Published in final edited form as:
Cancer Res. 2009 January 15; 69(2): 573–582. doi:10.1158/0008-5472.CAN-08-2088.

Gamma-Secretase Inhibitors Abrogate Oxaliplatin-Induced Activation of the Notch-1 Signaling Pathway in Colon Cancer Cells Resulting in Enhanced Chemosensitivity

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Abstract

Because Notch signaling is implicated in colon cancer tumorigenesis and protects from apoptosis by inducing pro-survival targets, it was hypothesized that inhibition of Notch signaling with gamma-secretase inhibitors (GSIs) may enhance the chemosensitivity of colon cancer cells. We first show that the Notch-1 receptor and its downstream target Hes-1 is upregulated with colon cancer progression, similar to other genes involved in chemoresistance. We then report that chemotherapy induces Notch-1, as oxaliplatin, fluorouracil (5-FU), or SN-38 (the active metabolite of irinotecan), induced Notch-1 Intracellular Domain (NICD) protein and activated Hes-1. Induction of NICD was caused by an increase in the gamma-secretase protein subunits, nicastrin and presenilin-1, as suppression of nicastrin with small interfering RNA (siRNA) prevented NICD induction after oxaliplatin. Subsequent, inhibition of Notch-1 signaling with a sulfonamide GSI (GSI34) prevented the induction of NICD by chemotherapy and blunted Hes-1 activation. Blocking the activation of Notch signaling with GSI34 sensitized cells to chemotherapy and was synergistic with oxaliplatin, 5-FU, and SN-38. This chemosensitization was mediated by Notch-1, as inhibition of Notch-1 with siRNA, enhanced chemosensitivity whereas overexpression of NICD increased chemoresistance. Downregulation of Notch signaling also prevented the induction of pro-survival pathways, most notably PI3K/Akt, after oxaliplatin. In summary, colon cancer cells may upregulate Notch-1 as a protective mechanism in response to chemotherapy. Therefore, combining GSIs with chemotherapy may represent a novel approach for treating metastatic colon cancers by mitigating the development of chemoresistance.

Keywords

Notch; oxaliplatin; colon; chemosensitivity; Akt

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Note: There are no potential conflicts of interest to disclose in this study.

Introduction

Although significant advances have occurred in the treatment of metastatic colorectal cancers with the introduction of novel chemotherapies combined with targeted agents, the overall survival rate remains low, as metastatic cancers eventually develop resistance to standard treatments through the activation of pro-survival tumor pathways. Given their role in cellular proliferation, many of these pro-survival pathways actually play important roles during development, including the Notch pathway, which is increasingly being studied as a novel mechanism for tumorigenesis (reviewed by 1, 2). Although originally found to be overexpressed in T-cell leukemias through an oncogenic translocation, the Notch pathway has now been shown to be activated in multiple tumors, including colon cancers (3). Further contributing to oncogenesis, activation of the Notch pathway induces pro-survival signals that have been associated with resistance to chemotherapy (4). However, the relationship between Notch activation and sensitivity of tumor cells to cytotoxic agents in colon cancer has not been examined. Notch signaling could contribute to chemoresistance by protecting the cell from apoptosis, as it activates targets involved in cellular survival, such as phosphoinositide kinase-3 (PI3K)/Akt (5, 6, 7), Bcl-X_L (7), and survivin (8). Consequently, activation of Notch-1 may increase chemoresistance, as overexpression of Notch-1 increases the resistance of T cells to etoposide (7), breast cancers to melphalan and mitoxantrone (9), cervical cancers to doxorubicin (5), and lung cancers to cisplatin and paclitaxel (4).

The activation of the Notch pathway occurs when specific ligands like Jagged-1 (JAG-1) bind to four related transmembrane receptors, Notch-1 through Notch-4. This binding activates the gamma-secretase protein complex, which cleaves the Notch-1 receptor in the transmembrane domain to release the cytoplasmic portion known as the Notch-1 intracellular domain (NICD). After translocating into the nucleus, the NICD binds three cofactors, including CSL (CBF-1/Suppressor of Hairless/Lag-1), MAML-1 (Mastermind-like-1) and p300/CBP, to create a complex that acts as a transcriptional co-activator. Notch signaling then induces the expression of multiple targets involved in cellular proliferation, such as cyclin D1 (10) and c-Myc (11). Overexpression of the Notch receptor and its ligands has now been identified in multiple cancers, including breast (12), ovarian (13), prostate (14), brain (15), and sarcoma (16, 17). Finally, overexpression of Notch elements in tumors is correlated with poor clinical outcome. For example, overexpression of Notch-1 has been associated with decreased time to recurrence in breast cancers (18), and increased expression of JAG-1 is associated with higher rates of recurrence in prostate cancers (14).

Consequently, multiple groups have studied the effects of inhibiting Notch signaling by targeting the gamma-secretase protein complex, which cleaves the Notch receptor to activate the pathway (reviewed by 2). It was first shown that inhibition of the gamma-secretase complex suppresses the growth of T-cell ALL lines, which leads to apoptosis (19). Gamma-secretase inhibitors (GSIs) have now been used to inhibit the growth of multiple tumors, including sarcoma (16), medulloblastoma (15), and breast cancer (18). Therefore, because of the role that Notch signaling plays in chemoresistance, it was hypothesized that inhibition of Notch-1 may sensitize colon cancer cells to chemotherapy. Although previous groups have reported that GSI treatment can enhance the apoptotic effect of taxanes in colon cancers (20), and of doxorubicin and melphalan in multiple myeloma cells (21), we now report for the first time that the Notch-1 pathway is actually activated in colon cancer cells in response to chemotherapy as a novel mechanism to increase chemoresistance. Downregulation of Notch-1 signaling with GSIs sensitizes colon cancer cells to chemotherapy, whereas overexpression of NICD increases chemoresistance. We then report that the mechanism for this potentiation of chemosensitivity by Notch inhibition may be related to downregulation of pro-survival pathways. Therefore, we propose that inhibition of Notch-1 signaling may be a novel strategy to prevent the development of chemoresistance in advanced colon cancers.

Materials and Methods

Microarray data analysis

Gene chip microarrays were constructed from 308 normal and malignant colon tissues obtained at the time of surgical resection, including normal colon mucosa (10%), colonic polyps (15%), primary colon cancers (55%), liver metastases (13%), and lung metastases (6%), using Affymetrix U133A arrays (Affymetrix, Santa Clara, CA). Data preprocessing and statistical analysis were carried out in R (http://www.r-project.org) and Bioconductor (http://www.bioconductor.org). The expression intensities were normalized using the Robust Multiarray Average method (22), which includes background adjustment, quantile normalization across arrays, and probe-level expression measure summarization using median polish on the log2 scale, for each probe set. Differential expression analysis was performed to identify putative genes between sample groups. An Empirical Bayes t test was applied to each gene (23), as a *p*-value cutoff of 0.01 was used to select differentially expressed genes ($p \leq 0.01$). The following sample groups were compared respectively: normal mucosa vs. primary polyp vs. primary colon cancer, primary tumor stage I vs. II vs. III vs. IV, and primary tumor vs. lung metastasis.

