

Targeting the p38 MAPK pathway inhibits irinotecan resistance in colon adenocarcinoma

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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 to 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 p38 α and β , 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 to non-responder 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.

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). Cetuximab indication is restricted to patients with wild type KRAS, since 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 (TopoI), a nuclear enzyme needed for replication and transcription by unwinding supercoiled DNA (3). SN38 interferes with TopoI activity by trapping TopoI-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 the 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 TopoI have been extensively reported in cell lines (7-10). 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) (13). p38 belongs to a class of Serine/Threonine MAPK (14) and is composed of four isoforms (α , β , γ and δ) with >60% overall sequence homology and >90% identity within the kinase domains. p38 α and p38 β are expressed in most tissues and are both sensitive to pyridinyl imidazole inhibitors (such as SB202190), whereas p38 γ and p38 δ 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 MKK6 (16). Phosphorylated p38 then activate a wide range of substrates that include transcription factors, protein kinases, 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, while in others the activation of the MAPK p38 pathway is described as a tumor suppressor. For example, p38 α expression is down-regulated 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 p38 α have been correlated with malignancy in follicular lymphoma, lung, thyroid, breast carcinomas, glioma and head and neck squamous cell carcinoma (19). In CRC, p38 α is required for cell proliferation and survival and its inhibition leads to cell cycle arrest and autophagic cell death (20).

Here, we have examined the functional role of the four p38 isoforms in the development of irinotecan resistance. We show that p38 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 p38 α and β overcome 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 p38 in tumor samples from patients who are refractory to irinotecan-based therapy compared to responder patients.

Material and methods

Cell lines and reagents

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

SN38 was provided by Sanofi-Aventis (Vitry-Sur-Seine, France). SB202190 was purchased from Sigma-Aldrich (St. Louis, MO, USA). Irinotecan, 5-fluorouracile and oxaliplatin 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, p38 α , p38 β , p38 γ , p38 δ , pATF2 (Cell Signaling Technology, Danvers, MA, USA), β -Actin (AbCam, Cambridge, MA, USA) and Topoisomerase I (1). Secondary antibodies were horseradish peroxidase-conjugated anti-mouse or anti-rabbit antisera (Sigma Aldrich, St. Louis, MO, USA).

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, Danvers, MA, USA) and then with a FITC-conjugated anti-rabbit antiserum (maker). DAPI was used to stain the nuclei. Coverslips were then mounted in Moviol and images were recorded using a 63XNA objective and a Leica inverted microscope.

Retroviral infection

HCT116-s and HCT116-SN6 cells that stably express the four constitutively active p38 isoforms were obtained by retroviral gene transduction of the pMSCV vector in which

constitutively active (CA) variants of p38 α , p38 β , p38 γ and p38 δ (22) had been previously subcloned. 24 hours after transduction, cells were selected with 1 μ g/mL of hygromycin and then stable clones were pooled.

HCT116-s and HCT116-SN6 cells expressing ShRNAs targeting *luciferase*, p38 α , p38 β , p38 γ and p38 δ mRNA were obtained by retroviral gene transduction of the pSIREN vector in which the ShRNAs were cloned. Cells were selected with 1 μ g/mL of puromycin and then stable clones were pooled.

Kinase assay

The p38 kinase assay was performed using the non-radioactive p38 MAPK Assay Kit from Cell Signaling Technology (Danvers, MA, USA) as previously described (13).

In vivo experiments

Xenografts: Female athymic *nu/nu* mice were purchased from Harlan Laboratories (Gannat, France) and used at 6–8 weeks of age. 3×10^6 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 1000 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 (one 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 μ mol/kg daily for 12 days when tumors reached 100 mm³.

Irinotecan + SB202190 were administered when tumors reached 100 mm³: 4 injections (one every 4 days) of irinotecan at 40 mg/kg + SB202190 at 0.05 μ mol/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 μ l of lysing buffer (150 mM NaCl, 10mM Tris pH 7.4, 1 mM EDTA, 1mM EGTA, 0.5% NP40, 1% Triton, 2 mM PMSF, 100 mM NaF, 10 Mm Na₃VO₄ 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 micro-array (TMA) including samples from 21 metastatic CRC patients was constructed as previously described (24), using three malignant tissue cores (0.6-mm diameter)/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 four or six cycles of chemotherapy. Nine patients showed a decrease $\geq 50\%$ of their metastatic lesion and were classified as responders and 12 patients, with a decrease $< 50\%$ or with an increase in size of lesions, were classified as non-responders. Three- μm thin microns sections of the TMA were de-paraffinized and rehydrated in graded alcohols. Following epitope retrieval treatment in EDTA buffer (pH 9) and neutralization of endogenous peroxidase, TMA sections were incubated overnight at $+4^\circ\text{C}$ with the anti-phospho-p38 MAPK (Thr180/Tyr182) (Cell Signaling, Beverly, MA), followed by a standard detection system (FLEX+, Dako, Glostrup, Denmark). 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. Lastly, 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 non-parametric 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 mm^3 using the Kaplan–Meier method. Survival curves were compared using the log-rank test. Statistical analysis was performed using STATA 11.0 software (StatCorp, College Station, TX, USA).

