The CXCR2 antagonist, SCH-527123, demonstrates antitumor activity and sensitizes cells to oxaliplatin in preclinical colon cancer models

Yan Ning1*, Melissa J. Labonte1*, Wu Zhang1, Pierre O. Bohanes1, Armin Gerger1, Dongyun Yang2, Leonor Benhaim1, David Paez1, David O. Rosenberg3, Kalyan C. Nagulapalli Venkata3, Stan G. Louie4, Nicos A. Petasis3,5, Robert D. Ladner6, Heinz-Josef Lenz1,5,7

1Division of Medical Oncology, Sharon A. Carpenter Laboratory; 2Department of Preventive Medicine; 3Department of Chemistry; 4School of Pharmacy; 5Norris Comprehensive Cancer Center; 6Department of Pathology, Keck School of Medicine, University of Southern California, Los Angeles, CA 90089.

7To whom correspondence should be addressed: Heinz-Josef Lenz, M.D., Norris Comprehensive Cancer Center, Keck School of Medicine, 1441 Eastlake Ave, Suite 3456, University of Southern California, Los Angeles, CA 90089; Tel:(323)865-3955; Fax (323)865-0061; E-mail: lenz@usc.edu.

This study was funded by the NIH grant 5 P30CA14089-27S1, the Kroha/Casner Family Foundation, Dhont Family and Wunderglo Foundation.

*Both authors contributed equally to this work.

Running title: SCH-527123 combined with oxaliplatin increases antitumor activity

Key words: SCH-527123; oxaliplatin; colon cancer; CXCR2; Interleukin-8
Abstract

Colorectal cancer (CRC) is the second most common cause of cancer-related death in the United States. Recent studies showed that interleukin-8 (IL-8) and its receptors (CXCR1 and CXCR2) are significantly upregulated in both the tumor and its microenvironment, and act as key regulators of proliferation, angiogenesis and metastasis. Our previous study demonstrated that IL-8 overexpression in CRC cells triggers the upregulation of the CXCR2-mediated proliferative pathway. The aim of this study was to investigate if the CXCR2 antagonist, SCH-527123, inhibits CRC proliferation and if it can sensitize CRC cells to oxaliplatin both in vitro and in vivo. SCH-527123 demonstrated concentration dependent anti-proliferative effects in HCT116, Caco2 and their respective IL-8 overexpressing variants CRC cell lines. Moreover, SCH-527123 was able to suppress CXCR2 mediated signal transduction as demonstrated through decreased phosphorylation of the NF-κB/MAPK/AKT pathway. These findings corresponded with decreased cell migration and invasion, while increased apoptosis in CRC cell lines. In vivo results verified that SCH-527123 treatment decreased tumor growth and microvessel density when compared with vehicle treated tumors. Importantly, these preclinical studies demonstrated that the combination of SCH-527123 and oxaliplatin resulted in a greater decrease in cell proliferation, tumor growth, apoptosis and angiogenesis that was superior to single agent treatment. Taken together, these findings suggest that targeting CXCR2 may block tumor proliferation, migration, invasion and angiogenesis. In addition, CXCR2 blockade may further sensitize CRC to oxaliplatin treatment.

Introduction

Colorectal cancer (CRC) is the leading cause of death from gastrointestinal malignancies, resulting in approximately 51,690 deaths in 2012 (1). Since 2005, no new
chemotherapeutic agents have been approved by the Food and Drug Administration for
treatment of patients with metastatic CRC, resulting in a significant need to develop more
effective targeted drugs aiming at both the tumor and its microenvironment. Recent
studies have identified that certain chemokines and their receptors act as key regulators of
CRC progression and may be important targets for novel drug development strategies (2).

Interleukin-8 (IL-8), a member of the neutrophil-specific C-X-C subfamily of
chemokines, acts on endothelial cells via binding onto either CXCR1 or CXCR2 to
promote invasion, proliferation and angiogenesis (2-5). IL-8 expression is upregulated by
hypoxia, cytokines and other environmental stresses, which are mediated by transcription
factors, including NF-κB and AP-1 (6, 7). The upregulation of CXCR2 has also been
correlated with promotion of tumorigenesis and angiogenesis in lung, melanoma and
ovarian cancers (8-10). Previous studies by our group and others have shown that IL-8
and its receptor CXCR2 are significantly upregulated in the tumor and its
microenvironment in CRC (11-13). These studies demonstrated that expression levels of
IL-8 and CXCR2 were associated with tumor proliferation, progression and sensitivity of
oxaliplatin-based therapy in CRC cell line models and genetic variants in IL-8 and
CXCR2 both predict tumor recurrence and oxaliplatin efficacy in patients (11, 14, 15). It
is well known that oxaliplatin-based chemotherapy is a standard-of-care agent used most
commonly in combination with 5-fluorouracil (5-FU) in patients with CRC (16).
Overexpression of IL-8 level in CRC cells decreased sensitivity to the cytotoxic effects of
oxaliplatin and contributed to oxaliplatin chemoresistance (11). Therefore, targeting IL-8
or CXCR2, in addition to having a direct antiangiogenic and antitumor effect, may also
increase chemosensitivity to oxaliplatin.
Recently, our group focused on inhibitors of the IL-8/CXCR2 pathway, which will have the potential not only to have antitumor activity, but to increase the efficacy of already available cytotoxic and targeted drugs, such as oxaliplatin, for patients with CRC by targeting the tumor and its microenvironment. SCH-527123, as a novel and selective antagonist of the CXCR2, has demonstrated efficacy in the treatment of inflammatory diseases (17). Moreover, Singh et al showed that SCH-527123 treatment inhibited human melanoma cancer growth and CRC liver metastases by decreasing tumor cell proliferation, angiogenesis and enhancing the apoptosis of malignant cells (18, 19).