Cell lines

The human colon adenocarcinoma cell lines HCT116, SW620, SW480, HT29, and LS513 (American Type Culture Collection, Manassas, VA) were maintained in McCoy's 5a medium (HCT116, HT29), Leibovitz's L-15 (SW480, SW620), or RPMI-1640 (LS513), as recommended by ATCC. Cultures were supplemented with 10% heat-inactivated fetal bovine serum and penicillin-streptomycin (100 units/ml) or 2 mM L-glutamine (SW480, SW620). All cell lines were maintained at 37° C in 5% carbon dioxide and were tested to rule out *Mycoplasma* contamination.

Drugs

GSI34, a sulfonamide analog, was derived from GSIs as described (24). GSI34 was dissolved in DMSO, stored at -20° C, and diluted in media before use so that the final concentration of DMSO was 0.1% or less in all experiments. The drugs oxaliplatin (Sanofi-Aventis, Bridgewater, NJ), and 5-FU (Pharmacia, Kalamazoo, MI) were obtained from the MSKCC Research Pharmacy. SN-38, the active metabolite of irinotecan, was generously provided by Dr. J. Patrick McGovren (formerly at Pharmacia and Upjohn, Peapack, NJ). Drugs were used at concentrations equal to or less than the IC_{50} specific to each cell line.

Constructs and small interfering RNA (siRNA)

The pGL2-*Hes-1*-luciferase and pCS2-*NICD* constructs were generously provided by Dr. Raffi Kopan (Washington University, St. Louis, MO). The pGL2 and *Renilla* vectors were obtained from Promega (Madison, WI). siRNA to the following genes were used: Notch-1 from Santa Cruz Biotechnology (Santa Cruz, CA), nicastrin from Santa Cruz and Cell Signaling Technology (Danvers, MA), and Akt1/2 from Cell Signaling. A minimum of two different siRNA sequences were chosen for each gene. Commercially-available siRNA to random noncoding sequences were used for control transfections (Santa Cruz).

Protein immunoblot assays

Cell lines were treated with oxaliplatin (0.5 or 1 μM), SN-38 (2 to 20 nM), or 5-FU (1 to 10 μM), combined with either GSI34 (1 to 10 μM) or 0.1% DMSO (as a control) for 24 to 48 hours. Total protein lysates were prepared. For isolation of nuclear and cytoplasmic fractions, the Pierce NE-PER extraction kit was used (Rockford, IL). Proteins were probed with the following primary antibodies: Notch-1, cyclin D1, and Hes-1 (all from Santa Cruz);

NICD, Phospho-Akt^{Ser473}, Akt, DNA-dependent protein kinase (DNA-PK), mammalian target of rapamycin (mTOR), Phospho-S6$^{Ser235/Ser236}$ ribosomal protein, total S6 ribosomal protein, nicastrin, cyclin D1, and presenilin (all from Cell Signaling); and survivin and Bcl-X_L (Pharmingen-BD Biosciences, San Jose, CA). Equal protein loading was confirmed by probing for α/β-tubulin expression (Cell Signaling). Appropriate secondary antibodies conjugated to horseradish peroxidase were used, including anti-mouse or anti-rabbit IgG (GE-Healthcare, United Kingdom), and proteins were visualized with Amersham ECL Chemiluminescence (GE-Healthcare). Films were digitized with a Microtek scanner (Carson, CA), and images were processed with Photoshop software (Adobe, San Jose, CA).

Hes-1 luciferase assays

Cell lines were co-transfected with pGL2-*Hes-1* luciferase reporter (1 μg/well) and the *Renilla* reporter pRL-*CMV* (0.1 μg/well) using Fugene (Roche, Switzerland). After 8 to 12 hours, cells were treated with oxaliplatin (0.5 or 1 μM), GSI34 (10 μM), or both drugs for 48 hours. Cells were also treated with SN-38 (2 or 20 nM), 5-FU (1 or 8 μM), GSI34 (10 μM) alone, or the combination of GSI34 with SN-38 or 5-FU for 48 hours. After 6, 12, 24, or 48 hours of drug treatment, total cell lysates were harvested using the Dual-Reporter luciferase assay kit (Stop-and-Glo, Promega), and luciferase activity was quantified on a luminometer (Turner Design, Sunnyvale, CA). Luciferase values were standardized by *Renilla* pRL-*CMV* co-transfection.

Clonogenicity assays

Cell lines, in single cell suspension, were plated and treated for 48 hours with oxaliplatin (0.5 to 2 μM), GSI34 (10 μM), or both drugs. Drug-containing media was then removed, and the cells were allowed to grow for a minimum of 2 weeks to form colonies. Colonies were stained with 0.01% crystal violet (Sigma) and quantified in an automated colony counter (ColCount, Oxford-Optronics, England).

Apoptosis assays

Apoptosis was assessed by quantitative confocal fluorescence microscopy. Briefly, following drug treatment, cells were fixed in 4% paraformaldehyde and stained with 4'-6-diamidino-2-phenylindole or DAPI (Sigma). Cells with fragmented nuclei under confocal fluorescence microscopy (*40x* magnification) were measured as apoptotic. A total of 500 nuclei from five different high-power fields were assessed for each condition.

Viability assays

HCT116 cells were plated in 96-well plates and treated with GSI34 (0 to 40 μM), oxaliplatin (0 to 2 μM), or both drugs for 24 to 72 hours. Viability was assessed with the sulforhodamine B (SRB) assay. Briefly, following drug treatment, cells were stained with 0.4% SRB (Sigma), fixed with 10% trichloroacetic acid, washed with 1% acetic acid, solubilized in 10 mM Tris-buffer, and absorbance was measured at 490 nM on a spectrophotometer (SpectraMax, Molecular Devices, Sunnyvale, CA).

Transfection of siRNA

Cell lines were transfected with siRNA for Notch-1 (Santa Cruz) at 60 pmol/L or with siRNA for Akt (Cell Signaling) at 50 nM using Oligofectamine (Invitrogen, Carlsbad, CA). Cells were also transfected with control siRNA consisting of random sequences (Santa Cruz) at 60 pmol/L. For protein immunoblotting, total cell lysates were harvested at 48 hours. For Hes-1 luciferase assays, cells were first transfected with the Hes-1 luciferase reporter for 12 hours and were then transfected with siRNA (Notch-1, Akt, or control) for 48 hours. For colony formation assays, cells were transfected with siRNA for 12 hours and then

oxaliplatin (0.5 to 2 μM), SN-38 (0.2 to 20 nM), or media was added for 48 hours. Colonies were stained after 14 days.

Transfection of NICD

Colon cancer cell lines were transfected with pCS2-*NICD* (0.5 to 2 μg) or pCS2 vector (0.5 to 2 μg). To confirm protein expression of NICD, total cell lysates were harvested after 24 to 48 hours for immunoblotting. For luciferase experiments, the *HES-1* luciferase reporter was transfected for 12 hours, and the cells were then transfected with NICD for 48 hours. For colony formation assays, cells were transfected with NICD for 48 hours, media was removed, and colonies were allowed to grow for 14 days.

Biostatistical analysis

All experiments were conducted in duplicate and were repeated at least twice. Statistical significance was analyzed with Student's T-test ($p<0.05$).