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 to 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 (Figure 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) (Figure 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 four 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 (Figure 1C). In order 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 Figure 1). Similarly to HCT116 SN38-resistant clones, all SW48 resistant clones displayed higher p38 phosphorylation, as compared to the parental SW48-s clone further suggesting that this feature could be a hallmark of SN38 resistance.

The p38 α and β 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 constitutively active form of the upstream p38 activating kinase MKK6 in HCT116 cells. Constitutively active MKK6 rendered cells more resistant to SN38 treatment (Supplementary Figure 2), confirming the notion that activation of p38 can induce SN38 resistance. Since p38 has four isoforms (α , β , γ and δ), we then overexpressed or down-regulated 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 four p38 isoforms was down-regulated by ShRNA in SN38-sensitive HCT116-s cells (Figure 2A, left panels), 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 *p38 α* was silenced (Figure 2B, left panel), as shown by their lower IC₅₀ (50% inhibitory concentration) than in control cells (1.1 nM and 1.9 nM, respectively, p: 0.002). Silencing of the β , γ or δ isoform had no impact on SN38 sensitivity in HCT116-s cells.

Then, HCT116-s cells were infected with retroviruses expressing a constitutively active (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 (Figure 2A, right panel). No difference in the IC₅₀ of the different cell lines was observed (Figure 2C). We then tested whether co-expression of the α and β isoforms could affect HCT116-s cell sensitivity to SN38 and found that these cells became more resistant to SN38 as evidenced by a higher IC₅₀ (Figure 2D). This result indicates that overexpression of both p38 isoforms α and β can influence sensitivity of HCT116-s cells to SN38.

Silencing of the four 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 (Figure 2E). Like for HCT116-s cells, only down-regulation of *p38 α* sensitized HCT116-SN6 cells to SN38 treatment (Figure 2F) (IC₅₀: 3.6 nM and 10 nM respectively, p-value: 0.0003). In addition, overexpression of CA p38 β (Figure 2G) or p38 α + β (Figure 2H) rendered HCT116-SN6 cells more resistant to SN38 treatment (β overexpression; IC₅₀: 19.4 nM and 10 nM respectively, p-value: 0.0018 and α + β overexpression; IC₅₀: 16.125 nM and 9 nM, p-value: 0.0028). These results indicate that co-expression of constitutively active p38 α and β can further enhance the level of resistance of resistant clones.

Since alterations of Topo1 (9, 28-29) can confer resistance to camptothecin derivatives (30-32), we then investigated whether overexpression of CA p38 β or down-regulation of p38 α in HCT116-SN6 and HCT116-s cells influenced TopoI expression (western blotting) and activity (ICE bioassay). No changes in TopoI expression or activity were detected (Supplementary Figure 3), indicating that p38 effect on cell sensitivity to SN38 is not linked to quantitative alteration of TopoI expression or to a difference in the number of TopoI-DNA complexes.

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

Since p38 α and β 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 α and β , 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 (Figure 3A). When SN38 was associated with SB202190, the IC₅₀ in both sensitive and resistant cells was decreased of two folds in comparison to cells treated only with SN38 (Figure 3A). The effect of SB202190 on p38 activity was then monitored by kinase assay (Figure 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 p38 α and β activity sensitizes even SN38-resistant cells to SN38 *in vitro*.

SB202190 effect on SN38 sensitivity was tested on two other colon adenocarcinoma cell lines (HT29 and SW480) that have different genotypes. Whereas the HCT116 cell line has wild type p53 and mutated KRAS, HT29 cells have mutated p53 and wild type KRAS and SW480 cells show mutated p53 and KRAS. SRB assays were carried out in HT29 and SW480 cells treated with SN38 alone or in combination with SB202190 (Figure 3C). Like for the different HCT116 cell lines, the association of SN38 with SB202190 also lowered the IC₅₀ of HT29 (IC₅₀: 9.8 nM and 5.5 nM respectively, p=0.005) and SW480 cells (IC₅₀: 5.5 nM and 2.8nM respectively, p=0.0013), indicating that SB202190 effect on SN38 sensitivity is independent of the p53 and KRAS status. Conversely, when constitutively active (CA) α and β isoforms were co-expressed in SW480 or in HT29 cells, we found that cells became more resistant to SN38 (Figure 3D), further strengthening the role of both p38 α and β isoforms in the development of SN38 resistance.