In this study, we demonstrate that treatment with SCH-527123 alone and in combination with oxaliplatin is effective in synergistically inhibiting proliferation, angiogenesis, and enhancing chemosensitivity in CRC cells and xenografts. Taken together, these findings suggest that CXCR2 antagonists, such as SCH-527123, may be important therapeutic candidates in treating CRC through attenuating the IL-8/CXCR2 signaling cascade, which influences disease progression and modulates the response to oxaliplatin sensitivity.

Materials and Methods

Compounds and Reagents

SCH-527123 was obtained from Schering Plough (Whitehouse Station, NJ) and synthesized in Petasis group. Compound purity was verified by NMR and LC/MS. Oxaliplatin was purchased from Sigma-Aldrich (St. Louis, MO). CellTiter96 Aqueous One Solution was purchased from Promega (Madison, WI). Recombinant human IL-8 (rhIL-8) was purchased from R&D systems (Minneapolis, MN). Protease inhibitor cocktail was purchased from Roche Molecular Biochemical (Indianapolis, IN).
Cell Lines

The human HCT116 and Caco2 CRC cells were purchased from American Type Culture Connection (ATCC, Lockville, MD) in August 2008 (no authentication was done by the authors). HCT116 and Caco2 IL-8 overexpressing isogenic cell lines (E2 and IIIe) were generated as previously described (11). HCT116 and Caco2 cell lines were maintained in McCoy’s5A and MEM media, respectively, and supplemented with 10% fetal bovine serum (Lonza, East Rutherford, NJ), 5% penicillin/streptomycin, sodium pyruvate and L-Glutamine (Mediatech, Manassas, VA). IL-8 overexpression cells were maintained as described above with the addition of 5 μg/ml blasticidin (Invitrogen, Carlsbad, CA). Cells were maintained in an incubator at 37°C with 5% CO₂. The cell lines were routinely tested to confirm that they were Mycoplasma free by using Mycoalert mycoplasma detection Kit (Lonza).

ELISA

The quantification of IL-8 protein was determined using the Quantikine IL-8 ELISA kit (R&D systems) according to manufacturers instructions. More information is described in Supplement methods and materials.

TUNEL Assay

Cells were plated in adherent conditions in 6-well plates at 50,000 cells per well. After treatment, cells were fixed and stained for the presence of apoptotic cells by using the in Situ apoptosis detection kit (Trevigen, Gaithersburg, MD). Nuclei were counterstained with DAPI in red.

Growth Inhibition Assay and Drug Combination Analysis
Growth inhibition was measured as previously described (11, 20, 21). The combination effect was determined using the combination index (CI) analysis methods of Chou and Talalay (22) by utilizing CalcuSyn software (Biosoft, Ferguson, MO) which quantifies the degree of synergy between two agents that both induce a linear pharmacologic response. Fraction Affected (FA) was calculated from the percent growth inhibition: FA=(100-% growth inhibition)/100. CI values: <1=synergism; 1–1.2=additive and >1.2=antagonism. Results are representative of three independent experiments conducted in duplicate.

**Clonogenicity Assay**

The clonogenicity assay were performed as previously described (20). Cells were subsequently treated with SCH-527123 and oxaliplatin either alone or in combination for a period of 72h. Cells were washed and incubated in drug-free media for 3 weeks to allow colony formation. All experiments were performed in triplicate. Drug treated samples were compared directly to untreated controls set at 100%.

**siRNA Analysis**

siRNA analysis was performed as previously described (11). siRNAs against CXCR2 were purchased from Ambion (Austin, TX). Two different siRNA oligonucleotides [#14777/siRNA#1 and #4067/siRNA#2] were tested and siRNA#2 was used in the described knockdown experiments. Knockdown was validated by mRNA analysis at 72h post-transfection. siRNA-treated cells were normalized to negative control siRNA (NC siRNA) for Q-PCR and growth inhibition analyses.

**Quantitative reverse transcription-PCR**
Total RNA was extracted with Qiagen Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. IL-8 /CXCR2/ERCC1 primers were used for RT-PCR, and target genes were normalized to β-actin and quantified using the $2^{-\Delta\Delta C_T}$ method (23).

**Western Blot**

Cells were solubilized in cell lysis buffer containing a protease inhibitor cocktail. For tissue samples, Tissue Protein Extraction Reagent was used for tumor tissue protein extraction. Primary-rabbit-polyclonal antibodies were purchased from Cell Signaling Technology (Beverly, MA) and rabbit-anti-β-tubulin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Quantitative analysis of Western blots was performed by Image J.

