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

Raymond D. Meng,[1] Christopher C. Shelton,[2] Yue-Ming Li,[2] Li-Xuan Qin,[3] Daniel Notterman,[5] Philip B. Paty,[4] and Gary K. Schwartz[1]

[1]Laboratory of New Drug Development, Division of Solid Tumor Oncology, Department of Medicine, [2]Molecular Pharmacology and Chemistry Program, [3]Department of Epidemiology and Biostatistics, and [4]Colorectal Service, Department of Surgery, Memorial Sloan-Kettering Cancer Center, New York, New York, and [5]Department of Molecular Biology, Princeton University, Princeton, New Jersey

Abstract

Because Notch signaling is implicated in colon cancer tumorigenesis and protects cells from apoptosis by inducing prosurvival targets, it was hypothesized that inhibition of Notch signaling with γ-secretase inhibitors (GSI) may enhance the chemosensitivity of colon cancer cells. We first show that the Notch-1 receptor, as well as its downstream target Hes-1, is up-regulated with colon cancer progression, similar to other genes involved in chemoresistance. We then report that chemotherapy induces Notch-1, as oxaliplatin, 5-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 by oxaliplatin was caused by an increase in the activity and expression of γ-secretase complex, as suppression of the protein subunit 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. Down-regulation of Notch signaling also prevented the induction of prosurvival pathways, most notably phosphoinositide kinase-3/Akt, after oxaliplatin. In summary, colon cancer cells may up-regulate 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. [Cancer Res 2009;69(2):573–82]

Introduction

Although significant advances have occurred in the treatment of metastatic colorectal cancers with the introduction of novel chemotherapies and targeted agents, the overall survival rate remains low, as metastatic cancers eventually develop resistance to standard treatments through the activation of prosurvival tumor pathways. Given their role in cellular proliferation, many of these prosurvival pathways actually play important roles during development, including the Notch pathway, which is increasingly being studied as a novel mechanism for tumorigenesis (reviewed by refs. 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 prosurvival 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 (5–7), 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) or Delta-like-3 (DLL3) bind to four related transmembrane receptors, Notch-1 through Notch-4. This binding activates the γ-secretase protein complex, composed of four subunits: presenilin (PS1), nicastrin (NCT), anterior pharynx-defective (APH-1), and presenilin enhancer-2 (PEN-2). γ-Secretase then 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, 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 coactivator. Notch signaling then induces the expression of multiple targets involved in cellular proliferation, such as cyclin D1 (10) and c-Myc (11), and in cellular survival, as discussed. Overexpression of the Notch receptors and/or their 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 γ-secretase protein complex, which cleaves the Notch receptor to activate the pathway (reviewed by ref. 2). It was first shown that inhibition of the

Downloaded from http://aacrjournals.org/cancerres/article-pdf/69/2/573/2614585/573.pdf by guest on 23 May 2025

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Requests for reprints: Gary K. Schwartz, Laboratory for New Drug Development, Division of Solid Tumor Oncology, Department of Medicine, Memorial Sloan-Kettering Cancer Center, New York, NY 10065. Phone: 212-639-8324; Fax: 212-717-3320; E-mail: schwartg@mskcc.org.

©2009 American Association for Cancer Research.

doi:10.1158/0008-5472.CAN-08-2088

γ-secretase complex suppresses the growth of T cell ALL lines, which leads to apoptosis (19). γ-Secretase inhibitors (GSI) 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. Down-regulation 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 down-regulation of prosurvival pathways. Therefore, we propose that inhibition of Notch-1 signaling may be a novel strategy to increase the sensitization of colon cancer cells to chemotherapy.

Materials and Methods

Microarray data analysis. Gene chip microarrays were performed on 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). Data preprocessing and statistical analysis were carried out in R[6] and Bioconductor.[7] 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 $\log_2$ 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 versus primary polyp versus primary colon cancer, primary tumor stage I versus stage II versus stage III versus stage IV, and primary tumor versus liver metastasis.

Cell lines. The human colon adenocarcinoma cell lines HCT116, SW620, SW480, HT29, and LS513 (American Type Culture Collection, ATCC) 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 mmol/L 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 analogue, 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) and 5-FU (Pharmacia) were obtained from the Memorial Sloan-Kettering Cancer Center Research Pharmacy. SN-38, the active metabolite of irinotecan, was generously provided by Dr. J. Patrick McGovren (formerly at Pharmacia and Upjohn). Drugs were used at concentrations equal to or less than the IC_{50} specific to each cell line.

Constructs and small interfering RNA. The pGL2-*Hes-1*-luciferase and pCS2-*NICD* constructs were generously provided by Dr. Raffi Kopan (Washington University). The pGL2 and *Renilla* vectors were obtained from Promega. At least two different small interfering RNA (siRNA) sequences to each of the following genes were used: Notch-1 (Notch-1h and Notch-1h2) and JAG-1 (JAG-1h and JAG-1h2) from Santa Cruz Biotechnology, nicastrin from Santa Cruz and Sigma Chemical Company, and Akt1/Akt2 from Cell Signaling and Sigma. Four commercially available siRNA to random noncoding sequences (control siRNA-A, siRNA-B, siRNA-C, and siRNA-D) were used for control transfections (Santa Cruz).

Protein immunoblot assays. Cell lines were treated with oxaliplatin (0.5 or 1 μmol/L), SN-38 (2–20 nmol/L), or 5-FU (1–10 μmol/L), combined with either GSI34 (1–10 μmol/L) or 0.1% DMSO (as a control) for 24 to 48 h. Total protein lysates were prepared. For isolation of nuclear and cytoplasmic fractions, the Pierce NE-PER extraction kit was used. Proteins were probed with the following primary antibodies: Notch-1, cyclin D1, and Hes-1 (all from Santa Cruz); NICD, phosphorylated Akt^{Ser473}, Akt, DNA-dependent protein kinase (DNA-PK), mammalian target of rapamycin (mTOR), phosphorylated $S6^{Ser235/Ser236}$ ribosomal protein, total S6 ribosomal protein, nicastrin, cyclin D1, and presenilin (all from Cell Signaling); and survivin and Bcl-X_L (Pharmingen-BD Biosciences). The antibody to Notch-1 (Santa Cruz) is directed to an epitope at the COOH terminus, so it detects both the full-length and the cleaved cytoplasmic portions of Notch-1. The antibody to NICD (Cell Signaling) specifically probes for the cytosolic domain of Notch-1 when cleaved between Gly1743 and Val1744. Equal protein loading was confirmed by probing for α/β-tubulin expression (Cell Signaling). Appropriate secondary antibodies conjugated to horseradish peroxidase were used, including antimouse or antirabbit IgG (GE-Healthcare), and proteins were visualized with Amersham enhanced chemiluminescence (GE-Healthcare). Films were digitized with a Microtek scanner, and images were processed with Photoshop software (Adobe).

Hes-1 luciferase assays. Cell lines were cotransfected with pGL2-*Hes-1* luciferase reporter (1 μg/well) and the *Renilla* reporter pRL-*CMV* (0.1 μg/well) using Fugene (Roche). After 8 to 12 h, cells were treated with oxaliplatin (0.5 or 1 μmol/L), GSI34 (10 μmol/L), or both drugs for 48 h. Cells were also treated with SN-38 (2 or 20 nmol/L), 5-FU (1 or 8 μmol/L), GSI34 (10 μmol/L) alone, or the combination of GSI34 with SN-38 or 5-FU for 48 h. After 6, 12, 24, or 48 h 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). Luciferase values were standardized by *Renilla* pRL-*CMV* cotransfection.

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

Apoptosis assays. Apoptosis was assessed by quantitative confocal fluorescence microscopy. Briefly, after drug treatment, cells were fixed in 4% paraformaldehyde and stained with 4'-6-diamidino-2-phenylindole (DAPI; Sigma). Cells with fragmented nuclei under confocal fluorescence microscopy (magnification, 40×) 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 (0–40 μmol/L), oxaliplatin (0–2 μmol/L), or both drugs for 24 to 72 h. Viability was assessed with the sulforhodamine B (SRB) assay. Briefly, after drug treatment, cells were stained with 0.4% SRB (Sigma), fixed with 10% trichloroacetic acid, washed with 1% acetic acid, and solubilized in 10 mmol/L Tris buffer, and absorbance was measured at 490 nmol/L on a spectrophotometer (SpectraMax, Molecular Devices).