We then asked whether the inhibition of p38 α and β activity by SB202190 could affect also sensitivity to 5-FU or oxaliplatin in HCT116-s cells. Although both drugs induced p38 phosphorylation, while the total p38 levels were not affected (Figure 3E), SB202190 had no impact on 5-FU cytotoxicity, whereas it reduced oxaliplatin cytotoxicity (IC₅₀: and 350 nM for oxaliplatin and 550 nM for oxaliplatin + SB202190, p=0.005) (Figure 3F).

These data suggest that inhibition of p38 α and β 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 were treated, or not, with irinotecan. Without treatment, similar tumor growth was observed for both cell lines

(Figure 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 (Figure 4A, $p < 0.001$). Conversely, mice with HCT116-SN6 xenografts were less sensitive to irinotecan, since tumor size was only half of that of untreated mice at day 18 (300 mm^3 in irinotecan-treated mice and 600 mm^3 in untreated mice, $p = 0.001$) and tumor growth was not completely blocked like 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 *p38 α* had been down-regulated by ShRNA or CA *p38 β* overexpressed. As before, tumor growth before irinotecan treatment was similar in all groups (mice xenografted with HCT116-SN6-pMSCV, HCT116-SN6-CAp38 β , HCT116-SN6-ShLuc or HCT116-SN6-Shp38 α cells), (Supplementary Figure 4A), indicating that differential p38 expression did not influence tumor growth. However, HCT116-SN6-CAp38 β tumors were less sensitive to irinotecan treatment than control HCT116-SN6-pMSCV tumors (Supplementary Figure 4B). Conversely, HCT116-SN6-SHp38 α tumors were slightly more sensitive to irinotecan than control HCT116-SN6-ShLuc tumors (Supplementary Figure 4D). Overexpression of CA *p38 β* and down-regulation of *p38 α* were monitored by western blot analysis of xenografts at the end of the experiment (Supplementary Figure 4C and 4E). These *in vivo* results confirm that *p38 α* down-regulation sensitizes HCT116 cells to irinotecan treatment, while *p38 β* 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 *p38 α* and β 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 four 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 (Figure 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 behaving like SN38-sensitive HCT116-s cells (compare Figure 4A and 4B). This result provides evidence that inhibition of *p38 α* and β activity can reverse resistance to irinotecan *in vivo*.

Survival was significantly longer in the groups treated with irinotecan or SB202190 alone (median survival=12 days in both groups) than in untreated mice (NT) (median survival=7 days) (respectively $p = 0.016$, $p = 0.047$ and $p = 0.003$). 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, since 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 α phosphorylation was analyzed in a TMA constituted 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 non-responders (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 non-responders (Figure 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 to samples from non-responders 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 (Figure 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). Conversely, the threshold did not correctly classify the non-responders (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 non responder patients with a low level of activated p38 α .

DISCUSSION

In the present study, we have demonstrated the role of the two p38 isoforms α and β 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 (41). 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 (39) or in response to other chemotherapeutic agents such as 5-FU (37) or oxaliplatin (38). 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 p38 α and β isoforms inhibit the cytotoxic effect of SN38 in HCT116 colorectal cancer cells. In addition, pharmacologic inhibition of p38 α and β 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 on DNA repair and autophagy. First, p38 is one of the effector kinases of the DNA damage sensor system starting with the activation of ATM, ATR and DNA-PK (35). The constitutively active p38 in colon cancer cells may induce enhanced DNA repair in response to chemotherapy and lead to drug resistance. Second, Comes and collaborators have demonstrated that p38 α inhibits autophagy and cell death of CRC cells (36) demonstrating that p38 α 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 (40). More recently, Wen et al, showed that p38 inhibition enhances the sensitivity to Arsenic trioxide and Bortezomib in multiple myeloma (42). In line with these results, we show that the level of phosphorylated p38 α is higher in patients unresponsive to chemotherapy compared to 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 non responders were correctly classified, and in the other non responders, we could not detect high p38 α phosphorylation. Both isoforms α and β being involved in SN38 resistance, misclassified non responder patients might present high p38 β phosphorylation, a hypothesis that we could not test because of the absence of available antibody directed against the active form of p38 β . 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 p38 α and β 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 non-responder CRC patients. Further studies using larger cohorts of patients will be necessary to confirm this result.