RESULTS

Overexpression of Notch-1 in colon cancer progression

To support our hypothesis that Notch signaling is important for colon cancer development, we analyzed the expression of Notch genes, along with known chemoresistance genes, in progressive stages of colon cancers. Gene chip microarrays were constructed from 308 normal and malignant colon tissues obtained at surgical resection, including normal colon mucosa (10%), colonic polyps (15%), primary colon cancers (55%), liver metastases (13%), or lung metastases (6%), using Affymetrix U133A arrays. Correlation analysis identified genes differentially expressed between sample classes among a list of 44 genes related to the Notch pathway (Supplemental Fig. 1). The expression of *NOTCH1* increased from normal colon mucosa to Stage IV cancers with levels being highest in liver metastases, compared to normal colonic mucosa or liver parenchyma (trend test, $p<0.001$, Fig. 1*A*). In contrast, *NOTCH2* did not similarly increase with disease progression ($p<0.001$, Fig. 1*A*). The pattern of *NOTCH1* overexpression mirrored that of other genes involved in chemoresistance, including *BCL2*, *BIRC5* or survivin, and *CCND1* or cyclin D1 (Fig. 1*A*). In addition, the expression of the downstream target *HES1*, a target gene of Notch-1, increased with colon cancer progression (Fig. 1*B*). We then examined the levels of two genes that regulate Notch expression. *GSK3B*, (glycogen synthase kinase-3-beta), which positively regulates Notch-1 by preventing its phosphorylation (25), increases with colon cancer stage progression ($p<0.001$, Fig. 1*C*). In contrast, *NUMB*, a negative-regulator of Notch-1, is downregulated in advanced colon cancers ($p<0.001$, Fig. 1*C*). This Notch-1 overexpression may be ligand-independent, as the Notch ligand *JAG1* was not increased with colon cancer progression (Fig. 1*D*).

Oxaliplatin induces Notch-1 protein and activity

Because Notch-1 expression is increased with colon cancer progression, we hypothesized that it may play a role in chemoresistance. First, we examined if Notch-1 signaling is affected by chemotherapy by treating colon cancer cells with oxaliplatin, a platinum-derived chemotherapy drug that damages DNA through the formation of DNA adducts. In two colon cancer cell lines, HCT116 and SW620, oxaliplatin induced NICD protein in a dose-dependent manner (Fig. 2*A*, top). The induction was independent of p53 status, as HCT116 has wild-type p53 and SW620 has mutant p53. It was then examined if the increase in NICD protein could augment Notch-1 signaling by measuring the activity of a downstream target, *HES1*. HCT116 and SW620 cells were transfected with a luciferase construct containing the promoter of *HES-1* and were then treated with oxaliplatin. In both cell lines, similar to the

induction of NICD protein, oxaliplatin induced Hes-1 activity in a dose-dependent manner, although the degree of induction in SW620 cells was five-fold higher (Fig. 2*A*, bottom). The effect was not limited to these two lines, as oxaliplatin also increased Hes-1 activity in three other colon cancer lines, HT29 (mutant p53), LS513 (wild-type p53), and SW480 (mutant p53), although the degree of Hes-1 induction again varied (Fig. 2*B*). We then examined if the increase in NICD by oxaliplatin activated the Notch pathway by measuring downstream targets. In HCT116 cells, oxaliplatin induced Hes-1 protein, as suggested by the luciferase data, as well as the Notch-1 targets cyclin D1 and survivin (Fig. 2*C*). Finally, it was determined if other chemotherapies used to treat metastatic colon cancer can also activate Notch signaling. Both the antifolate fluorouracil (5-FU), a pyrimidine analog, and SN-38, the active metabolite of the topoisomerase I inhibitor, irinotecan, increased Hes-1 activity (Fig. 2*D*).

Oxaliplatin induces gamma-secretase protein expression

We then examined the possible mechanism underlying the induction of NICD protein by chemotherapy. NICD is produced when the Notch-1 receptor is cleaved by the gamma-secretase complex, composed of four subunits: presenilin (PS1), nicastrin (NCT), anterior pharynx-defective (APH-1), and presenilin enhancer-2 (PEN-2). Therefore, we hypothesized that chemotherapy induced NICD protein by increasing gamma-secretase activity. Treatment of HCT116 cells with oxaliplatin induced the expression of two subunits, PS-1 and NCT (Fig. 3*A*). We then downregulated gamma-secretase production by using siRNA to target NCT. Transfection of HCT116 cells with NCT siRNA inhibited the protein levels of NCT (Fig. 3*B*, top), which, effectively decreased Notch signaling, as measured by Hes-1 activity (Fig. 3*B*, bottom). In HCT116 cells, suppression of nicastrin protein with siRNA prevented the induction of NICD and cyclin D1 protein usually observed after oxaliplatin (Fig 3*C*).

Gamma-secretase inhibitors abrogate Notch-1 induction by chemotherapy to enhance colon cancer chemosensitivity

We then hypothesized that the increase in Notch-1 by oxaliplatin could be blocked if the cell lines were co-treated with a gamma-secretase inhibitor (GSI). Our group used a novel sulfonamide analog, GSI34, synthesized as described (24). First, treatment of HCT116 cells with GSI34 effectively blocked the production of NICD protein at baseline (Fig. 4*A*, top). In the presence of either GSI34 or 0.1% DMSO as a control, HCT116 cells were then treated with increasing doses of oxaliplatin. At each dose level, the addition of GSI34 abrogated the induction of NICD protein by oxaliplatin (Fig. 4*A*, top). It was then examined if GSI34 could inhibit the activation of Hes-1 by oxaliplatin. Co-treatment with GSI34 abrogated the induction of Hes-1 by oxaliplatin at 0.5 μM but did not completely suppress the induction at the higher dose of 1 μM (Fig. 4*A*). GSI34 also decreased Notch-1 induction by oxaliplatin in four other colon cancer cells: HT29, LS513, SW480, and SW620 (Fig. 4*B*). Finally, co-treatment of HCT116 cells with GSI34 suppressed the induction of Hes-1 by other chemotherapies, including 5-FU alone (Supplemental Fig. 2*A*) or 5-FU combined with oxaliplatin (Supplemental Fig. 2*B*).

Because it was hypothesized that activation of Notch-1 may protect colon cancer cells from chemotherapy, we examined if inhibition of Notch-1 could decrease chemoresistance. Cell lines were treated with oxaliplatin combined with GSI34 or DMSO for 48 hours, and viability was then measured in a colony forming assay. In HCT116 cells, treatment with oxaliplatin or GSI34 as single agents decreased colony formation by 40%, but the combination of GSI34 and oxaliplatin decreased colony formation by 60% (Student's t-test, p=0.034, Fig. 4*C*). Similar reductions in colony formation by GSI34 and oxaliplatin were observed in SW620 and LS513 cells (Supplemental Fig. 2*C*). GSI co-treatment also

enhanced the chemosensitivity of HCT116 to SN-38 and to 5-FU ($p<0.05$, Fig. 4*C*) Using isobologram experiments, we confirmed that the combination GSI34 and oxaliplatin synergistically decreased the IC_{50} in HCT116 cells (Supplemental Fig. 2*D*). It was then examined if colon cancer cells co-treated with oxaliplatin and GSI34 were undergoing enhanced apoptosis. Co-treatment of HCT116 cells with GSI34 and oxaliplatin significantly increased the percentage of apoptotic cells, compared to either treatment alone, as determined by DAPI staining for nuclear fragmentation ($p<0.05$, Fig. 4*D*).