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 nmol/L using Oligofectamine (Invitrogen). 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 h. For Hes-1 luciferase assays, cells were first transfected with the Hes-1 luciferase reporter for 12 h and were then transfected with siRNA (Notch-1, Akt, or control) for 48 h. For colony formation assays, cells were transfected with siRNA for 12 h and then oxaliplatin (0.5–2 μmol/L), SN-38 (0.2–20 nmol/L), or the medium was added for 48 h. Colonies were stained after 14 d. Serial transfections were done to limit any potential cytotoxicity and to standardize transfection procedures in all experiments.

[6] http://www.r-project.org
[7] http://www.bioconductor.org

Downloaded from http://aacrjournals.org/cancerres/article-pdf/69/2/573/2614585/573.pdf by guest on 23 May 2025

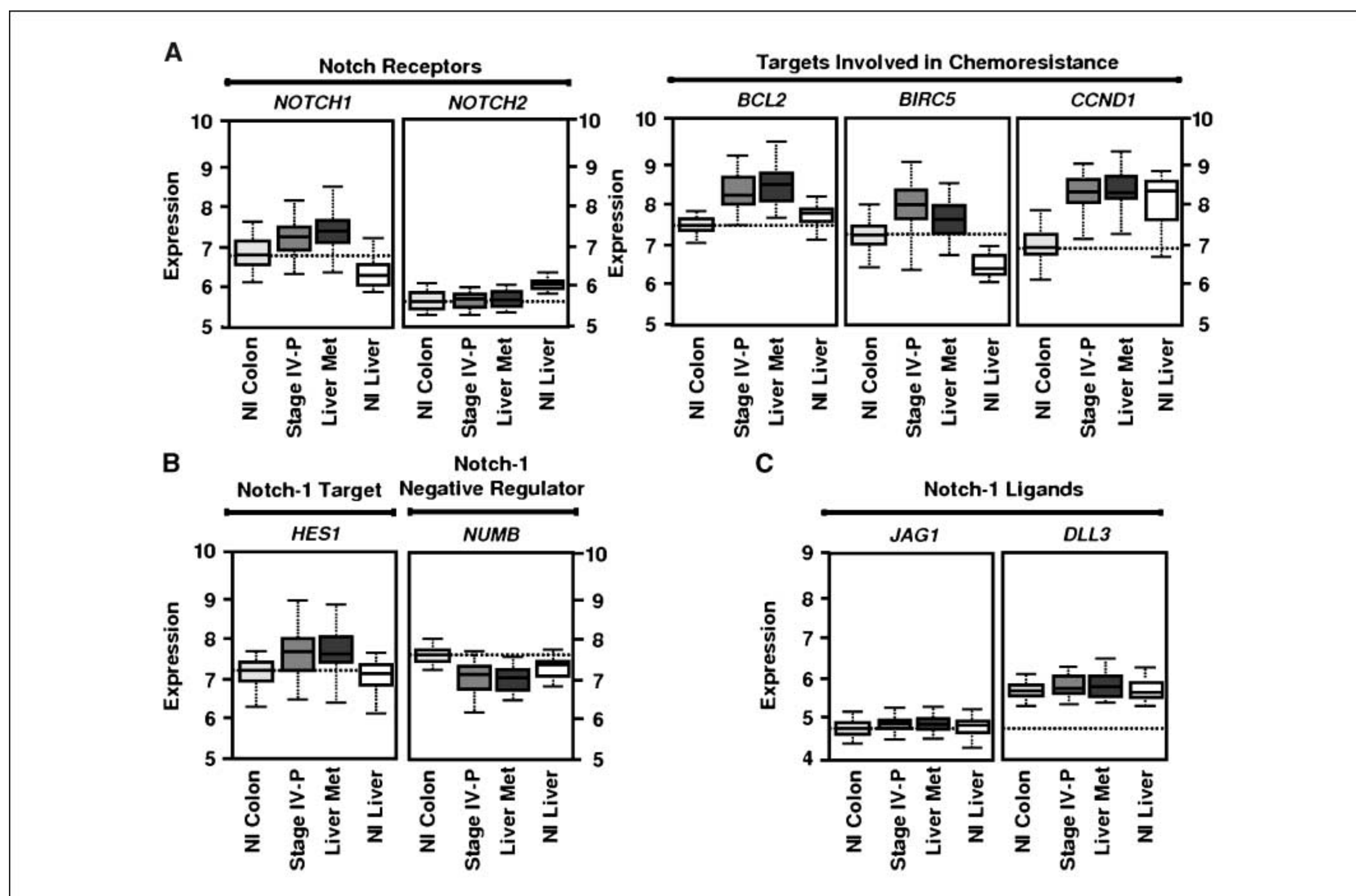

Figure 1. Overexpression of Notch-1 in colon cancer progression. *A,* in a microarray analysis of genes that are overexpressed during colon cancer progression from primary tumors to metastatic liver lesions, we identified several known chemoresistance genes, including *BCL2* (bcl-2), *BIRC5* (survivin), 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 primary and liver metastases) compared with normal colon mucosa or liver parenchyma. In contrast, a related Notch receptor, *NOTCH2*, is not increased in expression during colon cancer progression. Data are shown as box plots of mean relative gene expression ± SD. *B, HES1*, a downstream transcriptional target of Notch-1, is also overexpressed during colon cancer stage 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. *C,* expression of the Notch-1 ligands *JAG1* (*left*) and *DLL3* (*right*) is not increased during colon cancer progression.

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

γ-Secretase assays. The activity of the γ-secretase protein was measured using a modified *in vitro* assay, as previously published (25). In brief, cell lines were treated with oxaliplatin or transfected with control siRNA (random sequences) or siRNA targeting niastrin, as described above. Cell membranes were then solubilized with CHAPSO detergent (Sigma) to generate the catalytically active γ-secretase complex. Enzyme activity was then measured by incubating the solubilized protein complex with a recombinant substrate of γ-secretase, and target cleavage was detected by chemiluminescence with antibodies against the protein fragments.

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 performed from 308 normal and malignant colon tissues obtained at surgical resection, including normal colon mucosa (10%), colonic polyps (15%), primary colon cancers (55%), or liver metastases (13%), using Affymetrix U133A arrays. Correlation analysis identified genes differentially expressed between sample classes among a list of 44 genes related to the Notch pathway (Supplementary Fig. S1). The expression of *NOTCH1* increased from normal colon mucosa to stage IV metastatic cancers with levels being highest in liver metastases compared with normal colonic mucosa or liver parenchyma (trend test, $P < 0.001$; Fig. 1*A*). In contrast, *NOTCH2* did not similarly increase with disease progression, disproving the null hypothesis that there is a difference in *NOTCH2* expression 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*). *BIRC5* is not significantly associated with colon cancer disease progression, whereas *CCND1* tends to be up-regulated as disease progresses ($P < 0.001$). In addition, the expression of the downstream target *HES1*, a target gene of

Downloaded from http://aacrjournals.org/cancerres/article-pdf/69/2/573/2614585/573.pdf by guest on 23 May 2025

Notch-1, increased with colon cancer progression (Fig. 1*B, left*), and there is moderate correlation with *NOTCH1* (correlation coefficient = 0.29, $P < 0.001$). In contrast, *NUMB*, a negative-regulator of Notch-1, is down-regulated in advanced colon cancers ($P < 0.001$; Fig. 1*B, right*). Finally, the genes for the Notch ligands *JAG1* (Fig. 1*C, left*) and *DLL3* (Fig. 1*C, right*) are not increased with colon cancer progression.

Oxaliplatin induces NICD 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, first row*), whereas it slightly decreased the expression of full-length Notch-1 protein (Fig. 2*A, top, middle row*). 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 *HES1* 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 (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 with mutant p53, LS513 with wild-type p53, and SW480 with mutant p53 (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 5-fluorouracil (5-FU), a pyrimidine analogue, and SN-38, the active metabolite of the topoisomerase I inhibitor irinotecan, increased Hes-1 activity (Fig. 2*D*).