**In Vivo Studies**

Xenograft experiments were conducted in male C57Bl/6 Balb/c mice (Taconic Labs, Hudson, NY) that were 6-8-week old. Subcutaneous HCT116 and E2 xenografts were established and allowed to grow until they reached ~100mm³ (day0). Animals were randomized to treatment groups: vehicle, SCH-527123, oxaliplatin and combination of SCH-527123 and oxaliplatin (n=6/group). SCH-527123 was administered at 50mg/kg by oral gavage once daily. Oxaliplatin was administered at 7.5mg/kg by intraperitoneal injection every 4 days.

**Immunohistochemistry (IHC) and Quantitation of Microvascular Density (MVD)**

IHC was performed as previously described (11). Formalin-fixed and snap-frozen fragments of tumor specimens were paraffin embedded and sectioned to 4µm thicknesses. Primary antibody Rat-anti-mouse-CD31 (BD Pharmingen) was added. MVD was
quantified from 4 different tumor samples by counting the total number of CD31-positive vessels across the whole section of tumors for each experimental condition.

**Statistical Analysis**

For all analyses, the difference between each cell lines compared and/or treatment groups were evaluated using a two-tailed *t*-test or ANOVA from GraphPad software. *P*<0.05 was considered statistically significant.

**Results**

**IL-8 overexpression modulated CXCR2 mRNA level in CRC xenografts.**

To investigate the gene expression of IL-8 and CXCR2 from xenograft samples of the HCT116 and HCT116-IL-8-overexpressing cells (E2), which were established from subcutaneous injections in nude mice, qRT-PCR was used and data revealed that IL-8 and CXCR2 mRNA expression were significantly increased at 26-fold (*p*<0.005) and 2.5-fold (*p*<0.05), respectively, in E2 xenografts when compared with HCT116 xenografts (Fig. 1A). CXCR1 mRNA expression was not significantly different between the HCT116 and E2 xenografts.

**Knockdown of CXCR2 increased sensitivity to oxaliplatin in CRC cells.**

To examine the role of CXCR2 in CRC cell proliferation, CXCR2 siRNA was used to suppress CXCR2 expression in CRC cell lines. Two different CXCR2 siRNA oligonucleotides were tested. After 72h post-transfection, CXCR2 knockdown was validated by RT-PCR and qRT-PCR and siRNA#2 was used in subsequent CXCR2 siRNA knockdown experiments (Fig.1B). qRT-PCR analysis revealed that transfection of CXCR2 siRNA#2 reduced the mRNA expression of CXCR2 by 45%±0.039 and 59%±0.049 (siRNA#1 and siRNA#2) in HCT116 cells compared to mock-transfected
cells ($p<0.005$, Fig.1B). E2 cells transfected with control mock and negative control siRNA had no evidence of CXCR2 knockdown (Fig.1B). Our previously study demonstrated that IL-8 overexpression in HCT116 cells decreased sensitivity to the cytotoxic effects of oxaliplatin (11). Having confirmed CXCR2 mRNA knockdown, we examined if decreased CXCR2 mRNA expression in CRC cell lines can reduce the IL-8-induced chemoresistance to oxaliplatin. The growth profiles of HCT116, E2, Caco2 and Caco2-IIIe (IIIe) were analyzed after 24h treatment with CXCR2 siRNA, and then subsequently treated for 72h with increasing concentrations of oxaliplatin by growth inhibition assay (Fig.1C,D). There was a decrease in the IC$_{50(72h)}$ of the CXCR2 siRNA/oxaliplatin-treated cells when compared with mock and β-actin control siRNA/oxaliplatin-treated cells. Importantly, IC$_{50(72h)}$ of CXCR2 siRNA/oxaliplatin-treated cells that overexpressed IL-8 (E2 and IIIe) had significant differences than parental siRNA/oxaliplatin-treated cells. These results suggest that the knockdown of CXCR2 increases sensitivity to oxaliplatin in CRC cells, especially in IL-8-overexpressing CRC cells.

**SCH-527123 inhibited CRC proliferation, migration, and invasion.**

To investigate the impact of CXCR2 inhibitors, the effect of SCH-527123 was evaluated in CRC cells. Single agent efficacy of SCH-527123 was evaluated by the growth inhibition assays in the HCT116, E2, Caco2 and IIIe cells. Cells were treated with increasing concentrations of SCH-527123 for 72h and showed dose-dependent growth inhibitory activity with IC$_{50(72h)}$ values ranging from 18-40µM (Fig.2A). Importantly the IL-8 overexpressing cells demonstrated a higher IC$_{50(72h)}$ concentration of SCH-527123 when compared to parental cells [HCT116 and E2 ($p<0.005$): 28.9±0.02µM]...
and 39.5±0.01μM, respectively; Caco2 and IIIe(p<0.005): 18.8±0.03μM and 25.5±0.02μM, respectively] (Fig.2A). Therefore, we concluded that SCH-527123, decreased growth inhibitory activity in CRC cell lines. Next, the effect of SCH-527123 on inducing apoptosis was evaluated using a TUNEL assay in HCT116 and E2 cells. Data showed a significant increase in apoptosis in cells treated with SCH-527123, with a 10%±0.5 (p<0.005) (HCT116) and 25%±0.8 (p<0.005) (E2) increase in apoptotic cell after 4-day treatment compared to untreated control cells (Fig.2B). This result suggests that SCH-527123 resulted in a decrease of cell growth followed by induction of significant apoptosis in CRC cells. Moreover, we found that 25μM SCH-527123 was sufficient to block IL-8-mediated CXCR2 activation in all cell lines analyzed, where phosphorylation of downstream kinases (MAPK/NF-κB) of CXCR2 was reduced in a concentration-dependent manner (Supplement fig.1).