Notch-1 expression affects chemosensitivity

Because the gamma-secretase complex cleaves multiple transmembrane receptors besides Notch-1, including the other Notch receptors (Notch-2 to Notch-4), we wanted to confirm that the enhancement of chemosensitivity by GSI34 was mediated by Notch-1. We used multiple siRNA constructs to specifically inhibit Notch-1 to determine if the chemosensitivity observed with GSI34 could be replicated. Transfection of HCT116 cells with siRNA to Notch-1 for 24 hours decreased the expression of Notch-1 protein (Fig. 5*A*, top), which decreased the activity of Hes-1 ($p=0.014$, Fig. 5*A*, bottom), compared to cells transfected with control siRNA. Notch-1 siRNA also decreased the clonogenicity of HCT116 cells at 48 hours (Fig. 5*A*, right). It was then determined if Notch-1 siRNA could enhance chemosensitivity to oxaliplatin. Like the results with GSI34, the combination of Notch-1 siRNA and oxaliplatin decreased viability significantly, compared to either siRNA or chemotherapy alone ($p=0.011$, Fig. 5*A*, right).

Because inhibition of Notch-1 could enhance chemosensitivity, we then examined if overexpression of Notch-1 could instead increase colon cancer chemoresistance. HCT116 cells were transfected with an NICD construct for 24 hours, which significantly increased NICD protein (Fig. 5*B*, top), and activated Hes-1 (Fig. 5*B*, bottom). After transfection with NICD or empty vector for 12 hours, HCT116 cells were then treated with oxaliplatin for 48 hours. Overexpression of NICD did not significantly change the percentage of colonies, compared to vector-transfection (Fig. 5*B*, right). Although treatment with oxaliplatin decreased the percentage of colonies by 50%, overexpression of NICD protected against oxaliplatin, as no decrease in colony formation occurred (Fig. 5*B*, right). We then examined if Notch-1 expression could affect the chemosensitivity of HCT116 cells to SN-38. HCT116 cells were first transfected with either Notch-1 siRNA or with NICD, and the percentage of viable cells following treatment with SN-38 was then measured using the SRB viability assay. Again, Notch-1 siRNA enhanced chemosensitivity to SN-38, whereas overexpression of NICD increased resistance to SN-38 (Fig. 5*C*).

Gamma-secretase inhibitors decrease the induction of pro-survival factors, including Akt signaling, by oxaliplatin

We then investigated the mechanisms underlying how inhibition of Notch-1 signaling may enhance chemosensitivity. First, we examined if GSI co-treatment could decrease several pro-survival factors implicated in chemoresistance to oxaliplatin. Treatment of HCT116 cells with GSI34 decreased the baseline levels of Bcl-X_L and survivin and suppressed the induction of cyclin D1 by oxaliplatin (Fig. 6*A*, top). Second, because Notch-1 may activate PI3K/Akt signaling to protect against DNA damage (7), the expression of proteins in this pathway was examined. In HCT116 cells, co-treatment with GSI34 decreased the phosphorylation of Akt at Serine473 by oxaliplatin and also mildly decreased total Akt levels (Fig. 6*A*, bottom). We then examined two targets of Akt, mTOR and S6 ribosomal protein. Levels of total mTOR, phosphorylated S6 at Serine235/236, and total S6 protein levels were decreased following co-treatment with GSI34 and oxaliplatin (Fig. 6*A*, bottom). We then examined the expression of a kinase that may activate Akt, DNA-PK, as co-treatment with GSI34 and oxaliplatin decreased the protein levels of DNA-PK (Fig. 6*A*,

bottom). Because intact Akt signaling may play a role in chemoresistance, we determined if suppression of Akt with siRNA would affect chemosensitivity to oxaliplatin. siRNA to Akt effectively suppressed total Akt protein levels (Fig. 6*B*, top), and decreased the viability of HCT116 cells following oxaliplatin (Fig. 6*B*, bottom). These results suggest that inhibition of Notch-1 signaling may sensitize colon cancer cells to oxaliplatin by preventing the activation of pro-survival pathways, including Akt, after DNA damage.

DISCUSSION

In this study, we report that colon cancer cell lines can be rendered more sensitive to chemotherapy by downregulation of Notch-1 signaling. We first provide evidence that the Notch-1 pathway is overexpressed during colon cancer progression, similar to other genes involved in chemoresistance. Overexpression of Notch-1 has previously been reported in other solid tumors, but this marks the first report in colon cancers. The entire Notch pathway appears activated, as downstream targets, such as Hes-1, are also elevated, and overexpression of Hes-1 has been previously reported in primary colon tumors (26). In addition, Numb, a negative regulator of Notch, is suppressed during colon cancer development, a finding that has been reported in breast cancers (9). Whether other regulators of Notch signaling are also affected by chemotherapy, is currently being studied.

The effect of chemotherapy on the Notch-1 signaling pathway has not been previously studied. Our results indicate that chemotherapy in fact activates the Notch pathway in colon cancer cells by inducing NICD. The induction of NICD has been previously shown in neuroblastoma cells following treatment with histone deacetylase inhibitor valproic acid (27). Our data indicates that the induction of NICD in our colon cancer cell lines is not mediated by the *p53* gene. This is in contrast to keratinocytes in which it has been reported that Notch-1 is a p53 target (28). The differences may be related to tissue-specificity, as Notch-1 seems to function as a tumor suppressor in epithelial tissues (reviewed in 1). Interestingly, Numb has been reported to regulate p53, preventing its degradation (29). There does seem to be a link between p53 and Notch-1 in some tumor types, as restoration of p53 in human prostate and breast cancer cells, increased Notch-1 (30).

It has been reported that Akt activation can induce NICD production through induction of gamma-secretase (31), and that DNA damage from chemotherapy can increase the activity of gamma-secretase protein (32). Our results indicate that the induction of NICD by chemotherapy is due to an increase in the expression of components (PS-1 and NCT) of the gamma-secretase complex, resulting in an augmentation of gamma-secretase activity. This is consistent with previous studies showing that overexpression of PEN-2 can increase gamma-secretase activity (33). Using a new assay, we have preliminary data suggesting that the activity of gamma-secretase is indeed augmented by chemotherapy.[1] Moreover, NCT may be the key component of the complex, as it has been reported in mouse knockout models that gamma-secretase activity is dependent on NCT (34). As oxaliplatin increases NCT in the gamma-secretase complex, targeting of NCT with siRNA prevented the induction of NICD by oxaliplatin in the colon cancer cells. Interestingly, in endothelial cells, treatment with a GSI has been shown to suppress NICD production by VEGF (31).