Oxaliplatin induces γ-secretase protein complex activity and 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 γ-secretase complex; therefore, we hypothesized that chemotherapy induced NICD protein by increasing γ-secretase activity. First, we measured the activity of the enzyme complex after oxaliplatin treatment using an *in vitro* assay that uses a fluorescent-tagged substrate of γ-secretase. In two colon cancer cell lines, HCT116 (Fig. 3*A, left*) and HT29 (Fig. 3*A, right*), oxaliplatin treatment

Downloaded from http://aacrjournals.org/cancerres/article-pdf/69/2/573/2614585/573.pdf by guest on 23 May 2025

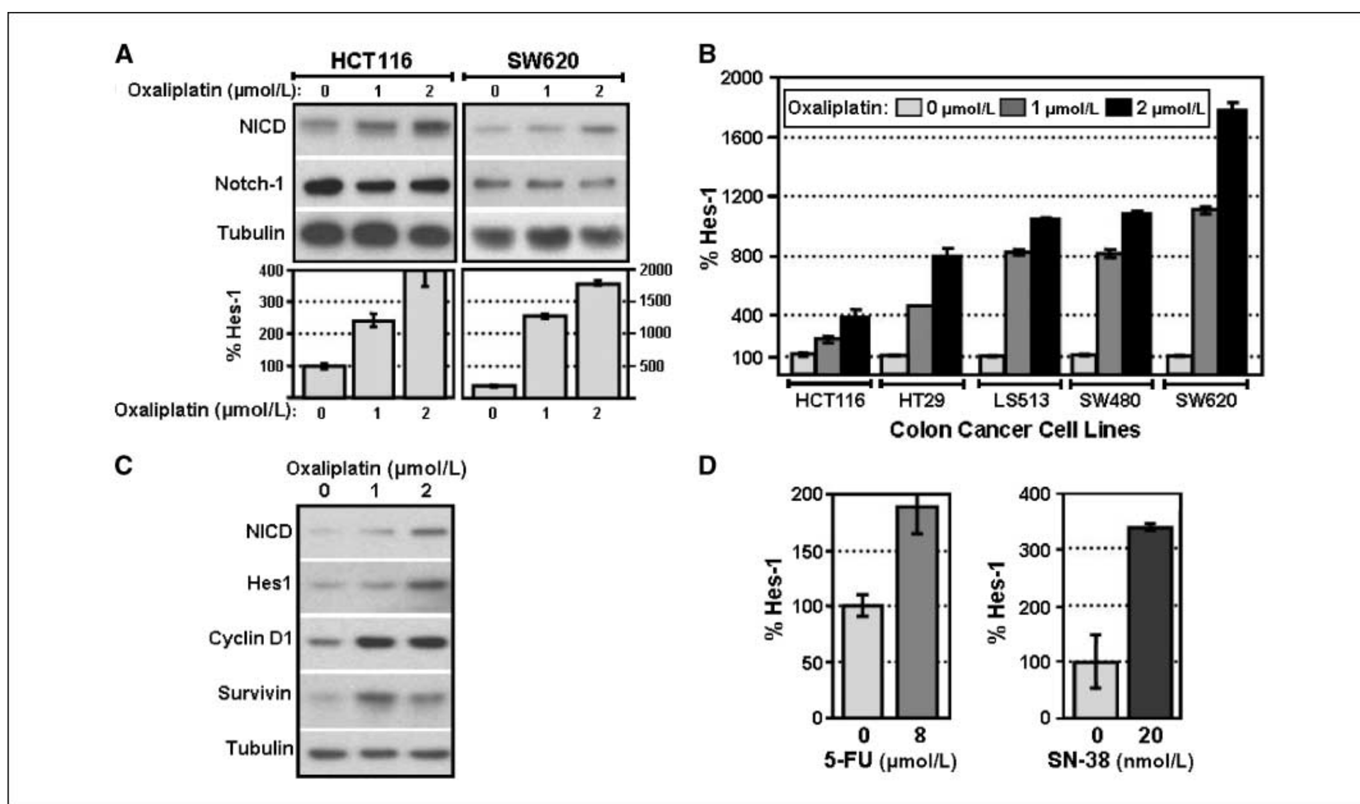

Figure 2. Oxaliplatin induces NICD protein and activity. *A,* the transcriptionally active NICD protein, but not full-length Notch-1 protein, increases with oxaliplatin in a dose-dependent manner in two colon cancer cell lines HCT116 and SW620 (*top*). The two cell lines were treated with oxaliplatin for 48 h, and total protein was immunoblotted for NICD or full-length Notch-1. Equal protein loading was confirmed by probing for tubulin. Next, using a luciferase reporter containing the promoter of the Notch-1 target *HES-1*, we report that oxaliplatin induces Hes-1 transcriptional activity in both colon cancer cell lines (*bottom*). All values were normalized for transfection efficiency. *Columns,* percentage of the untreated controls; *bars,* SE. *B*, Hes-1 luciferase activity was assayed in a panel of colon cancer cell lines after treatment with oxaliplatin (1 or 2 μmol/L) for 48 h. A statistically significant dose-dependent increase in Hes-1 luciferase activity was observed in each cell line. All values are standardized to the vehicle-treated group for each cell line. *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 shown by probing for α-tubulin protein expression. *D,* both the 5-FU (*left*) and SN-38, the active metabolite of irinotecan (*right* panel), increase the transcriptional activity of a Hes-1 luciferase reporter in HCT116 cells. All values were normalized for transfection efficiency. *Columns,* percentage of the untreated controls; *bars,* SE.

