemotherapy failure. To better elucidate the molecular mechanisms involved in resistance to irinotecan (and its active metabolite SN38), we established SN38-resistant clones derived from HCT-116 and SW48 cell lines. These clones show various levels (6- to 60-fold) of resistance to SN-38 and display enhanced levels of activated MAPK p38 as compared with the corresponding parental cells. Because four different isoforms of p38 have been described, we then studied the effect of p38 overexpression or downregulation of each isoform on cell sensitivity to SN38 and found that both α and β isoforms are involved in the development of resistance to SN38. In this line, we show that cell treatment with SB202190, which inhibits p38α and p38β, enhanced the cytotoxic activity of SN38. Moreover, p38 inhibition sensitized tumor cells derived from both SN38-sensitive and -resistant HCT116 cells to irinotecan treatment in xenograft models. Finally, we detected less phosphorylated p38 in primary colon cancer of patients sensitive to irinotecan-based treatment, compared with nonresponder patients. This indicates that enhanced level of phosphorylated p38 could predict the absence of clinical response to irinotecan. Altogether, our results show that the p38 MAPK pathway is involved in irinotecan sensitivity and suggest that phosphorylated p38 expression level could be used as a marker of clinical resistance to irinotecan. They further suggest that targeting the p38 pathway may be a potential strategy to overcome resistance to irinotecan-based chemotherapies in colorectal cancer. Cancer Res; 71(3); 1041–9. ©2010 AACR.

Introduction

Colorectal cancer (CRC) is the third most frequent malignancy in Western countries. Despite significant developments in CRC treatment, it still causes extensive mortality. Nowadays, the chemotherapy options for metastatic CRC patients consist in the combination of 5-fluorouracil/leucovorin (5-FU/LV) and irinotecan (FOLFIRI) or 5-FU/LV and oxaliplatin (FOLFOX) associated, or not, with the anti-VEGF antibody Bevacizumab or the anti-EGFR antibody Cetuximab (1). Bevacizumab indication is restricted to patients with wild type KRAS, as KRAS mutations predict resistance to treatment with anti-EGFR monoclonal antibodies (2). Therapeutic failure is mainly due to resistance to drug treatment. Thus, identifying the cellular mechanisms that lead to resistance is a crucial issue for improving CRC treatment and survival.

Irinotecan (CPT-11) is a derivative of camptothecin and its active metabolite is SN-38. Like other camptothecin derivatives, SN38 inhibits topoisomerase I (Topol), a nuclear enzyme needed for replication and transcription by unwinding supercoiled DNA (3). SN38 interferes with Topol activity by trapping Topol-DNA cleavage complexes, leading to lethal replication-mediated double strand breaks (3) that induce phosphorylation of the H2AX histone variant and activate the ATM-CHK2-p53, DNA-PK, and ATR-CHK1 signaling pathways.

Cellular mechanisms causing irinotecan resistance have been reported for each step of the CPT-11 pathway (4). Reduced intracellular drug accumulation (5) and alterations in CPT-11 and SN38 metabolism play important role in the development of resistance (6). In addition, quantitative and qualitative alterations of Topol have been extensively reported in cell lines (7–10, 27). Finally, alterations in the cellular responses to ternary complex formation that ultimately lead either to repair of DNA damage or to cell death (11, 12) can induce resistance to camptothecins. The clinical relevance of these resistance mechanisms is poorly documented, except for the high variability in patients’ metabolism of CPT-11, which represents a good response indicator. Moreover, there is a need to find new
markers that can predict patients’ response to irinotecan-based regimens and thus help to identify the patients who are most likely to respond to such treatments. We have previously shown that SN38-resistant HCT116 cells display endogenous activation of the p38 mitogen-activated protein kinase (MAPK; ref. 13). p38 belongs to a class of serine/threonine MAPK (14) and is composed of 4 isoforms (α, β, γ, and δ) with more than 60% overall sequence homology and more than 90% identity within the kinase domains. p38α and p38β are expressed in most tissues and are both sensitive to pyridinyl imidazole inhibitors (such as SB202190), whereas p38γ and p38δ have a more restricted pattern of expression and are insensitive to these inhibitors (15). p38 is activated through phosphorylation at the Thr180-Gly-Tyr182 motif by MKK3, MKK4, and M KK6 (16). Phosphorylated p38 then activate a wide range of substrates that include transcription factors, protein kinases, and cytosolic and nuclear proteins, leading to diverse responses such as inflammatory responses, cell differentiation, cell-cycle arrest, apoptosis, senescence, cytokine production, and regulation of RNA splicing (15). The role of p38 in cancer depends on the cell type and cancer stage: some studies have reported that p38 increases cell proliferation, whereas in others the activation of the MAPK p38 pathway is described as a tumor suppressor. For example, p38α expression is downregulated in lung tumors, suggesting that p38 loss could be related to tumor formation (17). Consistent with this, the expression of MKK4, a p38 activator, is reduced in advanced tumors (18). Conversely, increased levels of phosphorylated p38α have been correlated with malignancy in follicular lymphoma, lung, thyroid, and breast carcinomas, glioma, and head and neck squamous cell carcinoma (19). In CRC, p38α is required for cell proliferation and survival and its inhibition leads to cell-cycle arrest and autopapheic cell death (20).