Since IL-8 functions to promote cellular migration and invasion, we evaluated the effect of SCH-527123 on migration and invasion of CRC cells. Treatment with SCH-527123 (24h) significantly inhibited migration and invasion of HCT116 (24%±0.084%, p<0.05; 60%±0.003, p<0.005) and E2 (39%±0.01, p<0.005; 49%±0.004, p<0.005) cells when compared to control treated cells (Fig.2C). Similar results were observed after the addition of 10ng/ml RhIL-8 to HCT116 cells, where following treatment with 25μM SCH-527123 there was a significant inhibition of migration (59%±0.015, p<0.005) and invasion (57%±0.004, p<0.005) (Fig.2C, supplement fig.2).

**SCH-527123 in combination with oxaliplatin synergistically suppressed cell growth and survival.**
We next investigated the antiproliferative effects of combining SCH-527123 and oxaliplatin. All four cell lines were treated with increasing concentrations of SCH-527123 and oxaliplatin alone and in combination for 72h, then growth inhibition was measured by a MTS assay. The median-effect analysis method (22) was used in the evaluation of the combination drug effect. The effects of simultaneous treatment with both agents in HCT116 and Caco2 cell lines produced synergistic growth inhibition resulting in synergistic combination index (CI) value<1 for the majority of concentrations tested at 0.5 fraction affected (FA) (Fig.3A,3C. left). The combination of SCH-527123 (10-18µM) and oxaliplatin (0.25-1.5µM) in E2 and IIIe cell lines also produced synergistic growth inhibition resulting in FA range of 0.6-0.8 and synergistic CI value<1 (Fig.3B,3D. left). These results displayed that SCH-527123 synergistically increased sensitivity to oxaliplatin in HCT116 and Caco2 and IL-8 overexpressing cells, which was consistent with CXCR2 siRNA results described above.

To determine whether alterations in sensitivity to SCH-527123 and oxaliplatin observed by growth inhibition assay translated to changes in the ability of cells to recover from drug treatment, a clonogenicity assay was performed. All four cell lines were treated with SCH-527123 and oxaliplatin alone and selected combinations for 72h followed by outgrow in drug-free medium for 21 days. Combined drug analysis was performed using increasing concentrations of both agents. As shown in Fig. 3A-D (right) and supplement fig.2, in all cell lines, SCH-527123 and oxaliplatin alone resulted in a dose-dependent suppression of colony formation. Importantly, the combinations of SCH-527123 and oxaliplatin synergistically suppressed colony formation at all combinations tested in all cell lines (HCT116: 60%, E2: 64%, Caco2: 72%, IIIe: 60%). Therefore, these
findings suggest that targeting CXCR2 increases drug sensitivity of CRC cells to the one of the current CRC chemotherapies, oxaliplatin.

**SCH-527123 alone and in combination with oxaliplatin suppressed downstream signaling and modulated apoptotic markers in CRC cell lines.**

To evaluate the expression of CXCR2 and IL-8 in our CRC cell lines treated with SCH-527123 and oxaliplatin alone and combination, qRT-PCR analysis and ELISA assay were performed. Although there were no differences in the level of IL-8 mRNA expression in all cell lines with treatment (data not shown), IL-8 protein expression was significantly downregulated in HCT116, E2 and IIIe cells with both agents alone and combination (Fig.4A). These data suggest that SCH-527123-mediated antagonism may decrease IL-8 protein expression at the post-translational level.

To evaluate the effect of combination treatment on IL-8/CXCR2 downstream signaling, the activation of NF-κB/AKT/MAPK pathway was tested by Western blot. All four cell lines were treated with SCH-527123 and oxaliplatin alone and combination for 24h. Phospho-NF-κB/phospho-MAPK/phospho-AKT levels were significantly suppressed in all cell lines with combination treatment compared to untreated and single agents alone (Fig.4B). These results suggest that the effects of SCH-527123 alone and in combination with oxaliplatin may be mediated by NF-κB/AKT/MAPK signaling cascade. Furthermore, using immunoblotting assay, we observed NF-κB targeted anti-apoptotic gene BCL-2 expression, pro-apoptotic protein BAX expression, which acts as an antagonist of BCL-2 and promotes apoptosis by forming a heterodimer (BCL-2/BAX) and therefore losing the pro-apoptotic effect of BCL-2, and apoptotic protein Poly (ADP-ribose) polymerase (PARP) activation, which is involved in DNA repair (Fig.4C). After
exposure of all cells to SCH-527123 and oxaliplatin alone and combination, levels of BCL-2 protein expression decreased and BAX protein expression increased. HCT116 and E2 cells showed enhanced cleavage of PARP into 116-/89-kDa fragments by combination of both agents compared to oxaliplatin alone (Fig.4C). These findings suggest that SCH-527123 alone and in combination with oxaliplatin increased apoptosis in CRC cells.