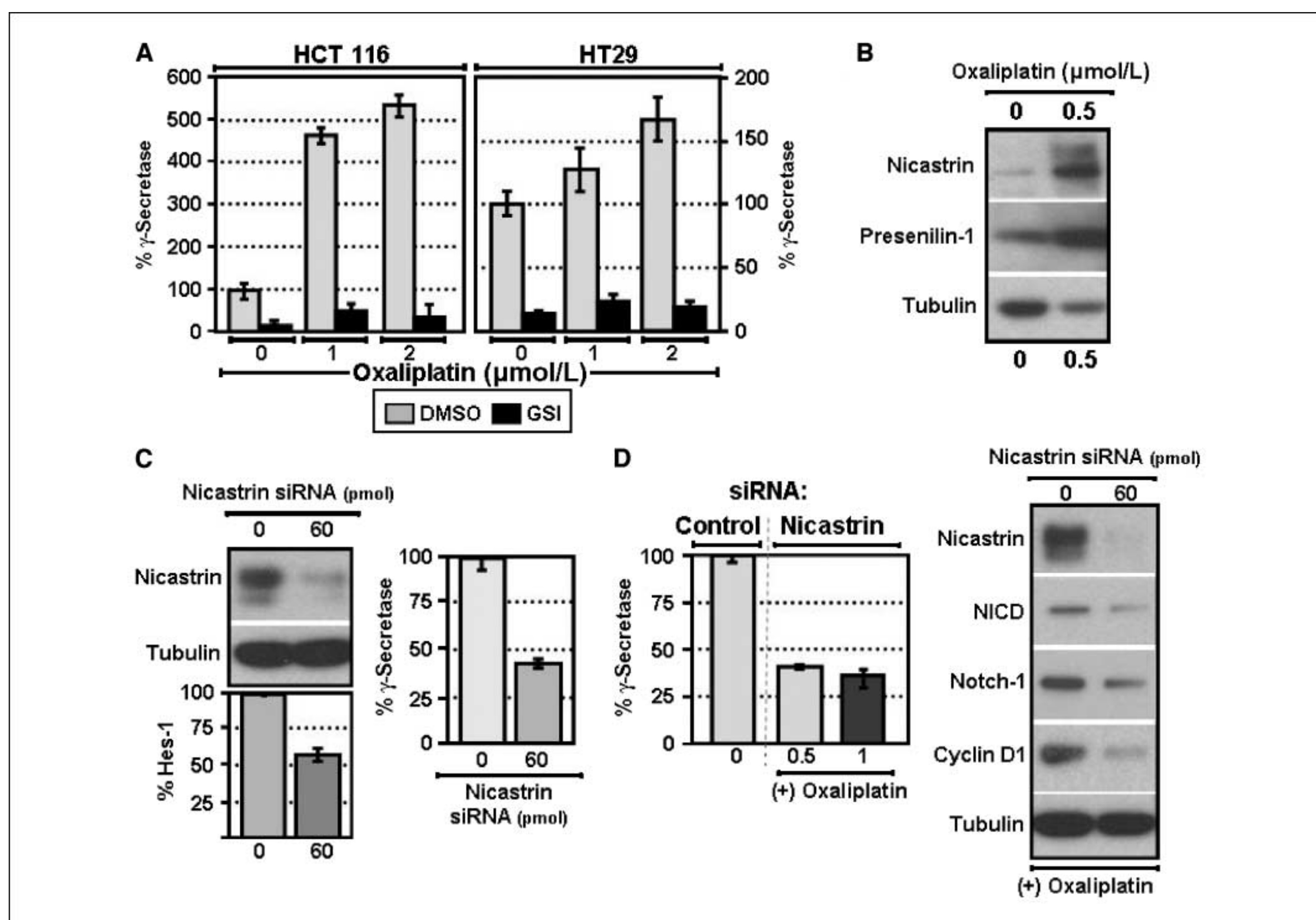

Figure 3. Oxaliplatin induces γ-secretase protein expression. *A,* oxaliplatin treatment (0.5 μmol/L) for 48 h (*gray columns*) of HCT116 cells (*left*) or HT29 cells (*right*) induces the activity of the γ-secretase protein complex, as measured with an *in vitro* substrate assay. Cotreatment with GSI34 (*black columns*) abrogates this induction of γ-secretase activity. Values are normalized to each cell line treated with media only. *Columns,* percentage of the untreated controls; *bars,* SE. *B,* in HCT116 colon cancer cells, oxaliplatin treatment (0.5 μmol/L) for 48 h induces the protein expression of two subunits of the γ-secretase protein complex, nicastrin and presenilin-1. Equal protein loading was confirmed by probing for tubulin expression. *C,* siRNA (60 pmol/L) to the nicastrin subunit of the γ-secretase complex suppresses nicastrin protein expression at 48 h (*top left*), which decreases the activity of a Hes-1 luciferase reporter in HCT116 cells (*bottom left*) and decreases activity of the γ-secretase complex (*right*). All values were normalized for transfection efficiency. *Columns,* percentage of the untreated controls; *bars,* SE. *D,* HCT116 cells were transfected with siRNA to nicastrin (60 pmol/L) for 24 h and then treated with oxaliplatin (0.5 or 1 μmol/L) for an additional 24 h. siRNA to nicastrin prevents the induction of γ-secretase activity by oxaliplatin (*left*) and abrogates the induction of nicastrin protein by oxaliplatin (*right*). Nicastrin siRNA also suppresses the increase in NICD and cyclin D1 protein expression by oxaliplatin (*right*). Total Notch-1 protein is also mildly decreased (*right*). Equal protein loading was shown by probing for tubulin.

increased γ-secretase activity in a dose-dependent manner compared with media-treated cells. This induction of γ-secretase could be abrogated by cotreatment with GSI34, a γ-secretase inhibitor, at each dose of oxaliplatin (Fig. 3*A, black columns*). We then examined if this induction in activity of the γ-secretase enzyme complex was caused by an increase in the expression of its components. Treatment of HCT116 cells with oxaliplatin induced the expression of two subunits of γ-secretase, PS1 and NCT (Fig. 3*B*). Because of the role that NCT may play in assembling the complex, we studied the effects of inhibiting NCT induction by using siRNA to target NCT. Transfection of HCT116 cells with NCT siRNA inhibited the protein levels of NCT (Fig. 3*C, top left*), which decreased the activity of the enzyme as measured with the *in vitro* assay (Fig. 3*C, bottom left*). This inhibition of γ-secretase activity effectively decreased Notch signaling, as measured by Hes-1 activity (Fig. 3*C, right*). In HCT116 cells, suppression of nicastrin protein with siRNA prevented both the induction of γ-secretase enzyme activity (Fig. 3*D, left*) and the induction of NICD and cyclin D1 protein previously observed after oxaliplatin (Fig. 3*D, right*). Full-length Notch-1 protein is also mildly decreased (Fig. 3*D, left*).

GSIs 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 cotreated with a GSI. Our group used a novel sulfonamide analogue 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. Again, oxaliplatin, in the absence of GSI (marked "−"), increases the levels of NICD protein (Fig. 4*A, top, first row*) but decreases the expression of full-length Notch-1 (*second row*). At each dose level, the addition of GSI34 (marked "+") abrogated the induction of NICD protein by oxaliplatin and results in the accumulation of full-length Notch-1 (Fig. 4*A, top*). It was then examined if GSI34 could inhibit the activation of the Notch-1 pathway by measuring Hes-1

Downloaded from http://aacrjournals.org/cancerres/article-pdf/69/2/573/2614585/573.pdf by guest on 23 May 2025

