Inhibition of JNK Sensitizes Hypoxic Colon Cancer Cells to DNA-Damaging Agents

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Abstract

Purpose: We showed previously that in HT29 colon cancer cells, modulation of hypoxia-induced stress signaling affects oxaliplatin cytotoxicity. To further study the significance of hypoxia-induced signaling through JNK, we set out to investigate how modulation of kinase activities influences cellular responses of hypoxic colon cancer cells to cytotoxic drugs.

Experimental Design: In a panel of cell lines, we investigated effects of pharmacologic and molecular inhibition of JNK on sensitivity to oxaliplatin, SN-38, and 5-FU. Combination studies for the drugs and JNK inhibitor CC-401 were carried out in vitro and in vivo.

Results: Hypoxia-induced JNK activation was associated with resistance to oxaliplatin. CC-401 in combination with chemotherapy demonstrates synergism in colon cancer cell lines, although synergy is not always hypoxia specific. A more detailed analysis focused on HT29 and SW620 (responsive), and HCT116 (nonresponsive) lines. In HT29 and SW620 cells, CC-401 treatment results in greater DNA damage in the sensitive cells. In vivo, potentiation of bevacizumab, oxaliplatin, and the combination by JNK inhibition was confirmed in HT29-derived mouse xenografts, in which tumor growth delay was greater in the presence of CC-401. Finally, stable introduction of a dominant negative JNK1, but not JNK2, construct into HT29 cells rendered them more sensitive to oxaliplatin under hypoxia, suggesting differing input of JNK isoforms in cellular responses to chemotherapy.

Conclusions: These findings demonstrate that signaling through JNK is a determinant of response to therapy in colon cancer models, and support the testing of JNK inhibition to sensitize colon tumors in the clinic. Clin Cancer Res; 1–10. ©2015 AACR.

Introduction

A salient characteristic of solid tumors is the presence of hypoxic regions, which form mostly as a result of accelerated cell growth and increased distance to blood vessels. Through neovascularization, oxygen influx to hypoxic regions can be restored, creating a complex tumor microenvironment, where continuous hypoxia can be followed by reoxygenation (1, 2). This phenomenon drives several processes associated with poor prognosis, including a more aggressive phenotype, resistance to chemotherapy and radiation, and increased genetic instability (3). A major transcriptional consequence of hypoxia is activation of hypoxia-inducible factors (HIF), which regulate multiple pathways involved in metabolic reprogramming, cell death, angiogenesis, epithelial to mesenchymal transition (EMT), and maintenance of cancer stem cells (4, 5). Thus, hypoxia is a legitimate target to augment outcomes of cancer therapy (6, 7). HIF, however, is not the sole factor mediating tumor responses to hypoxia. The breadth of these responses reflects involvement of various signaling pathways, resulting in activation of other major transcription factors, such as NF-κB, CREB, p53, and AP-1 (8).

The AP-1 transcription factor consists of members of Jun, Fos, ATF, and MAF protein families. By forming various homo- and heterodimers, AP-1 activates transcription of a broad range of genes involved in cell proliferation, differentiation, apoptosis, and other cellular functions (9). Activation of AP-1 occurs mostly through transcriptional induction and/or activating phosphorylation by MAPKs, which are the proximal members of three-tiered signal transduction cascades mediating cellular responses to various external and internal stimuli (10). Upstream activators of MAPKs, MAPK kinases (MAPKKs or MKKs), belong to a class of tyrosine protein kinases, and activate their corresponding targets after being induced, in turn, by multiple MAPKK kinases (MAPKKks, MEKKs, or MKKKs; ref. 11). Mitogenic signals are preferentially relayed through MEK1 and MEK2, which activate ERK1/2 (12), whereas stress signaling is mediated by MKK3/ MKK6 which phosphorylates p38 MAPK, and MKK4/MKK7 which activates JNK1/2/3 (13, 14). Signal transduction through MAP kinases is important for normal cell function, but in cancer ERKs, p38 MAPKs and JNKs can demonstrate both oncogenic and cancer suppressive features (15–17).