Here, we have examined the functional role of the four p38 isoforms in the development of irinotecan resistance. We show that p38α is activated by irinotecan treatment and that all the tested SN38-resistant HCT116 cell lines display higher p38 phosphorylation level than control or SN38-sensitive cells. Pharmacological inhibition of p38α and p38β overcomes irinotecan resistance and, therefore, could be used to prevent the development of a resistant phenotype in sensitive cells. Finally, we report higher level of phosphorylated p38α in tumor samples from patients who are refractory to irinotecan-based therapy compared with responder patients.

### Material and Methods

#### Cell lines and reagents

The HCT116 colon adenocarcinoma cell line from ATCC were grown in RPMI 1640 supplemented with 10% fetal calf serum (FCS) and 2 mM/L L-glutamine at 37°C under a humidified atmosphere with 5% CO2. The HCT116-s, SW48-s, and SN38-resistant clones were obtained as previously described (5; Supplementary Data).

SN38 was provided by Sanofi-Aventis. SB202190 was purchased from Sigma-Aldrich. Irinotecan, 5-fluorouracile, and oxalplatin were provided by Dr. F. Pinguet (CRLC Val d’Aurelle, France).

#### Drug sensitivity assay

Cell growth inhibition and cell viability after SN38 treatment were assessed using the sulforhodamine B (SRB) assay as previously described (5).

#### Western blot analyses

Western blot analysis of cell extracts from control and retrovirus-infected cells were performed as previously described (21). Primary antibodies were directed against phospho-p38, p38α, p38β, p38γ, p38δ, pATF2 (Cell Signaling Technology), β-actin (AbCam), and Topol (1). Secondary antibodies were horseradish peroxidase–conjugated antirabbit or anti-mouse (Sigma Aldrich).

#### Immunofluorescence

Cells were plated on 12-mm glass coverslips in culture dishes. They were fixed in 3.7% formaldehyde, blocked in PBS with 1% BSA and 0.3% Triton, and incubated with an anti- phospho p38 monoclonal antibody (Cell Signaling Technology) and then with a FITC-conjugated anti-rabbit antiserum (maker). 4′,6-diamidino-2-phenylindole (DAPI) was used to stain the nuclei. Coverslips were then mounted in Moviol, and images were recorded using a 63× NA objective and a Leica inverted microscope.

#### Retroviral infection

HCT116-s and HCT116-SN6 cells that stably express the 4 constitutively active (CA) p38 isoforms were obtained by retroviral gene transduction of the pMSCV vector in which CA variants of p38α, p38β, p38γ, and p38δ (22) had been previously subcloned. Twenty-four hours after transduction, cells were selected with 1 μg/mL of hygromycin and then stable clones were pooled.

HCT116-s and HCT116-SN6 cells expressing short hairpin RNA (shRNA) targeting luciferase, p38α, p38β, p38γ, and p38δ mRNA were obtained by retroviral gene transduction of the pSIREN vector in which the shRNAs were cloned. Cells were selected with 1 μg/mL of puromycin and then stable clones were pooled.

#### Kinase assay

The p38 kinase assay was performed using the nonradioactive p38 MAPK Assay Kit from Cell Signaling Technology as previously described (13).