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FIGURES LEGENDS

Figure 1: p38 is activated by phosphorylation in SN38-resistant HCT116 cells. **A:** Time course and dose-dependency analysis by Western Blot of phosphorylated p38 (pp38) in SN38-sensitive HCT116-s and SN38-resistant HCT116-SN6 cells. Equal loading is shown by tubulin. **B:** Detection of phospho-p38 by immunofluorescence in HCT116-s and HCT116-SN6 cells treated or not (NT) with SN38. **C:** Detection of phospho-p38 by immunofluorescence in SN38-sensitive HCT116-s and in SN38-resistant HCT116-A2, -SN50, -C8 and -G7 cells.

Figure 2: Analysis of the involvement of the different p38 isoforms in SN38 sensitivity. **A:** (left panels) Western blot analysis of p38 isoforms in SN38-sensitive HCT116-s cells transduced with ShRNAs directed against *p38 α* , *β* , *γ* or *δ* , or with the control ShRNA against *Luciferase* (ShLuc). (right panels) Western blot analysis (anti-HA antibody) of HCT116-s cells that express constitutively active (CA) p38 α , *β* , *γ* or *δ* (or EV, as a control). **B:** SRB assays to assess cytotoxicity of HCT116-s that stably express ShRNA targeting *p38 α* , *β* , *γ* , *δ* or *ShLuc* (control). **C:** SRB assay: HCT116-s that stably express the four CA p38 isoforms or EV as control. **D:** SRB assay: HCT116-s expressing p38 α + p38 β CA or EV as control. **E:** Western blot analysis of the four p38 isoforms in SN38-resistant HCT116-SN6 cells that stably express ShRNAs directed against *p38 α* , *β* , *γ* or *δ* , or the ShRNA control *ShLuc* (left panels), or that express the four CA p38 isoforms or EV (right panels). **F:** SRB assay on HCT116-SN6 cells that stably express ShRNA targeting *p38 α* , *β* , *γ* , *δ* or *ShLuc*. **G:** SRB assay on SN38-resistant HCT116-SN6 cells that stably express the four CA p38 isoforms or the EV control. **H:** SRB assay: HCT116-SN6 expressing p38 α + p38 β CA or EV as control. EV: Empty Vector. CA: constitutively active. ShRNA: Short Hairpin RNA.

Figure 3: SB202190 sensitizes colon adenocarcinoma cells to SN38 treatment. **A:** SRB assay on HCT116-s and HCT116-SN6 cells using SN38 alone, SB202190 (SB) alone or SN38 + SB202190 (SN38 + SB). **B:** p38 kinase assay to test p38 α activity in HCT116-s and HCT116-SN6 cells after treatment with SN38 (SN38), co-treatment with SN38 and SB202190 (SN38+SB) or no treatment (NT). **C:** SRB assay on SW480 and HT29 cells, using SN38 alone or SN38 + SB202190 (SN38+SB). **D:** SRB assay: SW480 or HT29 expressing p38 α + p38 β CA or EV as control. **E:** Western blot analysis of phospho-p38 expression in HCT116-s cells, treated or not with 10 μ M 5-FU or 5 μ M oxaliplatin (Oxali) for 24h. Equal loading is shown by β -Actin. **F:** SRB assay on HCT116-s cells, using 5-FU alone, 5-FU + SB202190 (5-FU+SB), oxaliplatin (Oxali) alone or oxaliplatin + SB202190 (Oxali+SB).

Figure 4: Effect of SB202190 on irinotecan cytotoxicity *in vivo* **A:** Tumor growth kinetics of mice xenografted with HCT116-s or HCT116-SN6 cells and treated or not with irinotecan. **HCT116-s:** NT vs irinotecan, $p < 0.001$, **HCT116-SN6:** NT vs irinotecan, $p = 0.001$. **B:** Tumor growth kinetics of tumors in mice xenografted with SN38-resistant HCT116-SN6 cells and left untreated (NT), or treated with irinotecan alone, SB202190 alone, irinotecan + SB202190 (I+ SB202190). **C:** Kaplan-Meier survival curves for the four groups described in B. Tick marks indicate censored events.

Figure 5: High expression of phosphorylated p38 in non-responder patients. **A:** Phospho-p38 expression according to the clinical response. Phospho-p38 nuclear expression was assessed by immunohistochemistry in 21 primary tumors. QS is the QuickScore, calculated from the TMA phospho-p38 staining. Gray boxes represent the 25th to 75th percentiles with the medians as black lines, whiskers marking the lower and upper adjacent values, all outliers indicated by dots. **B:** Phospho-p38 expression in colorectal primary tumors from FOLFIRI responders (panels 1, 2, 3) and FOLFIRI non-responders (panels 4, 5, 6) (X100). **C:** Correlation between the expression of phospho-p38 and the response to chemotherapy. The QS threshold (147) is the phospho-p38 level correlated with the response.