In our earlier work, we found that hypoxic induction of AP-1 in colon cancer cell lines was associated with resistance to chemotherapy (18–20). We demonstrated that in HT29 colon adenocarcinoma, hypoxia activates p38 MAPK and JNK, whereas ERK1 and ERK2 are constitutively active, and cJun/AP-1 activation is mediated predominantly by JNK (20). We then studied upstream activators of JNK, MKK4, and MKK7, and showed not only the activation of both under hypoxia, but that selective downregulation of this signaling exerts differing effects on oxaliplatin.
Translational Relevance

The combination of cytotoxic chemotherapy and bevacizumab is the backbone of therapy for advanced colorectal cancer. Understanding the basis of resistance is the key to development of newer and more effective therapies. Our previous work suggests that the induction of hypoxia by bevacizumab contributes to its interaction with chemotherapy, and may increase cell kill while promoting the acquisition of resistance. We found that inhibition of the JNK pathway at the SEK kinase level increased the efficacy of both bevacizumab and oxaliplatin. In this paper, we show that direct inhibition of JNK sensitizes colorectal cancer cells of varying genetic background to hypoxia and cytotoxic drugs, and that JNK inhibition may be a promising therapeutic approach in this disease. Furthermore, molecular disruption of JNK isoforms suggests that additional specificity might result from selective inhibition of JNK1.

cytotoxicity: MKK4 deficiency results in higher sensitivity to oxaliplatin, whereas downregulation of MKK7 renders HT29 cells more resistant to the drug in vitro and in vivo (21). These data suggested that hypoxic signaling through MKK4 could contribute to oxaliplatin resistance, while uninterrupted signaling to AP-1 through the MKK7/JNK module is essential for oxaliplatin cytotoxicity in hypoxic HT29 cells. Accordingly, targeting separate components of JNK signaling pathway or its downstream targets could clarify conflicting data concerning the role of JNK in chemotherapeutic resistance in general (22) and yield novel approaches to enhance oxaliplatin cytotoxicity in particular.

Here, we study the effects of JNK inhibition on sensitivity to oxaliplatin, SN-38, and 5-FU in a panel of 12 colon cancer cell lines. Our data show that inhibition of JNK by CC-401 enhances cytotoxicity of the chemotherapy in vitro. Sensitization to oxaliplatin was confirmed in vivo, and downregulation of JNK1, but not JNK2, by dominant negative constructs rendered hypoxic HT29 cells more sensitive to oxaliplatin. Our findings support further testing of JNK inhibitors in the clinic.

Materials and Methods

Cells and reagents

All human colon cancer cell lines were from ATCC. HCT116p53+/− was kindly provided by Dr. B. Vogelstein (John Hopkins Kimmel Cancer Center, Baltimore, MD) and BE cells were from Dr. B. Giovanella (St. Joseph’s Hospital Cancer Research Laboratory, Houston, TX.) Cells were grown in DMEM medium supplemented with 10% FBS and antibiotic-antimycotic (Invitrogen). SP600125 was purchased from Biomol, medium supplemented with 10% FBS and antibiotic-antimycotic (Forma Scientific, Inc.) filled with a gas mixture consisting of 5% CO2, 9% H2, and 86% N2. Oxygen content (below 0.5%) was monitored by PROOX 110 oxygen sensor (BioSpherix). Cells were plated in 100-mm glass Petri dishes to a density of 2 × 104 cells per dish and subjected to hypoxia within 36 hours. The cells were harvested at various time points for further experiments.

Plasmids and isolation of stably transfected cell lines

The HA-tagged dominant negative mutants of JNK1 and JNK2 (HA-JNK1-APF and HA-JNK2-APF, respectively) cloned into retroviral pLNCX vector were kindly provided by Dr. Tomas Berl (University of Colorado, Denver, CO). To isolate cell lines stably expressing empty vector and dnJNK1 or dnJNK2, HT29 cells were transfected using Fugene 6 transfection reagent (Roche Applied Sciences), according to the manufacturer’s recommendation and cultivated at low density in media containing G418 (0.75 mg/mL, Invitrogen); surviving colonies were isolated, propagated, and assessed for expression of tag protein.

Hypoxic treatment

Cells were exposed to acute hypoxia in an anaerobic chamber (Forma Scientific, Inc.) filled with a gas mixture consisting of 5% CO2, 9% H2, and 86% N2. Oxygen content (below 0.5%) was monitored by PROOX 110 oxygen sensor (BioSpherix). Cells were plated in 100-mm glass Petri dishes to a density of 2 × 104 cells per dish and subjected to hypoxia within 36 hours. The cells were harvested at various time points for further experiments.