#### In vivo experiments

**Xenografts.** Female athymic nu/nu mice were purchased from Harlan Laboratories and used at 6 to 8 weeks of age. About 3 × 106 tumor cells were injected subcutaneously (s.c.) into the left flank of each mouse. Tumors were detected by palpation and measured periodically with calipers. Mice were euthanized when the tumor volume reached 1,000 mm3.

**Irinotecan and SB202190 treatment.** Irinotecan stock solution was diluted in 0.9% sodium chloride and 40 mg/kg were administered intraperitoneally (i.p.) to tumor-bearing mice according to the following schedule: 4 injections (1 every 4 days) starting when tumors reached 100 mm3. Mice in the
control group received 0.2 mL of 0.9% sodium chloride solution according to the same schedule. SB202190 stock solution was diluted in 0.9% sodium chloride and administered i.p. at 0.05 μmol/kg daily for 12 days when tumors reached 100 mm³.

Irinotecan + SB202190 were administered when tumors reached 100 mm³; 4 injections (1 every 4 days) of irinotecan at 40 mg/kg + SB202190 at 0.05 μmol/kg for 12 days.

**Protein extraction from xenografts**

Xenografts from nude mice were isolated and proteins extracted as follows. Tumors were cut and lysed in 500 μL of lysis buffer (150 mmol/L NaCl, 10 mmol/L Tris, pH 7.4, 1 mmol/L EDTA, 1 mmol/L EGTA, 0.5% NP40, 1% Triton, 2 mmol/L PMSF, 100 mmol/L NaF, 10 mmol/L Na₂VO₄, and a cocktail of protease inhibitors) and then homogenized with beads using the MixerMill apparatus. Extracts were centrifuged and proteins in the supernatants were quantified with the Bradford assay and loaded on SDS-PAGE gels.

**Detection of phosphorylated p38 by immunohistochemistry in clinical samples**

A tissue microarray (TMA) including samples from 21 metastatic CRC patients was constructed as previously described (24), using 3 malignant tissue cores (0.6-mm diameter) per tumor. Tissue samples were from patients of a previously published prospective series (25) and were all chemotherapy-naive at the time of surgery of their primary tumor. They all subsequently received the FOLFIRI regimen as first-line chemotherapy. Tumor response was evaluated according to the WHO recommendations after each of the 4 or 6 cycles of chemotherapy. Nine patients showed a decrease of 50% or more of their metastatic lesion and were classified as responders, and 12 patients, with a decrease of 50% or more of their metastatic lesion and were classified as nonresponders. Three-micrometer sections of the tumor. They all subsequently received the FOLFIRI regimen as first-line chemotherapy. Tumor response was evaluated according to the WHO recommendations after each of the 4 or 6 cycles of chemotherapy. Nine patients showed a decrease of 50% or more of their metastatic lesion and were classified as responders, and 12 patients, with a decrease of 50% or more of their metastatic lesion and were classified as nonresponders. Three-micrometer sections of the TMA were deparaffinized and rehydrated in graded alcohols. Following epitope retrieval treatment in EDTA buffer (pH 9), TMA sections were incubated overnight at 4°C with the anti-phospho-p38 MAPK (Thr¹⁸⁰/Tyr¹⁸², Cell Signaling Technology), followed by a standard detection system (FLEX++; Dako). Phospho-p38 signals were observed both in the nucleus and cytoplasm of tumors cells, but only the nuclear staining was taken into account. Briefly, each spot in the TMA sections received a score for the percentage of marked cells and for the staining intensity. Data were then consolidated into a single score as the mean of the triplicate score. Finally, we defined a Quick Score (QS) by multiplying the intensity grade by the percentage of stained nuclei.

**Statistical analysis**

Continuous variables were presented as medians (range) and compared between populations with the nonparametric Wilcoxon rank-sum test. Qualitative variables were compared using the Fisher’s exact test. Differences were considered statistically significant when $P < 0.05$. Survival rates were estimated from the date of the xenograft to the date when the tumor reached a volume of 200 mm³, using the Kaplan–Meier method. Survival curves were compared using the log-rank test. Statistical analysis was performed using STATA 11.0 software (StatCorp).