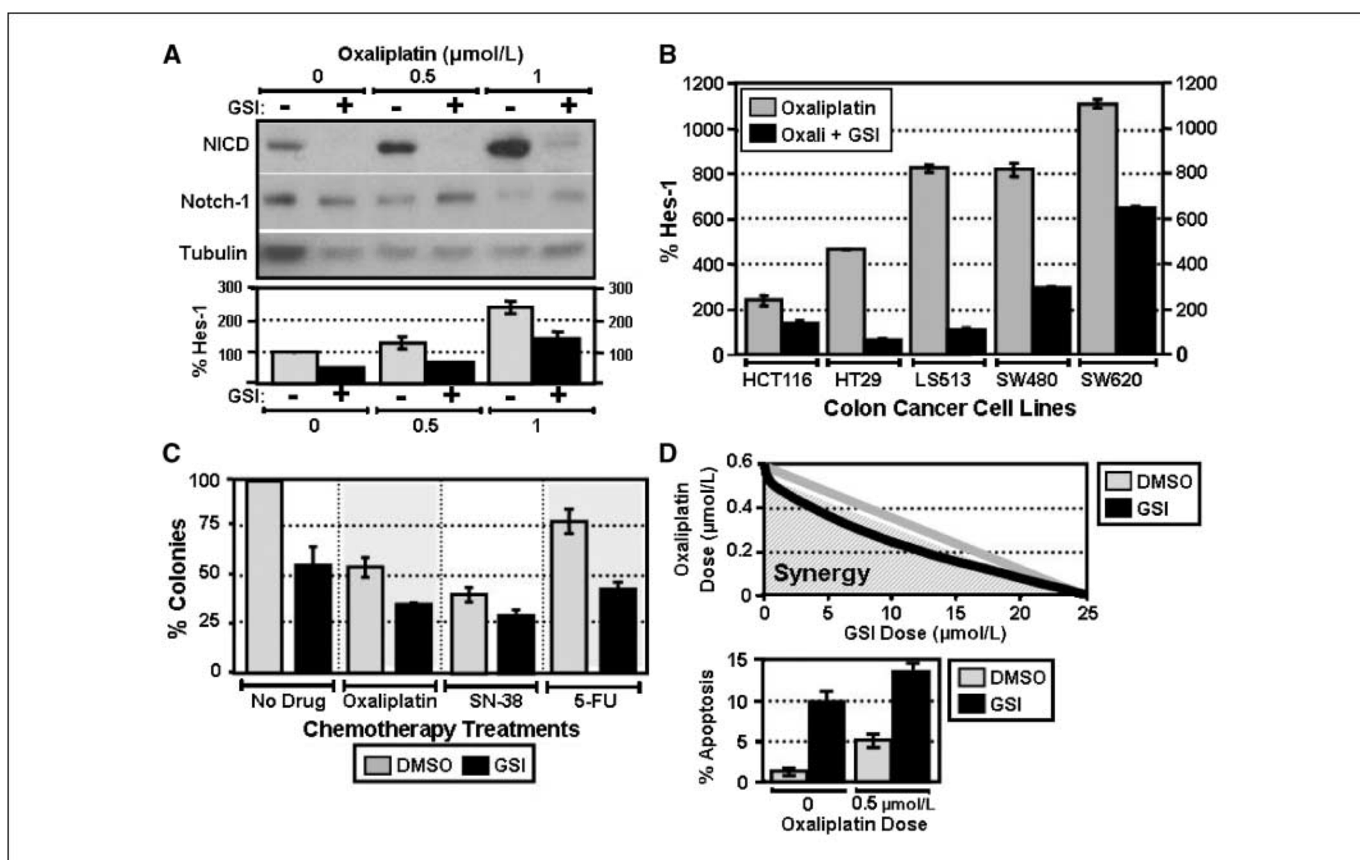

Figure 4. GSIs lessen Notch-1 induction by oxaliplatin. *A,* HCT116 cells were cotreated with oxaliplatin and GSI34 at 10 µmol/L (+) or DMSO for 48 h (−). Whereas oxaliplatin induced NICD expression, cotreatment with GSI34 lessened this induction (*top*). In contrast, the expression of full-length Notch-1 was not affected by oxaliplatin, whereas cotreatment with GSI34 increased levels (*bottom*). Finally, GSI34 (*bottom, black columns*) lessened the induction of Hes-1 activity by oxaliplatin (*gray columns*). *B,* a panel of colon cancer cells was treated with oxaliplatin (1 µmol/L) combined with either GSI34 (10 µmol/L) or 0.1% DMSO for 48 h to assess Hes-1 activity. GSI34 (*black columns*) abrogated the induction of Hes-1 by oxaliplatin in each colon cancer cell line. *C,* HCT116 were treated with 0.1% DMSO, oxaliplatin (1 µmol/L), GSI34 (10 µmol/L), or the combination of both drugs for 48 h, and colonies were counted after 14 d. Although GSI34 or oxaliplatin decreased colony formation, the combination of both drugs most significantly suppressed viability (Student's *t* test, $P = 0.034$). The combination of GSI34 with 5-FU (8 µmol/L) or SN-38 (20 nmol/L) also suppressed colonies more than either treatment alone. *Columns,* percentage of colonies in the DMSO group; *bars,* SE. *D*, to create an isobologram, HCT116 cells were treated with increasing ratios of oxaliplatin (up to 0.6 µmol/L) and GSI34 (up to 25 µmol/L) for 48 h. The percentage of colonies determined the IC_{50} values for each ratio, which were plotted (*black line*) and compared with the additive effect (*gray line*), to suggest a synergistic interaction. To measure apoptosis, HCT116 cells were cotreated with oxaliplatin (0.5 µmol/L) and either GSI34 at 10 µmol/L (*black columns*) or 0.1% DMSO (*gray columns*) for 48 h and stained with DAPI. The number of apoptotic cells was increased by the combination treatment ($P < 0.05$). *Columns,* percentage of cells with nuclear fragmentation; *bars,* SE.

activity. Cotreatment with GSI34 abrogated the induction of Hes-1 by oxaliplatin at 0.5 µmol/L but did not completely suppress the induction at the higher dose of 1 µmol/L (Fig. 4*A, bottom*). GSI34 also decreased Notch-1 induction by oxaliplatin in four other colon cancer cells: HT29, LS513, SW480, and SW620 (Fig. 4*B*). Finally, cotreatment of HCT116 cells with GSI34 suppressed the induction of Hes-1 by other chemotherapies, including 5-FU alone (Supplementary Fig. S2*A*) or 5-FU combined with oxaliplatin (Supplementary Fig. S2*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 (Supplementary Fig. 2C). GSI cotreatment also enhanced the chemosensitivity of HCT116 to SN-38 and 5-FU ($P < 0.05$; Fig. 4*C*). Using isobologram experiments, we confirmed that the combination of GSI34 and oxaliplatin synergistically decreased the IC_{50} in HCT116 cells (Fig. 4*D, top*). It was then examined if colon cancer cells cotreated with oxaliplatin and GSI34 were undergoing enhanced apoptosis. Cotreatment of HCT116 cells with GSI34 and oxaliplatin significantly increased the percentage of apoptotic cells compared with either treatment alone, as determined by DAPI staining for nuclear fragmentation ($P < 0.05$, Fig. 4*D, bottom*).

Notch-1 expression affects chemosensitivity. Because the γ-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. First, 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 full-length Notch-1 protein, which down-regulated expression of NICD and a downstream

Downloaded from http://aacrjournals.org/cancerres/article-pdf/69/2/573/2614585/573.pdf by guest on 23 May 2025

Notch-1 target cyclin D1 (Fig. 5*A, top left*). siRNA to Notch-1 also decreased the baseline transcriptional activity of Hes-1 (P = 0.014; Fig. 5*A, bottom left*) compared with 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 significantly decreased viability compared with either Notch-1 siRNA alone or chemotherapy alone (P = 0.011; Fig. 5*A, right*). We then examined if the Notch ligand JAG-1 is required for chemosensitivity. HCT116 cells transfected with siRNA targeting JAG-1 show no enhanced sensitivity to oxaliplatin compared with cells transfected with control siRNA (Fig. 5*B*). Inhibition of the γ-secretase complex with siRNA targeting nicastrin, however, did increase the sensitivity of HCT116 cells to oxaliplatin (Fig. 5*B*).

Because inhibition of Notch-1 could enhance chemosensitivity, we then examined if overexpression of NICD could instead increase colon cancer chemoresistance. HCT116 cells were transfected with an NICD construct for 24 hours, which significantly increased NICD protein but did not affect full-length Notch-1 protein (Fig. 5*C, top left*). NICD transfection increased expression of the Notch-1 target cyclin D1 (Fig. 5*C, top left*) and activated Hes-1 (Fig. 5*C, bottom left*). After transfection with NICD or vector alone 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 with cells transfected with vector alone (Fig. 5*C, 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*C, 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 after 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*D*).

GSIs decrease the induction of prosurvival 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 cotreatment