Protein extract preparation and Western blotting

Total protein extracts were prepared as described in ref. (21), using cell lysis buffer (Cell Signaling Technology), supplemented with complete protease inhibitor cocktail (Roche) and 1 mmol/L PMSF (Sigma). Cells were lysed inside the hypoxia chamber, followed by incubation in a shaker for 30 minutes at 4°C, and centrifugation. Protein concentration of cleared cellular extracts was measured using the Bio-Rad Protein Assay (Bio-Rad).

For protein electrophoresis in SDS-polyacrylamide gels, protein extracts were used in amounts of 10 μg per lane. Western blotting was carried out according to standard procedures, using horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology) and the ECL-Plus detection system (Amerham). Results were analyzed with BioSpectrum 810 Imaging System using VisionWorksLS Image Acquisition and Analysis Software (UVP). The primary antibodies against actin, Chk1, Chk2 were from Santa Cruz Biotechnology; the remaining antibodies were purchased from Cell Signaling Technology. For IHC, primary antibodies against CD31 (Abcam) and against Ki-67 (Dako) were also used.

Cytotoxicity assays and calculation of combination indices

For assessment of cytotoxicity, cells were plated in 96-well plates (2,000 cells per well), and 24 hours later, various concentrations of chemotherapeutic drugs alone or in combination were added, immediately before hypoxic exposure for 24 hours, followed by cultivation in oxic conditions for an additional 48 hours. Cytotoxicity was measured using a standard MTT assay. The IC50 values shown are the means of at least three independent experiments done in triplicate. The combination index analysis was performed using the median effect method by Chou and Talalay (23, 24).

Analysis of DNA damage induction

To study the induction of DNA damage, the levels of phosphorylated H2AX were measured. Cells were seeded in 6-well plates (2 × 105 cells/plate) and 24 hours later subjected to hypoxia and/or drug treatment for 24 hours. Cells were collected (including any floating cells in the culture medium), washed twice with PBS, permeabilized on ice for 15 minutes with 1% formaldehyde, resuspended in 70% ethanol, and kept at −20°C overnight. Next, after two washes with PBS and one with 1% BSA-PBS, cells were resuspended in 100 μL of 1% BSA-PBS together.
with 5 μL of the p-H2AX (ser139) antibody (Alexa Fluor 647 labeled, BD Pharmingen) and incubated at RT for 2 hours in the dark before the addition of 300 μL of 1% BSA-PBS. The percentage of positive cells was then determined by flow cytometry (BD FACSCalibur), and results analyzed using FlowJo software (TreeStar).

**Tumor growth and antiangiogenic therapy**

To assess the efficacy of JNK signaling inhibition by CC-401 in antiangiogenic and oxaliplatin combination therapy in a mouse xenograft model, adult (8–10 weeks of age) female SCID mice (C.B.17 SCID) were used. To generate tumors, HT29 cells (1 × 10⁶ cells) were injected subcutaneously into the left flank of the mice. When the tumors reached approximately 200 mm³, mice were divided into eight groups (eight mice per group) for treatment with bevacizumab (Genentech, Inc.), oxaliplatin, CC-401, and the appropriate combinations of bevacizumab, oxaliplatin, and CC-401. Mice in the bevacizumab treatment group received 5 mg/kg of bevacizumab by intraperitoneal injection every 3 days for 21 days. The oxaliplatin treatment group was injected intraperitoneally with 5 mg/kg oxaliplatin per week for 2 weeks. The CC-401 treatment group was injected intraperitoneally 25 mg/kg for every 3 days. The combination treatment groups received bevacizumab (every 3 days, 5 mg/kg), oxaliplatin (weekly for 2 weeks, 5 mg/kg), and CC-401 (every 3 days, 25 mg/kg). The control group received saline intraperitoneally. Tumor volume and body weight were measured every 3 days. Tumor volume was calculated using the formula V = AB²/2, where A is the largest diameter and B is the smallest diameter. Tumor growth delay was calculated as the difference in the time for control and treated tumors to grow from 200 to 800 mm³. For tumor growth delay calculations, mice were continued to receive treatments until the tumor volume reached 800 mm³. For IHC, mice were sacrificed after treatments on day 9 for tumor processing and staining. All animal experiments were performed according to an approved University of Pennsylvania IACUC (Institutional Animal Care and Use Committee).