**Results**

The MAPK p38 is constitutively activated in SN38-resistant colon carcinoma cells and its phosphorylation is increased by SN38 treatment

We have already shown that HCT116-SN6 colon adenocarcinoma cells, which display a 6-fold resistance to SN38, present constitutive activation of the MAPK p38, compared with a sensitive clone HCT116-s (13). Now, we investigated whether SN38 treatment favors p38 activation in sensitive HCT116-s and SN38-resistant HCT116-SN6 cells. Western blot analysis using phospho-specific p38 antibodies showed an increase of p38 phosphorylation following SN38 treatment in both cell lines (Fig. 1A). In untreated cells, basal p38 phosphorylation was more pronounced in HCT116-SN6 than in HCT116-s cells, as previously reported. We confirmed these results by immunofluorescence (IF; Fig. 1B). Phospho-p38 staining (in green) appeared to be granular or punctuated as previously reported (26). These results indicate that p38 is activated by SN38 in both SN38-sensitive and -resistant HCT116 cells.

To determine whether p38 is constitutively activated only in the SN38-resistant HCT116-SN6 clone, we tested the level of p38 phosphorylation in 4 other resistant clones, HCT116-A2, -SN50, -C8, and -G7 by immunofluorescence. We found that in all SN38-resistant clones, basal p38 phosphorylation was higher than in HCT116-s cells (Fig. 1C). To determine whether this phenomenon could be observed in another colon cancer cell model, we used the SW48-sensitive cells and generated SN38-resistant clones (Supplementary Fig. 1). Similar to HCT116 SN38-resistant clones, all SW48 resistant clones displayed higher p38 phosphorylation, as compared with the parental SW48-s clone further suggesting that this feature could be a hallmark of SN38 resistance.

The p38α and p38β isoforms are involved in SN38 resistance

We then analyzed the potential functional impact of p38 phosphorylation on the development of resistance to SN38. To this aim, we expressed a CA form of the upstream p38 activating kinase MKK6 in HCT116 cells. CA MKK6 rendered cells more resistant to SN38 treatment (Supplementary Fig 2), confirming the notion that activation of p38 can increase SN38 resistance. Because p38 has 4 isoforms (α, β, γ, and δ), we then overexpressed or downregulated each isoform in HCT116-s and HCT116-SN6 cells to identify which one was involved in the development of resistance to SN38.

First, each of the 4 p38 isoforms was downregulated by shRNA in SN38-sensitive HCT116-s cells (Fig. 2A, left), and the effect of their silencing on the sensitivity to SN38 was tested with the SRB assay. SN38 cytotoxicity was stronger only in the cells in which p38α was silenced (Fig. 2B, left), as shown by their lower IC₅₀ (50% inhibitory concentration) than in control...
cells (1.1 and 1.9 nmol/L, respectively, \( P = 0.002 \)). Silencing of the \( \beta, \gamma, \) or \( \delta \) isoform had no impact on SN38 sensitivity in HCT116-s cells.

Then, HCT116-s cells were infected with retroviruses expressing a CA variant of each p38 isoform (22) and sensitivity to SN38 was again tested using the SRB assay. Expression of the CA p38 variants was monitored by Western blotting with an anti-HA antibody (Fig. 2A, right). No difference in the IC\(_{50}\) of the different cell lines was observed (Fig. 2C). We then tested whether coexpression of the \( \alpha \) and \( \beta \) isoforms could affect HCT116-s cell sensitivity to SN38 and found that these cells became more resistant to SN38 as evidenced by a higher IC\(_{50}\) (Fig. 2D). This result indicates that overexpression of both p38 isoforms \( \alpha \) and \( \beta \) can influence sensitivity of HCT116-s cells to SN38.

Silencing of the 4 p38 isoforms by shRNA and expression of the CA isoforms were then carried out in SN38-resistant HCT116-SN6 cells and were monitored by Western blotting as before (Fig. 2E). For HCT116-s cells, only downregulation of p38\( \alpha \) sensitized HCT116-SN6 cells to SN38 treatment (Fig. 2F; IC\(_{50}\) 3.6 and 10 nmol/L respectively, \( P = 0.0003 \)). In addition, overexpression of CA p38\( \beta \) (Fig. 2G) or p38\( \alpha + \beta \) (Fig. 2H) rendered HCT116-SN6 cells more resistant to SN38 treatment (\( \beta \) overexpression: IC\(_{50}\) 19.4 and 10 nmol/L, respectively, \( P = 0.0018 \) and \( \alpha + \beta \) overexpression: IC\(_{50}\) 16.125 and 9 nmol/L, \( P = 0.0028 \)). These results indicate that coexpression of CA p38\( \alpha \) and p38\( \beta \) can further enhance the level of resistance of resistant clones.