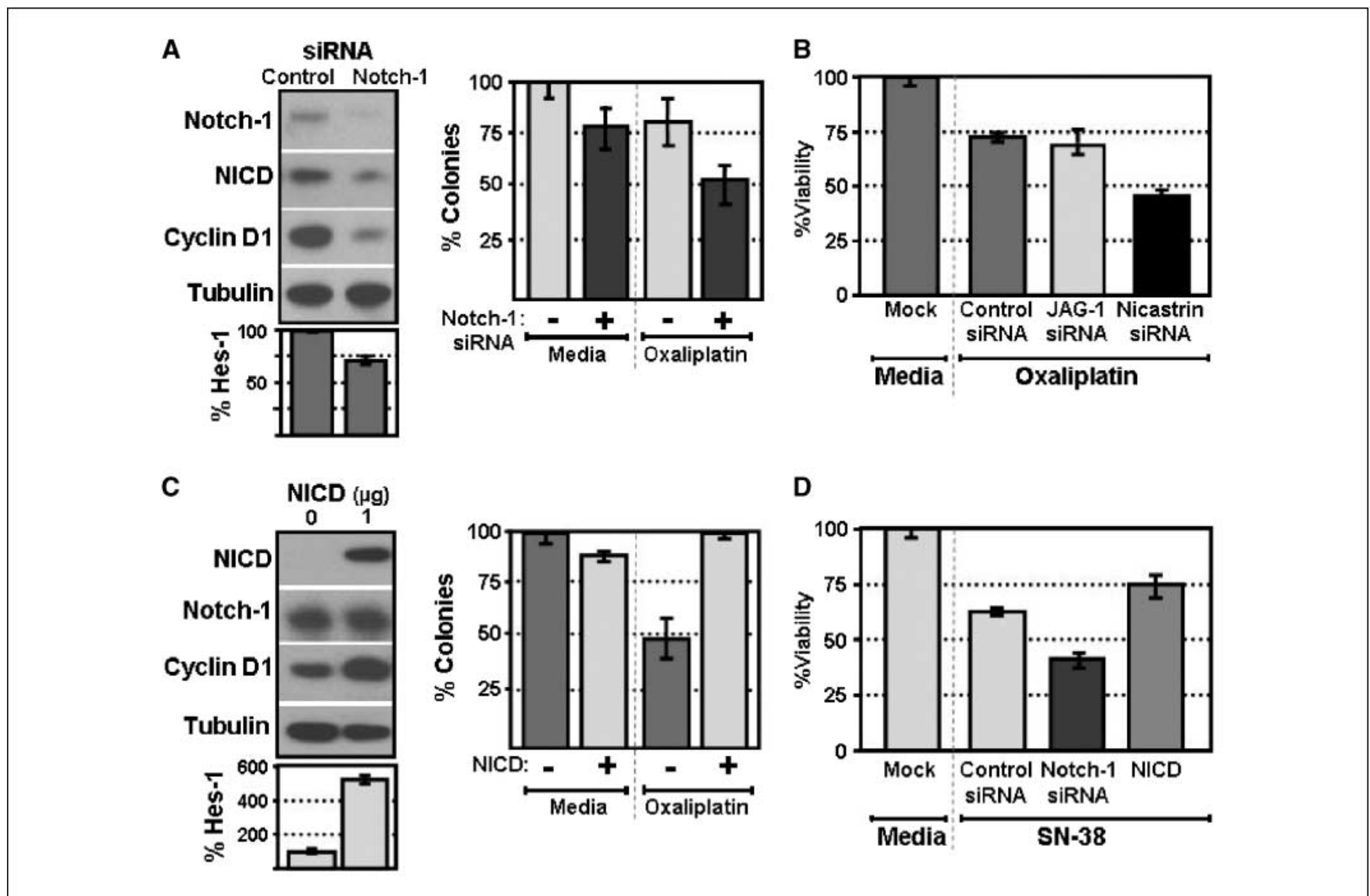

Figure 5. Notch-1 expression affects chemosensitivity. *A,* HCT116 cells were transfected with siRNA (60 pmol/L) to Notch-1 or to control sequences for 48 h. Notch-1 siRNA decreased Notch-1 expression, resulting in decreased NICD and cyclin D1 protein (*top left*). NICD transfection also decreased the activity of a Hes-1 luciferase reporter (*bottom left*). HCT116 cells were then transfected with siRNA to Notch-1 or control for 12 h before treatment with oxaliplatin (0.5 μmol/L) or media for another 48 h (*right*). The combination of Notch-1 siRNA and oxaliplatin decreased colony formation more than either treatment alone (P = 0.011). *B,* HCT116 cells were transfected with siRNA (60 pmol/L) to JAG-1, nicastrin, or control sequences for 12 h and then treated with oxaliplatin (0.5 μmol/L) for an additional 48 h. Whereas JAG-1 siRNA did not affect the chemosensitivity of HCT116 cells to oxaliplatin, nicastrin siRNA further sensitized the cells compared with control siRNA ($P < 0.05$). *C,* HCT116 cells were transfected with pCS2-*NICD* (1 μg) for 24 h, and NICD expression increased but not full-length Notch-1 protein (*top left*). NICD transfection also increased cyclin D1 (*top*) and enhanced Hes-1 luciferase activity after 48 h (*bottom*). HCT116 cells were then transfected with pCS2-*NICD* (2 μg) for 12 h and were treated with oxaliplatin (0.5 μmol/L) for 48 h. Expression of NICD protected the cells from oxaliplatin ($P < 0.05$). *D,* HCT116 cells were transfected with Notch-1 or control siRNA (60 pmol/L) and were treated with SN-38 (0.2 nmol/L) for 48 h. The combination of Notch-1 siRNA and SN-38 decreased viability significantly compared with either treatment alone (P = 0.022). In contrast, overexpression of NICD in HCT116 cells increased chemoresistance to SN-38.

Downloaded from http://aacrjournals.org/cancerres/article-pdf/69/2/573/2614585/573.pdf by guest on 23 May 2025