**SCH-527123 alone or in combination with oxaliplatin enhanced antiproliferative activity in tumor xenografts.**

To explore the enhanced anti-proliferative effects of SCH-527123 alone or in combination with oxaliplatin on tumor growth in vivo, and to assess the potential for SCH-527123 to compromise oxaliplatin activity, nude mice implanted with HCT116 or E2 cells were exposed to both single agents and their combination. SCH-527123 was administered at 50mg/kg/day, where the dose was determined by the in vitro IC50(72h), and previously published studies. Oxaliplatin was administered at 7.5mg/kg oxaliplatin every 4 days. Following 21 days of treatment, average tumor volume (TV) for the vehicle treated control, oxaliplatin and SCH-527123 single agent treatment groups were 831±133mm3, 382±69mm3 and 386±64mm3 respectively in the HCT116 xenografts. Interestingly, in the E2 xenografts, SCH-527123 alone decreased the TV with an average of 229±56mm3 and was more potent than oxaliplatin alone with a TV of 295±61mm3 where vehicle-treated control tumors had TV of 391±121mm3. Importantly, the combination displayed a significant decrease in TV [HCT116: 340±63mm3(p<0.05) and E2: 123±26mm3(p<0.05)], compared to either single agent alone from day13 through the end of the study (Fig.5A). Moreover, combination treatment did not cause any significant difference in body weight compared to vehicle-treated control (p=0.67, Fig.5B).
On day 21, the tumors were excised and evaluated for the mRNA expression of CXCR2 and IL-8 following treatment with either single agent alone or in combination by qRT-PCR. qRT-PCR analysis showed that the combination of SCH-527123 and oxaliplatin resulted in a significant reduction of CXCR2 and IL-8 mRNA in HCT116 (p<0.05) and E2 (p<0.005) xenografts when compared to vehicle-treated controls (Fig.5C). Measurement of circulating IL-8 levels in serum which were collected at the time of necropsy showed a dramatic decrease in both HCT116 and E2 tumor-bearing mice with agent alone and combination compared with vehicle-treated controls (Fig.5D).

Excision repair cross-complement group 1 (ERCC1) is a key element in the nucleotide excision repair pathway which has previously been associated with oxaliplatin resistance (24-26). Therefore, the effect of treatment on ERCC1 mRNA expression in the xenografts following SCH-527123, oxaliplatin alone and their combination was measured by qRT-PCR. The analysis confirmed that both the HCT116 and E2 xenografts displayed a significant decrease in ERCC1 mRNA expression following treatment with single agents alone and their combination in comparison with vehicle controls (Fig.5C).

The combination of SCH-527123 and oxaliplatin synergistically suppresses downstream signaling and angiogenic activity in xenograft model.

We examined the activity of NF-κB/AKT/MAPK by Western blot in each of the xenografts after SCH-527123 and oxaliplatin treatments at the end of the study. In HCT116 and E2 xenografts, SCH-527123 alone and in combination with oxaliplatin resulted in a significant decrease in the protein activation of phospho-NF-κB/phospho-AKT/phospho-MAPK (Fig.6A). These in vivo data are consistent with the in vitro data.
and confirm that SCH-527123 inhibits the NF-κB/AKT/MAPK signaling cascades in both models.

To establish whether the decreased tumorigenicity and growth of tumors was associated with decreased angiogenesis, IHC was utilized to measure the expression of CD31 (mouse endothelial cell-specific) to evaluate microvessel density (MVD) in the tumor specimens. MVD in the HCT116 xenografts was decreased following treatment with SCH-527123 [28%±0.04 (p=0.02)] and oxaliplatin [10%±0.05 (p=0.06)] when compared to vehicle-treated xenografts. Importantly, when used in combination, MVD were significantly decreased by 41%±0.05 (p=0.006) in HCT116 xenografts. Similarly, in the E2 xenografts, MVD was decreased by SCH-527123 by 44%±1.08 (p<0.05), oxaliplatin by 32%±0.58 (p<0.005) and their combination by 67%±0.6 (p<0.005) when compared to vehicle-treated xenografts (Fig.6B,C). These results demonstrate that SCH-527123 in combination with oxaliplatin resulted in a significant inhibition of angiogenic activity as determined by CD31 IHC expression.

Discussion
The role of IL-8 and CXCR2 in tumor development and progression has been well documented in a wide range of cancer types (8, 27-31). Our previous data and others have demonstrated that the IL-8/CXCR2 pathway plays a key role in mediating CRC development (11, 14, 15, 32). In follow-up to our previously published data in CRC, this study focused on the impact of inhibition of CXCR2 on the proliferation, survival, invasion and migration in CRC in vitro and in vivo models. To the best of our knowledge, this study demonstrates for the first time that the CXCR2 antagonist, SCH-527123, has significant antitumor activity in CRC pre-clinical models and can further sensitize colon...
cancer cells to oxaliplatin based treatment. Furthermore, our key findings showed that the antitumor activity of SCH-527123 resulted from inhibition of cancer cell growth, motility and angiogenesis through the NF-κB/AKT/MAPK signaling pathways, and this signaling could be further attenuated when SCH-527123 was co-administered with oxaliplatin.