**Tissue IHC**

IHC was conducted using the antigen retrieval protocol followed by primary antibody incubation previously described (25, 26). Rabbit anti-human p-JNK and p-cJun antibodies were used to detect the expression of p-JNK and p-cJun in tumors. For blood vessels, Ki-67 markers, and p-H2AX detection, antibodies against CD31, Ki-67, and p-H2AX were used. IHC images were visualized using a Leica DM2500 upright microscope with QImagingMicroPublisher 5.0 RTV color camera with objective lens PL FluotAR 20x/0.5 (Leica). Images were captured using iVision acquisition software, which were processed with Adobe Photoshop software (Adobe Systems). Tumor blood vessels densities were determined by counting per 40 high power field as described earlier (25, 26). For quantitative analysis, ImageJ software, measuring the intensity of staining through threshold analysis, coupled with the Color Deconvolution plug-in was used to quantify immunoreactivity in xenograft samples [http://www.dentistry.bham.ac.uk/landing/software/cdeconv/cdeconv.html].

**Statistical analysis**

Data were analyzed using the Student t test and ANOVA where appropriate. A P value less than 0.05 was accepted as a statistically significant difference when compared with corresponding control, and marked with an asterisk in figures. To assess effects of the drug’s combinations between oxic and hypoxic conditions, combinatorial indices (CI) were evaluated using two-way ANOVA models. In graphs, values are presented as mean plus SD.

**Results**

Hypoxia induces selective signaling through JNK and increases resistance to chemotherapy in a panel of colon cancer cell lines

We showed previously that induction of the JNK pathway in hypoxic HT29 colon adenocarcinoma cells was functionally associated with resistance, and that cells could be rendered sensitive by downregulation of MKK4 (SEK1; refs. 19, 21). To extend observations on this pathway, we evaluated induction of stress signaling through JNK by hypoxia in a panel of 12 colon cancer cell lines. Cells were subjected to hypoxia for 1, 3, 5, or 24 hours, and phosphorilation of c-Jun, as a marker of JNK induction, was measured by Western blotting (Fig. 1A). The levels of c-Jun phosphorylation, calculated by comparing band densities to the one from control sample, were established in at least two independent experiments for all of cell lines, and presented in Supplementary Table S1. We found that hypoxia induces signaling through JNK in a majority of cell lines, to varying degrees, and with cell-specific temporal characteristics. Induction of signaling in this pathway is an early response, which subsides by 24 hours of hypoxic exposure in a majority of cell lines; therefore, we designated lines with increased content of phospho-c-Jun by 5 hours of hypoxia as positive for hypoxia-induced JNK signaling. In the same cellular panel, we also assessed sensitivity to oxaliplatin, SN-38, and 5-FU under normal or hypoxic conditions using MTT assays. Our data show a wide variety of responses (IC₅₀ values for all drugs shown in Supplementary Table S2): under hypoxia more than a 2-fold increase in resistance to oxaliplatin was observed in 6 cell lines, sensitivity to SN-38 diminished in 9 cell lines, and 3 cell lines became more resistant to 5-FU (Fig. 1B). Figure 1B illustrates the relationship of hypoxia-induced resistance to chemotherapy and induction of JNK signaling by hypoxia, as assessed by c-Jun phosphorylation. Evidently, hypoxic resistance to chemotherapy is not associated with activation of JNK signaling in DLD1, HCT15, HCT116, and its p53-null derivative, HCT116p53⁻/⁻, but in the other eight cell lines, induction of the JNK pathway by hypoxia is associated with resistance. Therefore, we wished to find out whether inhibition of JNK would influence the efficacy of chemotherapy.