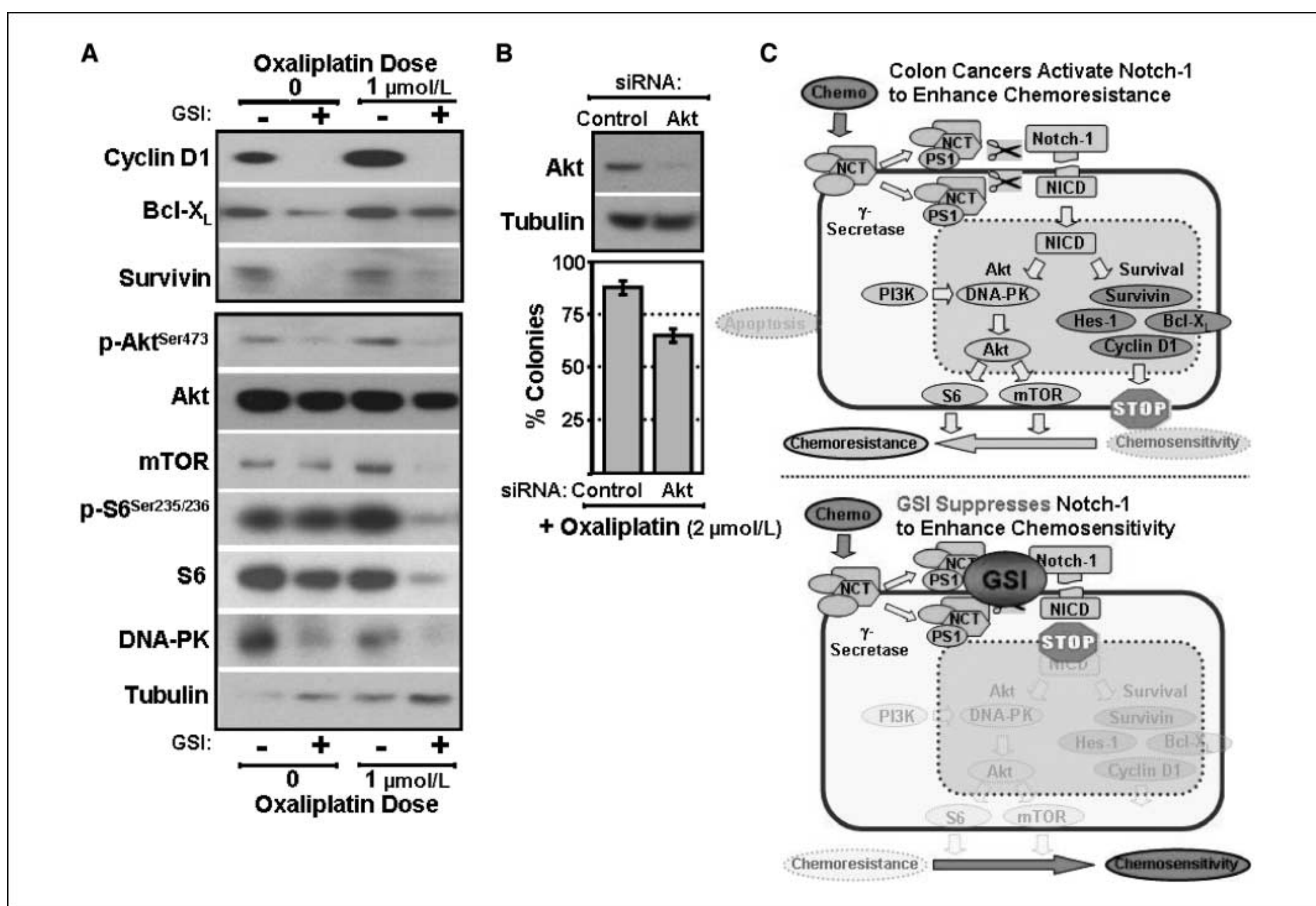

Figure 6. GSIs decrease the induction of prosurvival factors, including Akt signaling, by oxaliplatin. *A,* HCT116 cells were treated with both oxaliplatin and GSI34 (10 µmol/L) or DMSO for 48 h before total protein lysates were probed with antibodies, as indicated. GSI34 decreased the expression of Bcl-XL and survivin after oxaliplatin and blunted the increase in cyclin D1 (*top*). GSI34 also decreased phosphorylated Akt^{Ser473}, mTOR, and phosphorylated S6$^{Ser235/Ser236}$ proteins after oxaliplatin (*bottom*). Total S6 and DNA-PK proteins were also decreased by cotreatment with GSI34 but not by oxaliplatin. *B,* HCT116 cells were transfected with siRNA to Akt (50 nmol/L) for 48 h, decreasing total Akt protein (*top*). The percentage of colonies formed was also decreased by siRNA to Akt after oxaliplatin (*bottom*) compared with cells transfected with control siRNA ($P < 0.05$). *C,* after treatment with chemotherapy (oxaliplatin, 5-FU, or SN-38), colon cancer cells activate the Notch pathway by up-regulating the γ-secretase protein complex through induction of NCT and PS1 subunits to cleave more NICD (*top*). NICD proteins then activate targets involved in chemoresistance, such as PI3K/Akt, Bcl-XL, survivin, and cyclin D1. In contrast, down-regulation of Notch signaling chemosensitizes cells, as prosurvival targets are no longer activated (*bottom*). The Notch pathway can be suppressed if the colon cancer cells are cotreated with GSIs, which prevent the formation of the cleaved NICD by blocking the γ-secretase protein complex. Therefore, the colon cancer cell is more chemosensitive after inhibition of Notch signaling.

could decrease several prosurvival 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, cotreatment with GSI34 decreased the phosphorylation of Akt at Ser^{473} 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 Ser$^{235/236}$, and total S6 protein levels were decreased after cotreatment with GSI34 and oxaliplatin (Fig. 6*A, bottom*). We then examined the expression of a kinase that may activate Akt, DNA-PK, as cotreatment 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 after 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 prosurvival 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 down-regulation 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 seems 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).

Downloaded from http://aacrjournals.org/cancerres/article-pdf/69/2/573/2614585/573.pdf by guest on 23 May 2025

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 by DNA damage has been previously shown in neuroblastoma cells after treatment with the histone deacetylase inhibitor valproic acid (27). Our data indicate 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 ref. 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 γ-secretase (31) and that DNA damage from chemotherapy can increase the activity of γ-secretase protein (32). Our results indicate that the induction of NICD by chemotherapy may be due to an augmentation of γ-secretase activity from an increase in the expression of select components (PS-1 and NCT) of the γ-secretase complex. This is consistent with previous studies showing that overexpression of PEN-2 can increase γ-secretase activity (33). The observation that NCT is induced by chemotherapy is not entirely unexpected. NCT is an essential component of the γ-secretase complex, as it has been reported in mouse knockout models that γ-secretase activity is dependent on NCT (34). As oxaliplatin increases NCT in the γ-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 vascular endothelial growth factor (31).

If activation of Notch-1 signaling contributes to chemoresistance, then inhibition of the Notch pathway could sensitize cells to chemotherapy. Our results indicate that the viability of colon cancer cells was synergistically decreased by GSI34 in combination with multiple forms of chemotherapy, including oxaliplatin, SN-38, and 5-FU. This effect was associated with inhibition of NICD production and suppression of Hes-1 activity. Use of GSIs may then present a 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 (36). Therefore, it remains to be determined whether this effect of GSI on chemotherapy can be extended to all tumor subtypes.

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 UV irradiation (37). In fact, in lung cancer cells, the induction of Notch-1 sensitizes the cells to GSIs (38). Our studies indicate that, in colon cancer cells, the selective suppression of Notch-1 with siRNA enhances the effects of both oxaliplatin and SN-38. Similarly, overexpression of NICD protected against these two agents. This is consistent with a previous 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 seem multifactorial. First, GSI34 suppressed the expression of three prosurvival 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, as Notch-1 signaling has been implicated in AKT activation. For example, Notch-1 can activate Akt in T-cells (6), as well as in melanoma (42), leukemia (43), and cervical cancers (44). In addition, a recent microarray study identified overexpression of Akt and Notch signaling as hallmarks of gliomas with an eventual poor prognosis (45). NICD overexpression results in the phosphorylation of Akt at Ser^{473} (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 Ser^{473}. 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, cotransfection 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 GSIs (46). Interestingly, all of the colon cancer cell lines used in this study seem to have wild-type PTEN status, which would agree with the hypothesis from that study. Finally, there may be alternative mechanisms of chemosensitization by GSIs. For example, in Kaposi's sarcoma cell lines, it has been reported that treatment with GSIs 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 γ-secretase complex by increasing the expression of its subunits, including PS-1 and NCT. The increased protein subunits are then formed into multiple γ-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 prosurvival targets, such as Bcl-X_L, cyclin D1, and survivin. Consequently, suppression of Notch-1 signaling by blocking NICD production with GSIs down-regulates transcriptional targets involved in cellular survival, shifting the cell toward 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 trials (48).

Downloaded from http://aacrjournals.org/cancerres/article-pdf/69/2/573/2614585/573.pdf by guest on 23 May 2025

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

Received 6/3/2008; revised 10/17/2008; accepted 11/8/2008.

Grant support: Littlefield Foundation and American Association for Cancer Research (R.D. Meng and G.K. Schwartz); American Society of Clinical Oncology Foundation (R.D. Meng); William H. Goodwin, Alice Goodwin, and Commonwealth Foundation for Cancer Research (Y-M. Li); and Experimental Therapeutics Center of Memorial Sloan-Kettering Cancer Center (Y-M. Li).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