Previous data provides evidence that IL-8 is constitutively expressed in mCRC and primarily associated with the proliferation, metastasis and the induction of angiogenesis (11, 33). Important roles of its receptors, CXCR1 and CXCR2, have also been defined in CRC progression (34, 35). Our previous study demonstrated that knockdown IL-8 expression can inhibit CRC cell growth and metastasis. Conversely, IL-8 overexpression can increase CRC cell metastatic and angiogenic potential, as well as increasing chemoresistance to oxaliplatin (11). However, neutralizing antibodies against other chemokines [such as IL-6 (36), IL-5 (37)] also demonstrate similar results. Importantly, CXCR2 has been shown to interact and bind diverse ligands in addition to CXCR1, therefore, resulting in the targeting of IL-8/CXCR2 signaling demonstrating a more effective and broader inhibition in CRC development and progression.

Several small molecule inhibitors targeting IL8/CXCR2 signaling have been developed to suppress inflammatory diseases (38, 39). A recent study has demonstrated the potential of SCH-527123 in inhibiting human colon liver metastases using in vivo models (18). In our in vitro study, we demonstrated that SCH-527123 treatment inhibited cell proliferation and induced apoptosis in both HCT116 and Caco2 parental and IL-8 overexpressing CRC cells. The in vitro findings were validated in an in vivo study where mice were given SCH-527123 orally and exhibited a reduction in tumor volume when
compared with the vehicle-treated control group, which further supported the antiproliferative effect of SCH-527123. SCH-527123 also demonstrated a decrease in tumor vascular density when compared with the vehicle-treated control group. These findings indicated the targeting CXCR2 with antagonists such as SCH-527123 may be a promising therapeutic for CRC.

In prostate cancer cells it was reported that IL-8 signaling contributes to the intrinsic resistance of the cancer cells to undergo apoptosis in response to either environmental or chemical stress (40). One of the key investigations in this study was to evaluate the antiproliferative effects of combining the CXCR2 antagonist with cytotoxic chemotherapy. Oxaliplatin is a platinum-based DNA damaging chemotherapy that demonstrates clinical benefit for patients with high-risk stage II and stage III CRC, as well as patients with advanced disease (41, 42). However, cancer cells are frequently resistant to oxaliplatin (43). Our present study demonstrated CXCR2 to be a key mediator of IL-8-mediated chemoresistance to oxaliplatin and utilizing CXCR2siRNA to knockdown its expression in CRC cells sensitized them to oxaliplatin. These results led to the hypothesis that targeting IL-8/CXCR2 in combination with oxaliplatin may increase sensitivity to oxaliplatin, providing enhanced efficacy and eventually benefit in CRC treatment. Our novel observation showed that the combination of SCH-527123 and oxaliplatin resulted in synergistic suppression of CRC cell proliferation and survival with additive-to-synergistic effects. These synergistic antitumor properties were also observed in the IL-8-overexpressing cell lines, which were more insensitive to single agent SCH-527123 treatment. Our in vivo data confirmed the in vitro findings and showed that the combination of SCH-527123 and oxaliplatin significantly inhibits tumor growth when
compared to single agents alone. *ERCC1* mRNA levels have been shown to be predictive of oxaliplatin cytotoxicity in the HCT116 cell line (25) and a useful marker in predicting response to oxaliplatin-based treatment for CRC patients (44). High *ERCC1* mRNA expression has been demonstrated to be associated with resistance to oxaliplatin (26). Supplement fig.3 showed, in an oxaliplatin-resistant cell line (HCT116-OR, where resistance was induced by long-term culture in the presence of low dose oxaliplatin), or in E2 cell line, that both cell lines demonstrated an increased level of *ERCC1* mRNA expression. Our *in vivo* data showed that treatment with both agents in combination dramatically lowered *ERCC1* mRNA expression, which suggests that SCH-527123 may be associated with increased sensitivity to oxaliplatin through modulation of the DNA nucleotide excision repair pathway.

To elucidate the mechanism of action of SCH-527123, we analyzed the activation of the downstream pathway of IL-8/CXCR2. IL-8/CXCR2 has previously been shown to signal through AKT and MAPK pathways (45, 46). Wilson group demonstrated that IL-8/CXCR2 signaling confers resistance to oxaliplatin through NF-κB activity, which is an important determinant of cancer cell sensitivity to oxaliplatin (40). In this study, our findings suggest that SCH-527123 decreases NF-κB activity through IL-8/CXCR2 signaling, which is in turn responsible for enhancing sensitivity to oxaliplatin. Moreover, our study demonstrated that SCH-527123 in combination with oxaliplatin significantly increased apoptotic signaling in CRC cells. Therefore, we propose that inhibition of CXCR2/IL-8 signaling increases oxaliplatin sensitivity that is mediated partially by attenuating NF-κB activity and also inducing apoptosis. However, further investigations
are warranted to elucidate the mechanism of oxaliplatin sensitivity mediated through NF-κB activity and apoptosis.

In conclusion, our studies provide significant evidence that the CXCR2 antagonist, SCH-527123 demonstrates antitumor effects and increases sensitivity to oxaliplatin therapy in both in vitro and in vivo CRC models. The antitumor activity of SCH-527123 observed in CRC cells lines was shown to be due to decreased cell proliferation, migration, invasion and increased apoptosis. In addition, it was demonstrated that combination of SCH-527123 and oxaliplatin increased sensitivity to oxaliplatin in CRC cells in vitro and in vivo. Moreover, the combination of SCH-527123 and oxaliplatin synergistically inhibited in vivo tumor growth and vascularity. Based on these preclinical results, CXCR2 may represent a novel therapeutic target in CRC, that when targeted in combination with the DNA damaging agent, oxaliplatin will increase chemosensitivity.
Reference


Figure Legends

Figure 1: CXCR2 mRNA expression in CRC HCT116 and E2 xenografts and the influence of CXCR2 knockdown in CRC on growth of cells treated with oxaliplatin.