If activation of Notch-1 signaling contributes to chemoresistance, then inhibition of the Notch pathway should sensitize cells to chemotherapy. Our results indicate that the viability of colon cancer cells was synergistically decreased by GSI134 in combination with chemotherapy, including oxaliplatin, SN-38, and 5-FU. This was associated with inhibition of NICD production and suppression of Hes-1 activity. Use of GSIs may then present a

[1]Meng RD, Li YM, Schwartz GK. Unpublished observations.

novel means to both enhance the effects of chemotherapy and to delay chemoresistance in patients with metastatic disease, as oxaliplatin resistance has been correlated with colon cancer progression from an epithelial to an invasive phenotype (35). This effect of GSI may be tumor-specific. For example, in neuroendocrine cells, Notch-1 may act as a tumor suppressor; in fact, treatment with GSI's activates Notch-1 to suppress growth (36). Therefore, it remains to be determined whether this effect of GSI on chemotherapy can be extended to all tumor sub-types.

Because GSIs, as a class, also prevent the cleavage of other transmembrane proteins and because the IC_{50} of GSI34 in colon cancer cells was in the low micromolar range, we performed several siRNA experiments to clarify that the chemosensitivity induced by GSI34 was indeed mediated by inhibition of the Notch-1 receptor and not by an off-target effect. In HCT116 cells, transfection with siRNA to Notch-1 increases apoptosis by ultraviolet-irradiation (37). In fact, in lung cancer cells the induction of Notch-1 sensitizes the cells to GSIs (38). Our studies indicate that in the colon cancer cells the selective suppression of Notch-1 with siRNA and enhances the effects of both oxaliplatin and SN-38. Similarly, overexpression of overexpression of NICD protected against these two agents. This is consistent with the report that NICD overexpression protects against cisplatin in lung and liver cancer lines (4). Interestingly, the degree of sensitization to oxaliplatin by siRNA to Notch-1 was not as great as with GSI34. As GS134 targets all four Notch receptors, current experiments are now on-going using siRNA to knockdown expression of each Notch receptor to determine its effects on chemosensitivity.

The possible mechanisms by which GSIs enhance the effect of chemotherapy in colon cancer cells appear multifactorial. First, GSI34 suppressed the expression of three pro-survival factors that are targets of Notch-1 and have been reported to play a role in chemosensitivity to oxaliplatin: cyclin D1 (39), Bcl-X_L (40), and survivin (41). Second, we show that GSI34 can also affect the activation of the PI3K/Akt pathway by oxaliplatin. Notch-1 signaling has been implicated in AKT activation. For example, it has been known that Notch-1 can activate Akt in T-cells (6), as well as in melanoma (42), leukemia (43), and cervical cancer (44). In addition, a recent microarray study identified overexpression of Akt and Notch signaling as hallmarks of poor-prognosis gliomas (45). NICD overexpression results in the phosphorylated of Akt at Serine473 (4, 5, 42) and GSIs decrease phosphorylated Akt at this site in cervical cancer cells (44). In this study, we show that GSI34 abrogates the oxaliplatin-induced phosphorylation of Akt at Serine473. Likewise, two downstream targets of Akt, S6 and mTOR are also decreased by Notch inhibition. Other studies have also reported a link between Akt/mTor pathways and Notch-1. For example, in lymphoid cell lines, co-transfection with a dominant-negative Akt mutant decreased the protection mediated by Notch-1 against apoptosis (7), and the protective effect of NICD against p53-mediated apoptosis could be abrogated if mTOR was inhibited (4). In leukemia cells, inactivation of PTEN, which negatively regulates Akt activity, can cause resistance to GSI's (46). Finally, there may be alternative mechanisms of chemosensitization by GSI's. For example, in Kaposi's sarcoma cell lines, it has been reported that treatment with GSI's induces mitotic catastrophe (47).

Therefore, we propose a model for how Notch signaling may mediate chemoresistance. First, it is hypothesized that colon cancer cells activate Notch-1 in response to chemotherapy as a protective pathway (Fig 6*C*, top). Upon sensing DNA damage, the cancer cell augments the activity of the gamma-secretase complex by increasing the expression of its subunits, including PS-1 and NCT. The increased protein subunits are then formed into multiple gamma-secretase complexes, which cleave more Notch-1 receptors to produce NICD. After translocating into the nucleus, the increased NICD can activate the PI3K/Akt pathway or other pro-survival targets, such as Bcl-X_L, cyclin D1, and survivin. Consequently,

suppression of Notch-1 signaling by blocking NICD production with GSIs downregulates transcriptional targets involved in cellular survival, shifting the cell towards chemosensitivity (Fig. 6*C*, bottom). Therefore, our results suggest that in colon cancer Notch signaling may be an important modulator of cell survival, and Notch inhibition may provide a novel strategy to enhance the effects of chemotherapy in the treatment of patients with metastatic colon cancer. This model may be testable as Notch inhibitors enter clinical trial (48).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This research was supported by generous grants from the Littlefield Foundation and the American Association for Cancer Research (RDM and GKS), from the American Society of Clinical Oncology Foundation (RDM), from Mr. William H. Goodwin and Mrs. Alice Goodwin and the Commonwealth Foundation for Cancer Research (YML), and from the Experimental Therapeutics Center of MSKCC (YML).

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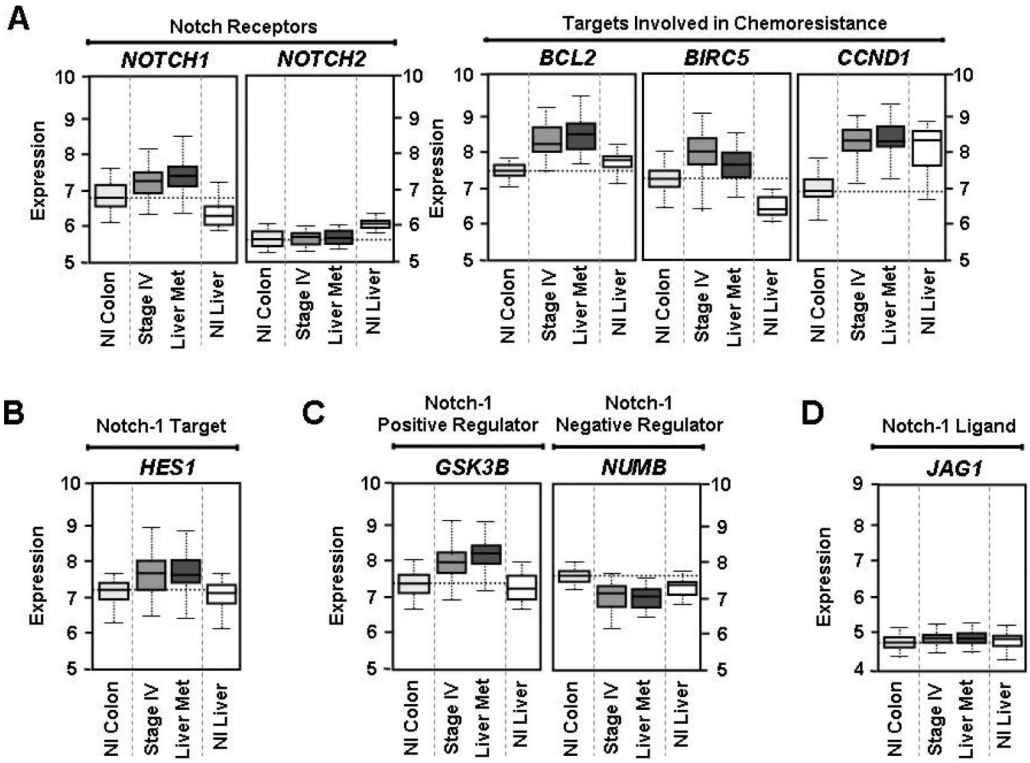

Figure 1. Overexpression of Notch-1 in Colon Cancer Progression

A, In a microarray analysis of genes that are overexpressed during colon cancer progression from primary tumors to metastatic liver lesions, we identified several known chemoresistance genes, including *BCL2* (bcl-2), *BIRC5* (survivin), and *CCND1* (cyclin D1), but also a novel target usually involved in developmental signaling, *NOTCH1*. The expression level of *NOTCH1* is enhanced in metastatic lesions (Stage IV and liver metastases), compared to normal colon mucosa or liver parenchyma. In contrast, a related Notch receptor, *NOTCH2*, is not increased in expression during colon cancer progression.