Because alterations of Topo1 (9, 28, 29) can confer resistance to camptothecin derivatives (30–32), we then investigated whether overexpression of CA p38\( \beta \) or downregulation of p38\( \alpha \) in HCT116-SN6 and HCT116-s cells influenced Topo1 expression (Western blotting) and activity (ICE bioassay; ref. 23). No changes in Topo1 expression or activity were detected (Supplementary Fig. 3), indicating that p38 effect on cell sensitivity to SN38 is not linked to quantitative alteration of Topo1-DNA complexes.

**Association of SB202190 with SN38 decreases resistance to SN38 in vitro**

Because p38\( \alpha \) and p38\( \beta \) appear to play a role in the development of resistance to SN38, we then assessed the effect of blocking concomitantly the activity of both isoforms with SB202190, a specific inhibitor of p38\( \alpha \) and p38\( \beta \), on the sensitivity to SN38 by SRB assay in HCT116-s and HCT116-SN6 cells. Incubation with SB202190 alone had similar effect on cell growth in both SN38-sensitive and SN38-resistant cells (Fig. 3A). When SN38 was associated with SB202190, the IC\(_{50}\) in both sensitive and resistant cells was decreased by 2-fold in comparison to cells treated only with SN38 (Fig. 3A). The effect of SB202190 on p38 activity was then monitored by kinase assay (Fig. 3B) that follows p38 activity on its target protein ATF2. Basal p38 activity was more pronounced in HCT116-SN6 than in HCT116-s cells, as we previously described (13). SN38 treatment enhanced p38 activity and this effect was inhibited by SB202190 treatment. These experiments show that sensitivity to SN38 is largely increased when SB202190 is added to the culture medium in both cell lines, indicating that...
inhibition of p38α and p38β activity sensitizes even SN38-resistant cells to SN38 in vitro.

SB202190 effect on SN38 sensitivity was tested on 2 other colon adenocarcinoma cell lines (HT29 and SW480) that have different genotypes. The HCT116 cell line has wild-type p53 and mutated KRAS, whereas HT29 cells have mutated p53 and wild-type KRAS and SW480 cells show mutated p53 and KRAS.
with SN38 alone or in combination with SB202190 (Fig. 3C). As for the different HCT116 cell lines, the association of SN38 with SB202190 also lowered the IC₅₀ of HT29 (IC₅₀: 9.8 and 5.5 nmol/L, respectively, *P* = 0.005) and SW480 cells (IC₅₀: 5.5 and 2.8 nmol/L respectively, *P* = 0.0013), indicating that SB202190 effect on SN38 sensitivity is independent of the p53 and KRAS status. Conversely, when CA α and β isoforms were coexpressed in SW480 or in HT29 cells, we found that cells became more resistant to SN38 (Fig. 3D), further strengthening the role of both p38α and p38β isoforms in the development of SN38 resistance.

We then asked whether the inhibition of p38α and p38β activity by SB202190 could also affect sensitivity to 5-FU or oxaliplatin in HCT116-s cells. Although both drugs induced p38 phosphorylation, total p38 levels were not affected (Fig. 3E), SB202190 had no impact on 5-FU cytotoxicity, whereas it reduced oxaliplatin cytotoxicity (IC₅₀: 350 nmol/L for oxaliplatin and 550 nmol/L for oxaliplatin + SB202190, *P* = 0.005; Fig. 3F).

These data suggest that inhibition of p38α and p38β by SB202190 enhances specifically the cytotoxic effect of SN38, independently of the type of colon cancer cell lines.