**Pharmacologic inhibition of JNK sensitizes colon cancer cell lines to chemotherapeutic drugs**

To investigate the effects of JNK inhibition on drug resistance under hypoxia, we carried out MTT assays using the small-molecule JNK inhibitors CC-401 and SP600125 in combinations with oxaliplatin, SN-38, and 5-FU, followed by calculation of combination indices. Our data show additive or synergistic effects of these combinations in the majority of cell lines tested [Table 1 (CC-401), Supplementary Table S3 (SP600125)], implying the importance of JNK pathway in drug resistance. However, not all of the effects are hypoxia specific in this setting. For example, the combination of CC-401 with oxaliplatin demonstrates higher synergy under hypoxia in HT29, SW620, HCT116, HCT15, and LoVo cell lines (5 out of 6), with median CI of 0.81 and 0.48 for
oxic and hypoxic conditions, respectively. Enhanced cytotoxicity of SN-38 is further significantly augmented by hypoxia only in HT29 cells, with median CI values for whole panel of 0.56 for normoxia and 0.54 in hypoxic cells. The cytotoxic interaction between CC-401 and 5-FU is also synergistic in oxic conditions (median CI = 0.88) and hypoxia (median CI = 0.64). Although higher synergism under hypoxia is observed in HT29, HCT116, and KM12 cells, we further narrowed the field of our study to oxaliplatin, because neither HCT116 nor KM12 demonstrates increased resistance to 5-FU under hypoxia (Fig. 1B). Combinations of the drugs with SP600125, while also demonstrating rather modest hypoxic specificity (Supplementary Table S3), still are mostly synergistic. Thus, inhibition of JNK signaling by CC-

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Oxaliplatin CI</th>
<th>SN-38 CI</th>
<th>5-FU CI</th>
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<tbody>
<tr>
<td>HT29</td>
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<td>0.56</td>
<td>0.36</td>
</tr>
<tr>
<td>SW620</td>
<td>0.64</td>
<td>0.56</td>
<td>0.36</td>
</tr>
<tr>
<td>HCT116</td>
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<td>0.56</td>
<td>0.36</td>
</tr>
<tr>
<td>KM12</td>
<td>0.78</td>
<td>0.56</td>
<td>0.36</td>
</tr>
<tr>
<td>HCT15</td>
<td>0.73</td>
<td>0.56</td>
<td>0.36</td>
</tr>
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</table>

Table 1. Combination of JNK inhibitor CC-401 with chemotherapy is synergistic in colon cancer cell lines.

Hypoxia induces DNA damage response in cell-specific manner

Platinum compounds result in DNA adduct formation, which in turn initiate several cellular responses. Involvement of JNK in the DNA damage response, by controlling directly or indirectly DNA damage repair and cell death, among other processes, was shown in multiple models (27). We therefore asked whether JNK inhibition by CC-401 would modify DNA damage responses induced by hypoxia, oxaliplatin, or both in HT29, SW620, and HCT116 cell lines. We showed that 1 × IC50 concentrations of CC-401 (3, 6.5 and 3.5 μmol/L for HT29, SW620 and HCT116, respectively) are sufficient to inhibit hypoxic JNK signaling and used them in all further experiments (Fig. 2A and B). For assessment of DNA damage, cells were subjected to hypoxia for 24 hours with or without oxaliplatin and the levels of phosphorylated p-
H2AX measured by FACS analysis. We used oxaliplatin at concentrations of 0.7, 0.4, and 0.9 μmol/L, for HT29, SW620, and HCT116 cells, respectively (1xIC50 for oxic conditions). We found (Fig. 2C) that hypoxia causes a significant increase in p-HA2X in all cell lines (4–5 fold, compared with untreated control), but JNK inhibition by CC-401 (one hour treatment before hypoxia) augments it further only in HT29 and SW620 cell lines. This observation serves to emphasize that in models in which JNK is activated by hypoxia, its inhibition may have therapeutic consequences. We also evaluated activation of Chk1 and Chk2 kinases at 24 hours of hypoxia, which was brisk with or without CC-401 (Fig. 2A and B). Our data do not permit a firm quantitative conclusion, but suggest that there is no inhibition of DNA damage signaling by CC-401. Together these results suggest that CC-401 may exert its synergistic activity by increasing DNA damage in "responsive" cell lines.