References

1. Roy M, Pear WS, Aster JC. The multifaceted role of Notch in cancer. Curr Opin Genet Dev 2007;17:52–9.
2. Shih IeM, Wang TL. Notch signaling, γ-secretase inhibitors, and cancer therapy. Cancer Res 2007;67:1879–82.
3. Katoh M, Katoh M. Notch signaling in gastrointestinal tract (review). Int J Oncol 2007;30:247–51.
4. Mungamuri SK, Yang X, Thor AD, Somasundaram K. Survival signaling by Notch1: mammalian target of rapamycin (mTOR)-dependent inhibition of p53. Cancer Res 2006;66:4715–24.
5. Nair P, Somasundaram K, Krishna S. Activated Notch1 inhibits p53-induced apoptosis and sustains transformation by human papillomavirus type 16 E6 and E7 oncogenes through a PI3K-PKB/Akt-dependent pathway. J Virol 2003;77:7106–12.
6. Rangarajan A, Talora C, Okuyama R, et al. Notch signaling is a direct determinant of keratinocyte growth arrest and entry into differentiation. EMBO J 2001;20:3427–36.
7. Sade H, Krishna S, Sarin A. The anti-apoptotic effect of Notch-1 requires p56lck-dependent, Akt/PKB-mediated signaling in T cells. J Biol Chem 2004;279:2937–44.
8. Wang Z, Banerjee S, Li Y, Rahman KM, Zhang Y, Sarkar FH. Down-regulation of notch-1 inhibits invasion by inactivation of nuclear factor-κb, vascular endothelial growth factor, and matrix metalloproteinase-9 in pancreatic cancer cells. Cancer Res 2006;66:2778–84.
9. Stylianou S, Clarke RB, Brennan K. Aberrant activation of notch signaling in human breast cancer. Cancer Res 2006;66:1517–25.
10. Ronchini C, Capobianco AJ. Induction of cyclin D1 transcription and CDK2 activity by Notch(ic): implication for cell cycle disruption in transformation by Notch(ic). Mol Cell Biol 2001;21:5925–34.
11. Weng AP, Millholland JM, Yashiro-Ohtani Y, et al. c-Myc is an important direct target of Notch1 in T-cell acute lymphoblastic leukemia/lymphoma. Genes Dev 2006;20:2096–109.
12. Reedijk M, Odorcic S, Chang L, et al. High-level coexpression of JAG1 and NOTCH1 is observed in human breast cancer and is associated with poor overall survival. Cancer Res 2005;65:8530–7.
13. Hopfer O, Zwahlen D, Fey MF, Aebi S. The Notch pathway in ovarian carcinomas and adenomas. Br J Cancer 2005;93:709–18.
14. Santagata S, Demichelis F, Riva A, et al. JAGGED1 expression is associated with prostate cancer metastasis and recurrence. Cancer Res 2004;64:6854–7.
15. Fan X, Mikolaenko I, Elhassan I, et al. Notch1 and notch2 have opposite effects on embryonal brain tumor growth. Cancer Res 2004;64:7787–93.
16. Curry CL, Reed LL, Golde TE, Miele L, Nickoloff BJ, Foreman KE. γ secretase inhibitor blocks Notch activation and induces apoptosis in Kaposi's sarcoma tumor cells. Oncogene 2005;24:6333–44.
17. Li Y, Rao PK, Wen R, et al. Notch and Schwann cell transformation. Oncogene 2004;23:1146–52.
18. Farnie G, Clarke RB, Spence K, et al. Novel cell culture technique for primary ductal carcinoma *in situ*: role of Notch and epidermal growth factor receptor signaling pathways. J Natl Cancer Inst 2007;99:616–27.
19. Weng AP, Nam Y, Wolfe MS, et al. Growth suppression of pre-T acute lymphoblastic leukemia cells by inhibition of notch signaling. Mol Cell Biol 2003;23:655–64.
20. Akiyoshi T, Nakamura M, Yanai K, et al. γ-Secretase inhibitors enhance taxane-induced mitotic arrest and apoptosis in colon cancer cells. Gastroenterology 2008;134:131–44.
21. Nefedova Y, Cheng P, Alsina M, Dalton WS, Gabrilovich DI. Involvement of Notch-1 signaling in bone marrow stroma-mediated *de novo* drug resistance of myeloma and other malignant lymphoid cell lines. Blood 2004;103:3503–10.
22. Irizarry RA, Hobbs B, Collin F, et al. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. Biostatistics 2003;4:249–64.
23. Smyth GK. Linear models and empirical bayes methods for assessing differential expression in microarray experiments. Stat Appl Genet Mol Biol 2004;3:Article 3. Epub 2004 Feb 12.
24. Lewis SJ, Smith AL, Neduvelil JG, et al. A novel series of potent γ-secretase inhibitors based on a benzobicyclo[4.2.1]nonane core. Bioorg Med Chem Lett 2005;15:373–8.
25. Li YM, Lai MT, Xu M, et al. Presenilin 1 is linked with γ-secretase activity in the detergent solubilized state. Proc Natl Acad Sci U S A 2000;97:6138–43.
26. Veenendaal LM, Kranenburg O, Smakman N, Klomp A, Borel Rinkes IH, van Diest PJ. Differential Notch and TGFβ signaling in primary colorectal tumors and their corresponding metastases. Cell Oncol 2008;30:1–11.
27. Stockhausen MT, Sjölund J, Manetopoulos C, Axelson H. Effects of the histone deacetylase inhibitor valproic acid on Notch signalling in human neuroblastoma cells. Br J Cancer 2005;92:751–9.
28. LeFort K, Mandinova A, Ostano P, et al. Notch1 is a p53 target gene involved in human keratinocyte tumor suppression through negative regulation of ROCK1/2 and MRCKα kinases. Genes Dev 2007;21:562–77.
29. Colaluca IN, Tosoni D, Nuciforo P, et al. NUMB controls p53 tumour suppressor activity. Nature 2008;451:76–80.
30. Alimirah F, Panchanathan R, Davis FJ, Chen J, Choubey D. Restoration of p53 expression in human cancer cell lines upregulates the expression of Notch1: implications for cancer cell fate determination after genotoxic stress. Neoplasia 2007;9:427–34.
31. Takeshita K, Satoh M, Ii M, et al. Critical role of endothelial Notch1 signaling in postnatal angiogenesis. Circ Res 2007;100:70–8.
32. Jin SM, Cho HJ, Jung MW, Mook-Jung I. DNA damage-inducing agent-elicited γ-secretase activity is dependent on Bax/Bcl-2 pathway but not on caspase cascades. Cell Death Differ 2007;14:189–92.
33. Seo SJ, Hwang DY, Cho JS, et al. PEN-2 overexpression induces γ-secretase protein and its activity with amyloid β-42 production. Neurochem Res 2007;32:1016–23.
34. Li J, Fici GJ, Mao CA, et al. Positive and negative regulation of the γ-secretase activity by nicastrin in a murine model. J Biol Chem 2003;278:33445–9.
35. Yang AD, Fan F, Camp ER, et al. Chronic oxaliplatin resistance induces epithelial-to-mesenchymal transition in colorectal cancer cell lines. Clin Cancer Res 2006;12:4147–53.
36. Greenblatt DY, Vaccaro AM, Jaskula-Sztul R, et al. Valproic acid activates notch-1 signaling and regulates the neuroendocrine phenotype in carcinoid cancer cells. Oncologist 2007;12:942–51.
37. Kim SB, Chae GW, Lee J, et al. Activated Notch1 interacts with p53 to inhibit its phosphorylation and transactivation. Cell Death Differ 2007;14:982–91.
38. Chen Y, De Marco MA, Graziani I, et al. Oxygen concentration determines the biological effects of NOTCH-1 signaling in adenocarcinoma of the lung. Cancer Res 2007;67:7954–9.
39. Hata T, Yamamoto H, Ngan CY, et al. Role of p21waf1/cip1 in effects of oxaliplatin in colorectal cancer cells. Mol Cancer Ther 2005;4:1585–94.
40. Hayward RL, Macpherson JS, Cummings J, Monia BP, Smyth JF, Jodrell DI. Enhanced oxaliplatin-induced apoptosis following antisense Bcl-xl down-regulation is p53 and Bax dependent: genetic evidence for specificity of the antisense effect. Mol Cancer Ther 2004;3:169–78.
41. Troiani T, Lockerbie O, Morrow M, Ciardiello F, Eckhardt SG. Sequence-dependent inhibition of human colon cancer cell growth and of prosurvival pathways by oxaliplatin in combination with ZD6474 (Zactima), an inhibitor of VEGFR and EGFR tyrosine kinases. Mol Cancer Ther 2006;5:1883–94.
42. Liu ZJ, Xiao M, Balint K, et al. Notch1 signaling promotes primary melanoma progression by activating mitogen-activated protein kinase/phosphatidylinositol 3-kinase-Akt pathways and up-regulating N-cadherin expression. Cancer Res 2006;66:4182–90.
43. Chan SM, Weng AP, Tibshirani R, Aster JC, Utz PJ. Notch signals positively regulate activity of the mTOR pathway in T-cell acute lymphoblastic leukemia. Blood 2007;110:278–86.
44. Ramdass B, Maliekal TT, Lakshmi S, et al. Coexpression of Notch1 and NF-κB signaling pathway components in human cervical cancer progression. Gynecol Oncol 2007;104:352–61.
45. Phillips HS, Kharbanda S, Chen R, et al. Molecular subclasses of high-grade glioma predict prognosis, delineate a pattern of disease progression, and resemble stages in neurogenesis. Cancer Cell 2006;9:157–73.
46. Palomero T, Sulis ML, Cortina M, et al. Mutational loss of PTEN induces resistance to NOTCH1 inhibition in T-cell leukemia. Nat Med 2007;13:1203–10.
47. Curry CL, Reed LL, Broude E, Golde TE, Miele L, Foreman KE. Notch inhibition in Kaposi's sarcoma tumor cells leads to mitotic catastrophe through nuclear factor-κB signaling. Mol Cancer Ther 2007;6:1983–92.
48. Deangelo DJ, Stone RM, Silverman LB, et al. A phase I clinical trial of the notch inhibitor MK-0752 in patients with T-cell acute lymphoblastic leukemia/lymphoma (T-ALL) and other leukemias [abstract]. ASCO Annual Meet Proc 2006;24:6585.

Downloaded from http://aacrjournals.org/cancerres/article-pdf/69/2/573/2614585/573.pdf by guest on 23 May 2025