B, *HES1*, a downstream transcriptional target of Notch-1, is also overexpressed during colon cancer stage progression.

C, A positive regulator of Notch-1, *GSK3B* (GSK-3-beta), which prevents the degradation of the NICD, is enhanced during colon cancer progression, whereas the expression of a negative Notch regulator *NUMB*, which binds the NICD and prevents its translocation into the nucleus, is suppressed in advanced colon cancers.

D, Expression of the Notch-1 ligand *JAG1* is not increased during colon cancer progression.

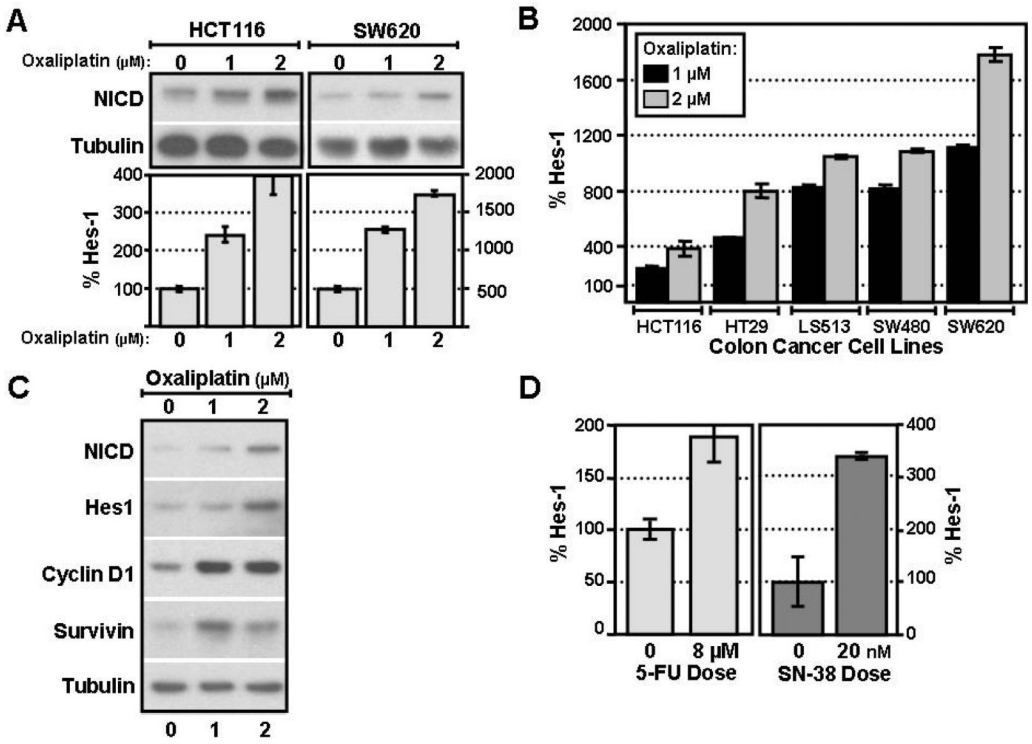

Figure 2. Oxaliplatin Induces Notch-1 Protein and Activity

A, The transcriptionally active Notch-1 intracellular domain (NICD) protein increased with oxaliplatin in a dose-dependent manner in two colon cancer cell lines HCT116 and SW620, with wild-type and mutant p53 respectively (top panel). The two cell lines were treated with increasing doses of oxaliplatin for 48 hours, and total protein was immunoblotted for NICD. Equal protein loading was confirmed by probing for α-tubulin. Next, using a luciferase reporter containing the promoter of the Notch-1 target *HES-1*. we report that oxaliplatin also induces Hes-1 transcriptional activity in a dose-dependent manner (bottom panel) in both colon cancer cell lines. Cells were first transfected with the Hes-1 luciferase construct and then total protein was harvested. All values were normalized for transfection efficiency and are expressed as a percentage of the untreated controls ± SEM

B, Hes-1 luciferase activity was assayed in a panel of colon cancer cell lines following treatment with oxaliplatin (1 or 2 μM) for 48 hours. A statistically significant dose-dependent increase in Hes-1 luciferase activity was observed in each cell line, although the degree of induction varied.

C, In HCT116 cells, the induction of NICD protein by oxaliplatin also increases the expression of downstream targets of Notch-1, including Hes1, cyclin D1, and survivin. Equal protein loading was demonstrated by probing for α-tubulin protein expression.

D, Both the chemotherapeutic antimetabolite fluorouracil (5-FU) and the topoisomerase I inhibitor irinotecan, metabolized to SN-38, increase the transcriptional activity of a Hes-1 luciferase reporter in HCT116 cells in a dose-dependent manner. All values were normalized for transfection efficiency and are expressed as a percentage of the untreated controls ± SEM.

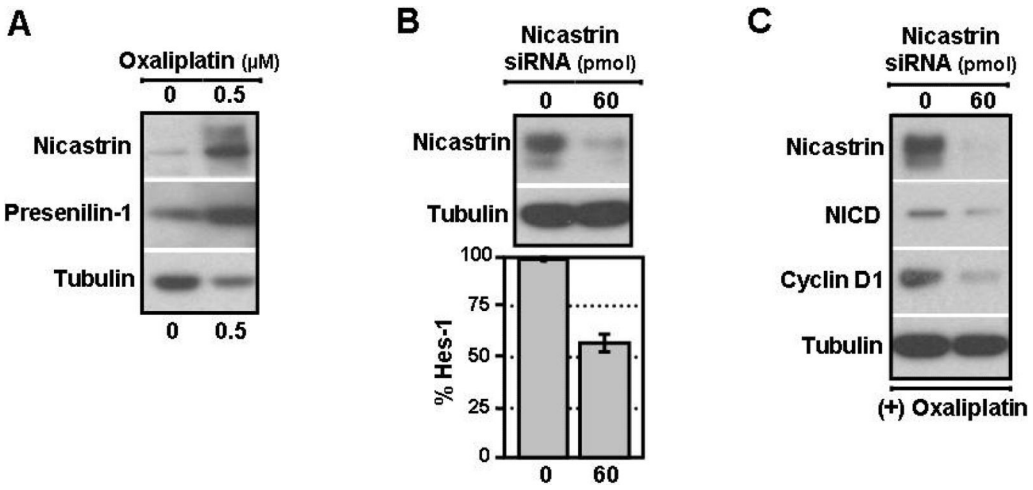

Figure 3. Oxaliplatin Induces Gamma-Secretase Protein Expression

A, In HCT116 colon cancer cells, oxaliplatin treatment (0.5 μM) for 48 hours induces the protein expression of two subunits of the gamma-secretase protein complex, nicastrin and presenilin-1. Equal protein loading was confirmed by probing for α-tubulin expression.