Figure 2. Hypoxia causes DNA damage in a cell-specific manner. A, cells were subjected to hypoxia with or without CC-401 at the dose of 1x IC50 (for HT29, HCT116, and SW620, respectively) for 6 or 24 hours, followed by Western blot analysis to assess c-Jun phosphorylation, or activation of Chk1 and Chk2 as indicators of DNA damage signaling. B, graph presents effects of CC-401 on c-Jun phosphorylation and activation of DNA-damage response under hypoxia, based on calculation of band densities normalized to actin; average values from at least two independent experiments are shown. C, cell lines were subjected to 24 hours of hypoxia with or without oxaliplatin at the dose of IC50 for oxic condition in the absence (black bars) or presence (striped bars) of CC-401. Percentages of p-H2AX–positive cells were established in three independent experiments and plotted as fold-increase compared with oxic control. *, P < 0.05.

JNK inhibition potentiates therapy in colon cancer models

To determine the effect of JNK inhibition on therapy with bevacizumab, oxaliplatin, and the combination in vivo, mice bearing HT29 xenografts were randomly divided into eight groups for treatments as follows: PBS-treated control, CC-401 alone, bevacizumab alone, bevacizumab and CC-401 in combination, oxaliplatin alone, oxaliplatin and CC-401 in combination, bevacizumab and oxaliplatin in combination, and bevacizumab,
Effect of CC-401 on the growth delay of HT29-derived tumors treated with bevacizumab, oxaliplatin, and CC-401 in combination. Figure 3 shows effects of these treatments on growth of the tumors. The tumor growth delay for 2 days was observed for CC-401 alone (Table 2). A much greater impact was seen with the combinations of CC-401 with oxaliplatin alone (6 vs. 18 days), bevacizumab alone (7 vs. 9 days), and with the combination of both (11 vs. 19 days). Therefore in all treatments, the strategy of incorporating CC-401 increased the tumor growth delay significantly, suggesting that JNK-signaling inhibition may have an impact on efficacy of both oxaliplatin and antiangiogenesis therapy (Table 2).

Tumors from mice treated as described above were processed for immunostaining of phospho-JNK and phospho-cJun proteins to assess JNK signaling in xenografts and effects of CC-401. IHC analysis revealed the cytoplasmic localization of p-JNK in control, bevacizumab-, and oxaliplatin-treated tumors (Fig. 4A), whereas p-cJun staining was nuclear in all samples (Fig. 4B). The staining of p-JNK was moderately induced in bevacizumab and oxaliplatin treatments as compared with control, and in the CC-401–treated samples, p-cJun content was significantly lower, consistent with effective JNK inhibition. To identify additional effects of combined treatments on HT29-derived xenografts, tumors were also stained for blood vessels, cell viability, and DNA damage using CD31, Ki-67, and p-H2AX antibodies. As evidenced in Fig. 4C, bevacizumab inhibited blood vessels density in the treated samples, with CC-401 and oxaliplatin exerting no effect on its antiangiogenic properties (Fig. 4C). Staining with cell Ki-67 revealed the lowest proliferation when bevacizumab, oxaliplatin, and CC-401 were combined (Fig. 4D). Finally, DNA damage was modestly elevated in combined treatments with CC-401 (Fig. 4E). Thus, the in vivo results were in accordance with in vitro studies in HT29 colon adenocarcinoma. We then sought to determine whether inhibition of JNK isoforms could affect oxaliplatin cytotoxicity in this cell line.

Table 2. Effect of CC-401 on the growth delay of HT29-derived tumors treated with bevacizumab, oxaliplatin, and the combination

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Tumor growth delaya</th>
</tr>
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<tbody>
<tr>
<td>CC-401</td>
<td>2</td>
</tr>
<tr>
<td>Bevacizumab</td>
<td>7</td>
</tr>
<tr>
<td>Bevacizumab + CC-401</td>
<td>9</td>
</tr>
<tr>
<td>Oxaliplatin</td>
<td>6</td>
</tr>
<tr>
<td>Oxaliplatin + CC-401</td>
<td>18</td>
</tr>
<tr>
<td>Bevacizumab + oxaliplatin</td>
<td>11</td>
</tr>
<tr>
<td>Bevacizumab + oxaliplatin + CC-401</td>
<td>19</td>
</tr>
</tbody>
</table>

NOTE: For tumor growth delay calculations, mice continued to receive treatments until the tumor volume reached 800 mm3. *Tumor growth delay was calculated as time (days) needed for treated tumors to grow from 200 to 800 mm3 minus the time needed for control tumor to grow to the same size.