**HCT116-SN6 cells are resistant to irinotecan in a xenograft model**

To evaluate the behavior of SN38-resistant and -sensitive HCT116 cell lines in vivo, HCT116-s and HCT116-SN6 cells were s.c. injected in nude mice and mice treated with or without irinotecan. Without treatment, similar tumor growth was observed for both cell lines (Fig. 4A), but they responded differently to irinotecan. In mice with HCT116-s xenografts, tumor growth was blocked by irinotecan, and at the end point (day 18), tumor size was 5-fold lower than in untreated mice (Fig. 4A, *P* < 0.001). Conversely, mice with HCT116-SN6 xenografts were less sensitive to irinotecan, as tumor size was only half of that of untreated mice at day 18 (300 mm³ in irinotecan-treated mice and 600 mm³ in untreated mice, *P* = 0.001) and tumor growth was not completely blocked as in the case of HCT116-s xenografts. These observations further confirm that HCT116-SN6 cells are less sensitive to irinotecan than HCT116-s.

We then analyzed the in vivo response to irinotecan in xenografts of HCT116-SN6 cells in which p38α had been downregulated by shRNA or CA p38β overexpressed. As before, tumor growth before irinotecan treatment was similar in all groups (mice xenografted with HCT116-SN6-pMSCV, HCT116-SN6-Cap38β, HCT116-SN6-ShLuc, or HCT116-SN6-Shp38α cells), (Supplementary Fig. 4A), indicating that differential p38 expression did not influence tumor growth. However, HCT116-SN6-Cap38β tumors were less sensitive to irinotecan treatment than control HCT116-SN6-pMSCV tumors (Supplementary Fig. 4B). Conversely, HCT116-SN6-Shp38α tumors were slightly more sensitive to irinotecan than control HCT116-SN6-ShLuc tumors (Supplementary Fig. 4D). Overexpression of CA p38β and downregulation of p38α were monitored by Western blot analysis of xenografts at the end of the experiment (Supplementary Fig. 4C and 4E). These in vivo results confirm that p38α downregulation sensitizes HCT116 cells to irinotecan treatment whereas p38β overexpression renders cells less sensitive to the drug.

**Association of SB202190 with irinotecan decreases resistance to therapy in vivo**

We then investigated the impact of inhibition of p38α and p38β activity on resistance to irinotecan in mice injected s.c.
with HCT116-SN6 cells. When tumors reached 100 mm$^3$, xenografted mice were divided in 4 groups: one group was left untreated, one was treated with irinotecan alone, one with SB202190 alone (32), and the last group with irinotecan and SB202190. SB202190 inhibited tumor growth as efficiently as irinotecan (Fig. 4B, $P = 0.792$), whereas the combination of irinotecan and SB202190 was statistically more effective than irinotecan ($P < 0.001$) or SB202190 alone ($P = 0.003$) and completely abolished growth of SN38-resistant xenografts, making HCT116-SN6 cells behave like SN38-sensitive HCT116-s cells (cf. Fig. 4A and B). This result provides evidence that inhibition of p38$\alpha$ and p38$\beta$ activity can reverse resistance to irinotecan in vivo.

Survival was significantly longer in the groups treated with irinotecan ($P = 0.016$) or SB202190 ($P = 0.047$) alone (median survival = 12 days in each group) than in untreated mice (NT; median survival = 7 days). Although irinotecan or SB202190 were efficient in inhibiting growth of HCT116-SN6 xenografts, as previously described (20), the irinotecan + SB202190 combination was much more effective, as survival was significantly better than with irinotecan alone ($P = 0.045$). This data shows an additive effect of SB202190 and irinotecan on tumor growth.

**Level of p38 phosphorylation is linked to impaired response to FOLFIRI therapy in CRC patients**

To address the clinical relevance of p38 activation on the response to treatment, the level of p38$\alpha$ phosphorylation was analyzed in a TMA composed of 21 primary colorectal carcinomas from metastatic CRC patients who received, following surgery, first line FOLFIRI chemotherapy. Nine patients were classified as responders (R) and 12 patients as nonresponders (NR) according to the WHO recommendations (25). TMA phospho-p38 signals were recorded to define a Quick Score (QS) that reflected the level of nuclear phospho-p38 expression in each clinical sample. Responders displayed less phospho-p38 staining than nonresponders [Fig. 5A; median QS (range): 105 (35; 248.9) and 160 (46.7; 180), respectively, $P = 0.0644$] as evidenced by immunohistochemistry in Figure 5B (compare samples from responders 1, 2, and 3 with samples from nonresponders 4, 5, and 6). This is in agreement with our in vitro results, where SN38-resistant HCT116-SN6 cells presented constitutive p38 activation.