B, siRNA (60 pmol/L) to the nicastrin protein subunit of the gamma-secretase complex suppresses nicastrin protein expression at 48 hours (top panel), which subsequently decreases the activity of a Hes-1 luciferase reporter in HCT116 cells (bottom panel). All values were normalized for transfection efficiency and are expressed as a percentage of the untreated controls ± SEM.

C, HCT116 cells were transfected with siRNA to nicastrin (60 pmol/L) for 24 hours and then treated with oxaliplatin (1 μM) for an additional 24 hours. siRNA to nicastrin not only abrogates the induction of nicastrin protein previously observed with oxaliplatin treatment, but also suppresses the increase in NICD and cyclin D1 protein usually observed after oxaliplatin. Equal protein loading was demonstrated α-tubulin protein expression.

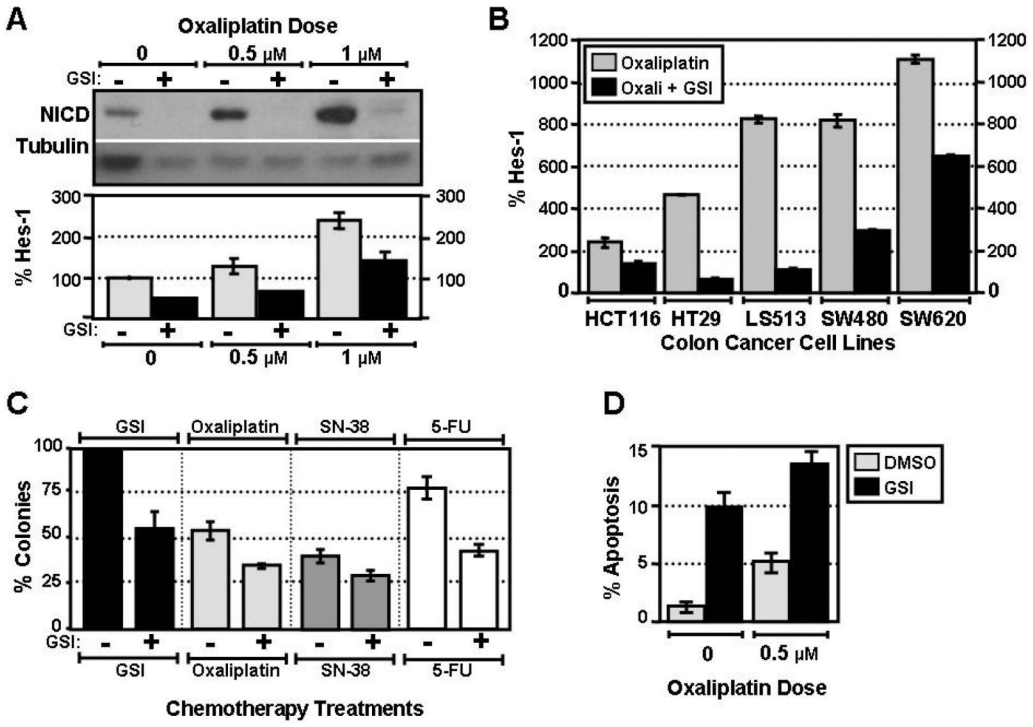

Figure 4. Gamma-Secretase Inhibitors Abrogate Notch-1 Induction by Oxaliplatin

A, HCT116 colon cancer cells were co-treated with increasing doses of oxaliplatin and either the gamma-secretase inhibitor (GSI) GSI34 at 10 μM (indicated by "+" signs) or 0.1% DMSO for 48 hours (indicated by "-" signs). Whereas oxaliplatin induced NICD expression in a dose-dependent manner, co-treatment with GSI34 abrogated this induction at each dose. Correspondingly, GSI34 (bottom panel, black bars) also abrogated the induction of Hes-1 luciferase activity by oxaliplatin at both dose levels (bottom panel, light gray bars).

B, A panel of colon cancer cell lines was treated with oxaliplatin (1 μM) combined with either GSI34 (10 μM) or 0.1% DMSO for 48 hours to assess Hes-1 activity. GSI34 (black bars) abrogated the induction of Hes-1 by oxaliplatin in each colon cancer cell line, although the effect lessened in cell lines with high induction of Hes-1, such as SW620.

C, HCT116 were treated with 0.1% DMSO control, oxaliplatin (1 μM), GSI34 (10 μM), or the combination of both drugs for 48 hours, and colonies were counted after 14 days. Although GSI34 and oxaliplatin both decreased colony formation, the combination of both drugs produced the most significant suppression of viability (Student's t-test, p=0.034). Similarly, the combination of GSI34 with either 5-FU (8 μM) or SN-38 (20 nM) also suppressed colony formation more than either treatment alone. All values are expressed as a percentage of colonies formed in the DMSO controls ± SEM.

D, HCT116 cells were co-treated with oxaliplatin (0.5 μM) and with either GSI34 (10 μM) or 0.1% DMSO for 48 hours and stained with 4'-6-diamidino-2-phenylindole (DAPI). As visualized by confocal immunofluorescence microscopy, the number of cells with fragmented nuclei, suggestive of apoptotic cells, were increased by the combination treatment (p<0.05). Values are expressed as the percentage of cells with nuclear fragmentation ± SEM.