A. qRT-PCR determination of IL-8/CXCR2 mRNA expression in HCT116 and E2 xenografts. B. HCT116 cells were transfected with mock, β-actin siRNA and CXCR2 siRNA. Top, Q-PCR results are representative at 72h post-transfection. Bottom, qRT-PCR results are representative of mean±SD from triplicate samples and presented as mRNA fold change relative to mock transfected control. C,D. All cell lines were transfected with mock, β-actin siRNA and CXCR2 siRNA, and then treated with increasing concentrations of oxaliplatin for 72h.

Figure 2: SCH-527123 decreases cell proliferation, migration and invasion and increases apoptosis in CRC cells.

A. Growth inhibition assay. IC_{50 (72h)} of SCH-527123 were determined using GraphPad Prism software. B. After 72h treatment, the number of apoptotic cells was evaluated, and apoptotic cells (blue) in HCT116 and E2 were detected in SCH-527123-treated cells compared with controls, in which mostly viable cells (red) were present. C. Quantitation of migration and invasion after SCH-527123 treatment. Histogram shows fold change over the number of HCT116 and HCT116-rhIL-8 and E2 cells that migrated or invaded compared to no treatment.

Figure 3: SCH-527123 combined with oxaliplatin synergistically suppresses CRC cell proliferation and survival.

A-D (Left panel). Growth inhibition assay. A-D (Right panel). Clonogenicity assay. All cell lines were treated with increasing concentrations of SCH-527123 and oxaliplatin
alone and in combination for 72h. Data is presented as histograms of the mean percentage of colony formation compared with untreated controls (100%)±SD. The combined drug effects were analyzed using the combination index (CI) with fraction affected (FA) values for combinations.

**Figure 4: SCH-527123 in combination with oxaliplatin modulates protein expression of IL-8, PARP, BCL-2/BAX and decreased NF-κB/Akt/MAPK signaling activity in CRC cells.**

A. An ELISA assay was used to measure IL-8 production from all cell lines. The results were normalized to total cell numbers. Histograms represent the mean fold change ±SD of IL-8 overexpressing cells compared to parental cells. B and C. Western blot analysis. All cells were treated with 25μM SCH-527123 and 0.5μM oxaliplatin alone and in combination for 24h. β-tubulin was used to as a loading control and normalization protein for quantitation.

**Figure 5: Antitumor activity of SCH-527123 combined with oxaliplatin in HCT116 and E2 xenografts.**

A, Examination of tumor growth in xenografts of CRC cells. The graphs indicate the mean tumor growth rates ±SEM of each group. Statistical significance was determined by ANOVA using Graphpad Software, \( P<0.05 \). B, Mouse bodyweight represented as the percent initial body weight at day21 compared to day1. C, qRT-PCR was used to determine the level of IL-8, CXCR2 and ERCC1 mRNA in HCT116 and E2 xenografts with treatment. D, Serum IL-8 production was measured by ELISA, and the results were normalized to whole blood volume and presented as the mean±SD. The results are representative of a minimum of three independent experiments.
Figure 6: SCH-527123 in combination with oxaliplatin significantly suppressed NF-κB/Akt/MAPK downstream signaling and angiogenic activity in HCT116 and E2 xenografts.

A. Cell lysates of each tissue were immunoblotted with anti-phospho-NF-κB-p65/Akt/p44/42MAPK or anti-total NF-κB-p65/Akt/p44/42MAPK antibodies, β-tubulin as a loading control and normalization protein for quantitation. B,C. Immunohistochemistry and Quantitation of MVD (CD31) from tumor tissue slides. In HCT116 and E2 tumor specimens, rat-anti-mouse-CD31 antibodies were added to tissue sections. Data shown represent the mean±SD (*, P<0.05; **, P<0.005).
Figure 1.

A. 

B. 

C. 

D. 

HCT 116 72h

Mock NC SiRNA#1 SiRNA#2

CXCR2

β-actin

HCT116-parental 48 h

HCT116-parental 72 h

Mock Negative control siRNA CXCR2 siRNA#1 CXCR2 siRNA#2

CXCR2 mRNA fold change

HCT116 (Parental xenograft) E2 (IL-8 transfected xenograft)

Log[10] Oxaliplatin

% Control

HCT116 - Parental

HCT116-E2

Mock NC SiRNA#1 SiRNA#2

CXCR2 siRNA

HCT 116 72h

Mock NC SiRNA#1

CXCR2 siRNA

Caco2 Mock Transfected IC50=0.67+/-.09

Caco2 Negative control IC50=0.60+/-.03

Caco2 CXCR2 siRNA IC50=0.41+/-.05

Caco2-llle Mock Transfected IC50=0.88+/-.06

Caco2-llle Negative control IC50=0.82+/-.08

Caco2-llle CXCR2 siRNA IC50=0.384+/-.14

Caco2 - Parental

Caco2-llle

p < 0.0001

p = 0.08

Actin

CXCR2

β

SiRNA#2

HCT116 72h

Mock NC SiRNA#1

CXCR2 siRNA

CXCR2 mRNA fold change

HCT116-parental 48 h

HCT116-parental 72 h

Mock Negative control siRNA CXCR2 siRNA#1 CXCR2 siRNA#2

CXCR2 mRNA fold change

HCT116 (Parental xenograft) E2 (IL-8 transfected xenograft)