Downregulation of JNK1 sensitizes HT29 cells to oxaliplatin under hypoxia

For these studies, we isolated HT29-derived cell lines stably expressing dominant-negative, non-phosphorylatable mutants of either JNK1 (HTJ1.3) or JNK2 (HTJ2.2), with a cell line transfected with empty vector as control (HTLX). Our results show that under hypoxic exposure, phosphorylation of c-Jun is diminished in the cell line with impaired JNK2, while the protein level is comparable with that of parental cells. On the other hand, in HTJ1.3 cells both expression and phosphorylation levels of c-Jun are elevated (Fig. 5A). We assessed sensitivity of these HT29 derivatives to oxaliplatin, SN-38, and 5-FU, and found that JNK1 deficiency abrogates hypoxic resistance to oxaliplatin, whereas downregulation of JNK2 slightly increases resistance when compared with control cells (hypoxic IC50 of 0.8, 2.9, and 2.3 μmol/L, respectively). The effects of JNK modulation were less pronounced in hypoxic cells treated with SN38 (27, 25, and 34 nmol/L for HTLX, HTJ1.3 and HTJ2.2 cells) and downregulation of both JNK isoforms sensitized hypoxic HT29 to 5-FU (11, 8, and 5.5 μmol/L, respectively; Fig. 5B). These data suggest differing inputs of JNK isoforms in the sensitivity of colon cancer cell lines to chemotherapeutic drugs, and warrant further investigation to identify more selective approaches to the pathway.

Discussion

The JNK signaling pathway regulates a multitude of cellular processes involved in both proliferation and cell death, and its deregulation (most often activation) has been implicated in several diseases including neurologic and cardiac disorders, inflammation, and cancer (28). In many of these diseases, inhibition of overactivated JNK may be beneficial. In cancer, the effects of JNK inhibition, both pharmacologically and in genetically modified models, are opposing in many tumors, reflecting the interaction of the JNK pathway with molecular characteristics of the cancer and/or host. Activation of the JNK pathway in colonic tissues and biopsies was observed in inflammatory bowel disease and Crohn’s disease (28), but in colon cancer, it is less well described. A contribution of JNK to carcinogenesis by cooperation of JNK and β-catenin pathways inactivation of c-Jun was documented in APCmin mice (29). More studies revealed that JNK/β-catenin pathways cooperate at a transcriptional level through c-Jun/Tcf4 common target genes activated in cancer, such as c-jun itself, c-myc, cyclin D1, MMP7, and others (30). In chemically induced mouse liver cancer, JNK deficiency also leads to impaired proliferation, through an increase in the cell-cycle inhibitor p21, and reduced expression of c-Myc (31). Reduction in tumor formation and growth after JNK inhibition was observed in intestinal, lung, and ovarian cancer models, among others (32–34). On the other hand, JNK deficiency resulted in increased tumor formation in models of breast and hepatocellular cancer (35, 36), and in a genetically engineered mouse model of pancreatic cancer (37). In our study, JNK was variably activated both
constitutively and, even more so, in response to hypoxia in colon cancer cells (Fig. 1A). However, as with many therapeutic approaches, optimal translation of these findings to the clinic must recognize the potential for a deleterious effect in some tumors, and perhaps limit the duration of pathway inhibition to the period surrounding cytotoxic drug administration.

Our analysis of 12 cell lines suggests that JNK activation contributes to the survival of colon cancer cells, since its inhibition with CC-401 resulted in higher sensitivity to oxaliplatin, SN-38, and 5-FU in both normal and hypoxic conditions (Table 1). In a majority of cell lines, an increase in chemotherapy resistance under hypoxia was associated with JNK induction (Fig. 1B). Cell lines in which that was not the case (DLD1, HCT15, HCT116 and its derivative, HCT116p53/C0/C0) all have the mismatch repair deficiency genotype, representing a relatively small proportion of patients with metastatic colon cancer, which can differ from most common types in their behavior (38). Our data suggest that JNK could be a valid target to enhance efficacy of chemotherapy in colon tumors.