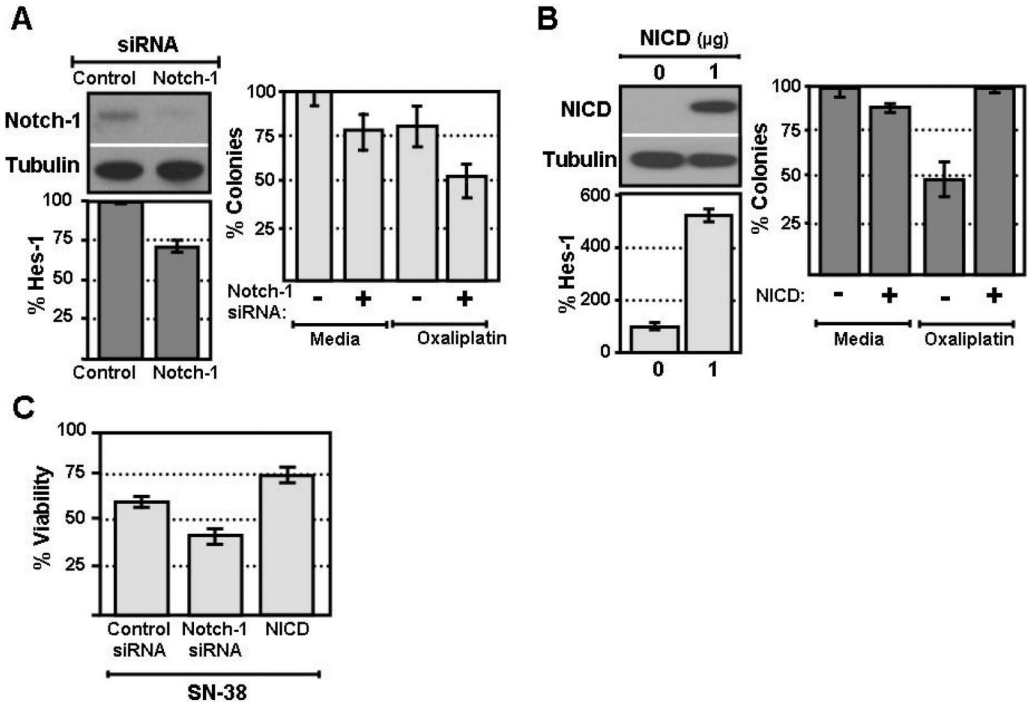

Figure 5. Notch-1 Expression Affects Chemosensitivity

A, HCT116 cells were transfected with siRNA (60 pmol/L) to Notch-1 or to control random sequences for 48 hours. Notch-1 siRNA decreases both the protein expression of Notch-1 (top panel) and the activity of a Hes-1 luciferase reporter (bottom panel). HCT116 cells were then transfected with siRNA to Notch-1 or to control sequences (as indicated) for 12 hours and then treated with either oxaliplatin (0.5 μM) or media for an additional 48 hours. The combination of Notch-1 siRNA and oxaliplatin treatment decreased colony formation more than either treatment alone (Student's t-test, p=0.011).

B, HCT116 cells were transfected with pCS2-*NICD* (1 μg) for 24 hours, and NICD expression increases in a dose-dependent manner (top panel). NICD overexpression also enhances Hes-1 luciferase activity after 48 hours (bottom panel). HCT116 cells were then transfected with pCS2-*NICD* (2 μg) or pCS2 empty vector for 12 hours and were treated with oxaliplatin (0.5 μM) or media alone for 48 hours. Whereas vector-transfected cells showed a decrease in colony formation, expression of NICD protected the cells from oxaliplatin (p<0.05).

C, HCT116 cells were transfected with siRNA to Notch-1 or to control sequence (both at 60 pmol/L), and were then treated with either SN-38 (0.2 nM) for 48 more hours. The combination of Notch-1 siRNA and SN-38 decreased viability significantly, compared to either treatment alone (p=0.022) as measured by the sulforhodamine B assay. In contrast, overexpression of NICD following transfection of pCS2-*NICD* (1 μg) in HCT116 cells increased chemoresistance to SN-38 (0.2 nM).

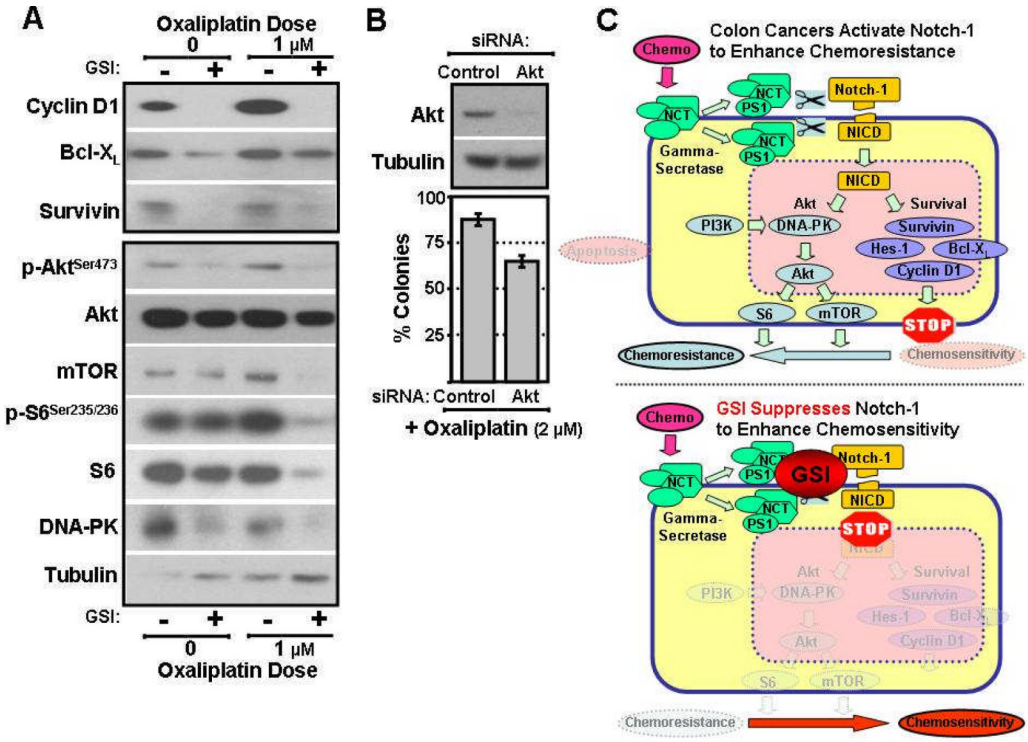

Figure 6. Gamma-secretase inhibitors decrease the induction of pro-survival factors, including Akt signaling, by oxaliplatin

A, HCT116 cells were treated with both oxaliplatin and either GSI34 (10 μM) or 0.1% DMSO for 48 hours before total protein lysates were probed with antibodies, as indicated. GSI34 decreased the expression of Bcl-X_L and survivin following oxaliplatin and blunted the increases in cyclin D1 after oxaliplatin (top panels). Co-treatment with GSI34 also decreased Phospho-Akt-Ser^{473}, mTOR, and Phospho-S6$^{Ser235/Ser236}$ proteins following oxaliplatlin (bottom panels). Total S6 and DNA-PK protein expression were also decreased by co-treatment with GSI34 but not by oxaliplatin. Equal protein loading was demonstrated by probing for tubulin expression.

B, HCT116 cells were transfected with siRNA targeting Akt (50 nM) for 48 hours, and total Akt protein levels were decreased (top panel). The percentage of colonies formed was also decreased by siRNA to Akt following oxaliplatin treatment (bottom panel), compared to cells transfected with control siRNA ($p<0.05$).

C, Following treatment with chemotherapy (oxaliplatin, 5-FU, or SN-38), colon cancer cells activate the Notch pathway by upregulating the gamma-secretase protein complex through induction of the nicastrin (NCT) and presenilin-1 (PS1) subunits to cleave more NICD (top panel). The NICD proteins then activate targets involved in chemoresistance, such as PI3K/Akt, Bcl-X_L, survivin, and cyclin D1. In contrast, downregulation of Notch signaling chemosensitizes cells as pro-survival targets are no longer activated (bottom panel). The Notch pathway can be suppressed if the colon cancer cells are co-treated with GSIs, which prevent the formation of the cleaved NICD by blocking the gamma-secretase protein complex. Therefore, the colon cancer cell is more chemosensitive after inhibition of Notch signaling.