Log[10] Oxaliplatin

% Control

HCT116 - Parental

HCT116-E2

Mock NC SiRNA#1 SiRNA#2

CXCR2 siRNA

HCT 116 72h

Mock NC SiRNA#1

CXCR2 siRNA

Caco2 Mock Transfected IC50=0.67+/-.09

Caco2 Negative control IC50=0.60+/-.03

Caco2 CXCR2 siRNA IC50=0.41+/-.05

Caco2-llle Mock Transfected IC50=0.88+/-.06

Caco2-llle Negative control IC50=0.82+/-.08

Caco2-llle CXCR2 siRNA IC50=0.384+/-.14

p < 0.05 p < 0.005

p < 0.0001 p = 0.08
Figure 2.

A. 

![Graph showing the effect of SCH-527123 on HCT116 cell survival.](image)

- Caco2 IC\(_{50}\) 18.78 +/- 0.02
- Caco2-Illc IC\(_{50}\) 25.45 +/- 0.02
- HCT116 IC\(_{50}\) 28.95 +/- 0.02
- HCT116-E2 IC\(_{50}\) 39.45 +/- 0.01

B. 

![Images showing HCT116 and HCT116-E2 cell lines with and without SCH-527123 treatment.](image)

C. 

![Bar graphs showing fold change in migration and invasion](image)

- Fold Change in Migration:
  - HCT116
  - HCT116 + IL-8
  - HCT116-E2

- Fold Change in Invasion:
  - HCT116
  - HCT116 + IL-8
  - HCT116-E2

- Vehicle
- SCH-527123 (25 \(\mu\)M)

**Caco2 IC\(_{50}\) 18.78 +/- 0.02**

**Caco2-Illc IC\(_{50}\) 25.45 +/- 0.02**

**HCT116 IC\(_{50}\) 28.95 +/- 0.02**

**HCT116-E2 IC\(_{50}\) 39.45 +/- 0.01**
Figure 3.

A. HCT116-parental

- Oxaliplatin
- SCH-527123
- Combo

B. HCT116-E2

- Oxaliplatin
- SCH-527123
- Combo

C. Caco2-parental

- Oxaliplatin
- SCH-527123
- Combo

D. Caco2-Ille

- Oxaliplatin
- SCH-527123
- Combo
Figure 4.

A. Graph showing IL-8 concentration (pg/ml/1x10^6/48h) for HCT116, HCT116-E2, Caco2, and Caco2-IIIe.


C. Western blots for PARP, BAX, BCL-2, and β-Tubulin for HCT116, HCT116-E2, Caco2, and Caco2-IIIe.

Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

Downloaded from mct.aacrjournals.org on April 2, 2017. © 2012 American Association for Cancer Research.
Figure 6

A. HCT116 xenograft vs. E2 xenograft

<table>
<thead>
<tr>
<th>Treatment</th>
<th>HCT116 xenograft</th>
<th>E2 xenograft</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCH-527123 Oxaliplatin</td>
<td>- + - +</td>
<td>- + - +</td>
</tr>
<tr>
<td>NF-κB</td>
<td>1 1.25 1.25 1.25</td>
<td>1 1.2 1.2 1</td>
</tr>
<tr>
<td>P-NF-κB</td>
<td>1 0.85 0.28 0.1</td>
<td>1 0.6 0.01 0.1</td>
</tr>
<tr>
<td>P42/44</td>
<td>1 1.5 2 2.5</td>
<td>1 1.1 1.25 1.25</td>
</tr>
<tr>
<td>P-p42/44</td>
<td>1 0.2 0.6 0.2</td>
<td>1 0.7 0.2 0.2 0.02</td>
</tr>
<tr>
<td>Akt</td>
<td>1 1.6 0.4 0.1</td>
<td>1 2 0.2 0.1</td>
</tr>
<tr>
<td>P-Akt</td>
<td>1 1.5 1.4 1.5</td>
<td>1 0.9 0.85 0.85</td>
</tr>
<tr>
<td>β-Tubulin</td>
<td>1 1.25 1.5 1.4</td>
<td>1 0.9 0.85 0.85</td>
</tr>
</tbody>
</table>

B. Vehicle, SCH-527123, Oxaliplatin, Combo

C. Mean microvessel density

Downloaded from mct.aacrjournals.org on April 2, 2017. © 2012 American Association for Cancer Research.
Molecular Cancer Therapeutics

The CXCR2 antagonist, SCH-527123, demonstrates antitumor activity and sensitizes cells to oxaliplatin in preclinical colon cancer models

Yan Ning, Melissa J LaBonte, Wu Zhang, et al.

Mol Cancer Ther Published OnlineFirst March 5, 2012.

Updated version
Access the most recent version of this article at:
doi:10.1158/1535-7163.MCT-11-0915

Supplementary Material
Access the most recent supplemental material at:
http://mct.aacrjournals.org/content/suppl/2012/03/08/1535-7163.MCT-11-0915.DC1

Author Manuscript
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.