The outcome of JNK inhibition in cell lines also varied with the DNA-damaging stimulus involved, especially under hypoxic conditions. Although it was almost uniformly additive or synergistic in all cell lines, significant hypoxia-specific enhancement of SN-38 and 5-FU toxicity by CC-401 was shown only in one (HT29) and three (HT29, HCT116, and KM12) cell lines, out of six, respectively. By far, the best hypoxic interaction with JNK inhibition was observed for oxaliplatin: five cell lines demonstrate clear hypoxia-specific synergism of these compounds in combination (Table 1). The basis for difference is, most likely, the variability of cellular responses to DNA damage (39) induced by particular chemotherapy, which in turn depend on genetic makeup of the cells. In our panel, synergism of oxaliplatin in the combination with CC-401 is seemingly based on DNA damage extent and ensuing enhancement of cell death, either by apoptosis or necrosis (40, 41). In addition, the inhibition of JNK by CC-401 could also attenuate induction of protective hypoxia-induced autophagy (26) in some cell lines, but this needs to be confirmed further. On the basis of the data presented here, the increase in DNA
damage consequent upon JNK inhibition may be the most important driver of oxaliplatin sensitization in hypoxic colon cancer cell lines.

Combinations of bevacizumab, the VEGF-binding monoclonal antibody, with oxaliplatin, irinotecan, and 5-FU are effective in colorectal cancer treatment (42). By inhibiting the tumor vasculature, bevacizumab and other angiogenic agents cause substantial alterations in the tumor microenvironment (43). We have previously demonstrated that bevacizumab treatment induces hypoxia in colon cancer xenografts, but tumor shrinkage as a consequence of treatment is variable (25, 44) and depends on susceptibility to hypoxia-induced cell death (25). In this study, the growth of HT29-derived xenografts showed noticeable delay when mice were treated with bevacizumab or oxaliplatin alone (Fig. 3), but the best results were achieved in triple treatment, when oxaliplatin and CC-401 were combined in a bevacizumab-induced hypoxic environment (Table 2; Fig. 4C). These data support a positive impact of JNK inhibition on cytotoxicity of oxaliplatin in hypoxic tumors, and support clinical testing of the combination.

The complexity of cellular responses to JNK pathway inhibition in varying contexts led to interest in development of better pharmacologic JNK inhibitors, including those against specific isoforms (45, 46). These inhibitors may facilitate an understanding of the distinctive, and sometime opposing, functions of JNK isoforms (47, 48). At this point, however, a specific small-molecule inhibitor of JNK1 or JNK2 is not readily available. To investigate the effect of JNK isoform inhibition on cytotoxicity of the chemotheraphy, we performed initial experiments using dominant-negative constructs of JNK1 and JNK2 in HT29 cells. This approach has the potential to lower the ability of JNKs to substitute for each other, since nonphosphorylatable proteins would compete with corresponding endogenous kinase for binding both to upstream activators and to downstream targets. The fact that inhibition of either JNK isoform does not significantly alter sensitivity to SN38 under hypoxia, and leads to increased sensitivity to 5-FU, suggests differing input of JNK isoforms in signal transduction induced by each drug, and points to the benefit of pan-JNK inhibition used alongside 5-FU treatment. In the case of oxaliplatin, however, there is an obvious difference in cellular responses: inhibition of JNK1 in HT29 completely abolishes hypoxia-induced resistance to the drug, whereas inhibition of JNK2 results in a slightly opposite effect. This result suggests a prosurvival function for JNK1 under these circumstances, which could include induction of protective autophagy, DNA damage repair, or other cellular pathways. Although in our preliminary experiments, inhibition of JNK2 led to a slight increase in resistance to oxaliplatin under hypoxia, diminished expression, and activation of c-Jun (Fig. 5A) could nevertheless be of a benefit considering the pro-oncogenic features of this protein as a part of AP-1. Our data suggest that inhibition of JNK1 would be more advantageous when combined with oxaliplatin for treatment of solid tumors, but unless specific inhibitors for each isoform is available, pan-inhibition of JNK would work as well, because there is little downside form JNK2 inhibition.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Conception and design: I.A. Vasilevskaya, M. Selvakumaran, S.R. Goldstein, J.D. Winkler, P.J. O’Dwyer
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