These data were then analyzed using the ROC curves and the Youden index to define a threshold (147) that separated the QS depending on the response to treatment (Fig. 5C). Eight of the 9 responders were correctly classified (QS lower than 147; sensitivity = 89%). Thus, the correlation between low p38 phosphorylation and good response to FOLFIRI regimen was almost significant ($P = 0.067$, Fisher’s test). Conversely, the threshold did not correctly classify the nonresponders (specificity = +58%). This result means that high p38 phosphorylation is a marker of impaired response, and that other mechanisms may also be involved in resistance to irinotecan treatment, explaining the clinical response of nonresponder patients with a low level of activated p38$\alpha$.

**Discussion**

In the present study, we have demonstrated the role of the 2 p38 isoforms $\alpha$ and $\beta$ in the resistance to irinotecan. Moreover we have shown that inhibition of p38 enhances the efficacy of irinotecan-based chemotherapies.

We have shown that p38 phosphorylation is increased in SN38 resistant clones derived from HCT116 and SW48 cells, suggesting that p38 activation might represent a general resistance mechanism. Even though p38 constitutive activation was never reported in irinotecan-resistant cells, we could reproducibly detect a low level of p38 phosphorylation in our resistant clones. This low level of p38 activation may not be
sufficient to induce the negative regulatory controls such as protein phosphatases explaining the permanent constitutive activation (39). Moreover, we report that treatment with SN38 induces p38 activation both in SN38-sensitive and -resistant HCT116 cells. Activation of p38 MAPK by SN38 has already been observed in MCF-7 breast cancer cells (37), or in response to other chemotherapeutic agents such as 5-FU (35) or oxaliplatin (36). In these studies, p38 activation was shown to be involved in the cytotoxic effect of the drugs, but the authors did not discriminate between the four p38 isoforms. Here, we could provide experimental evidences showing that activation of both p38α and p38β isoforms inhibit the cytotoxic effect of SN38 in HCT116 colorectal cancer cells. In addition, pharmacologic inhibition of p38α and p38β restores sensitivity to irinotecan in a xenograft model.

p38 involvement in resistance to SN38 or irinotecan may be explained by its role in cell survival. Indeed, p38 mediates colon cancer cell survival pathway, in particular, via its role in DNA repair and autophagy. First, p38 is one of the effectors of the DNA damage sensor system starting with the activation of ATM, ATR and DNA-PK (33). The CA p38 in colon cancer cells may induce enhanced DNA repair in response to chemotherapy and lead to drug resistance. Second, according to Comes and colleagues, p38α inhibits autophagy and cell death of CRC cells (34) demonstrating that p38α activity is necessary for their survival.

Detection of p38 activation in clinical samples has already been shown in reports studying the resistance to treatment. Gutierrez and colleagues demonstrated that p38 activation contribute to tamoxifen resistance in ER-positive breast tumors (38). More recently, Wen and colleagues showed that p38 inhibition enhances the sensitivity to arsenic trioxide and bortezomib in multiple myeloma (40). In line with these results, we show that the level of phosphorylated p38α is higher in patients unresponsive to chemotherapy compared with responders, suggesting that p38 activation is correlated with irinotecan resistance in CRC. Indeed, 8 of the 9 responders exhibit low phospho-p38 expression and were correctly classified. Half of the nonresponders were correctly classified, and in the rest of the nonresponders, we could not detect high p38α phosphorylation. Both isoforms α and β being involved in SN38 resistance, misclassified nonresponder patients might present high p38β phosphorylation, a hypothesis that we could not test because of the absence of available antibody directed against the active form of p38β. In addition, other mechanisms of resistance, independent of p38 activation may be present in this patient’s population. Altogether, our data show, for the first time, that phosphorylation status of the MAPK p38α and p38β isoforms may be a marker of resistance to irinotecan-based chemotherapies in CRC. They further suggest the use of p38 inhibitors as an adjuvant therapy to potentiate the efficacy of irinotecan-based chemotherapies in nonresponder CRC patients. Further studies using larger cohorts of patients will be necessary to confirm this result.

Disclosure of Potential Conflicts of Interests

No potential conflicts of interests were disclosed.

Grant Support

Funds for this research were provided by INSERM and La Ligue Contre le Cancer.

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Received July 26, 2010; revised November 18, 2010; accepted November 23, 2010; published OnlineFirst December 15, 2010.

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