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Research Article

# Synergistic Activity of the Src Family Kinase Inhibitor Dasatinib and Oxaliplatin in Colon Carcinoma Cells Is Mediated by Oxidative Stress

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## Abstract

**Chemotherapeutic regimens for the treatment of colorectal cancer generally include oxaliplatin, although inherent and acquired resistance is common. One potential mediator of oxaliplatin sensitivity is the nonreceptor protein tyrosine kinase, Src, the activity of which correlates with disease stage and patient survival. Therefore, we investigated the effects of Src inhibition using the tyrosine kinase inhibitor dasatinib on oxaliplatin sensitivity. We show that oxaliplatin acutely activates Src and that combination treatment with dasatinib is synergistic in a cell-line dependent manner, with the level of Src activation correlating with extent of synergy in a panel of six cell lines. Intracellular reactive oxygen species (ROS) are generated after oxaliplatin treatment, and ROS potently activates Src. Pretreatment with antioxidants inhibits oxaliplatin-induced Src activation. In oxaliplatin-resistant cell lines, Src activity is constitutively increased. In a mouse model of colorectal liver metastases, treatment with oxaliplatin also results in chronic Src activation. The combination of dasatinib and oxaliplatin results in significantly smaller tumors compared with single-agent treatment, corresponding with reduced proliferation and angiogenesis. Therefore, we conclude that oxaliplatin activates Src through a ROS-dependent mechanism. Src inhibition increases oxaliplatin activity both *in vitro* and *in vivo*. These results suggest that Src inhibitors combined with oxaliplatin may have efficacy in metastatic colon cancer and may provide the first indication of a molecular phenotype that might be susceptible to such combinations.** [Cancer Res 2009;69(9):3842–9]

## Introduction

Metastatic colorectal cancer remains incurable for patients with surgically unresectable disease. The approval of new chemotherapy agents, such as the third-generation platinum analogue, oxaliplatin, has led to improved outcomes for patients with metastatic disease. Nevertheless, these patients inevitably develop refractory disease, with overall survival only ~2 years.

Oxaliplatin is a platinum-based chemotherapeutic agent that forms platinum-DNA adducts that block DNA replication, leading to cell cycle arrest and cell death (1). Platinum-based compounds also induce cytotoxicity through oxidative stress (2–4) and may lead to generation of reactive oxygen species (ROS) both directly and indirectly (5, 6). Resistance to platinum agents occurs through several mechanisms, including decreased platinum influx, improved base excision repair, and/or increased detoxification by glutathione and metallothionein (1).

Reversing resistance has been proven challenging in part due to the inability to pharmacologically modulate these pathways. Recently, however, Src family kinases, for which inhibitors are in trial, have been implicated in drug resistance (7). Src is the prototype of this nine-member family and is activated by numerous growth stimulatory, migratory, and stress pathways (8). Src activity increases in >70% of colon tumors relative to adjacent mucosa, with the highest activity observed in metastases (9, 10), and correlates inversely with patient survival (11). Although Src has been implicated in a myriad of cellular processes that are deregulated in cancer, current evidence suggests that Src activation is critical to mechanisms regulating tumor progression and metastasis [refs. 12–14; reviewed by Summy and Gallick (8)]. As a result, coupled with the recent availability of relatively nontoxic Src family kinase inhibitors, numerous clinical trials have been initiated using small-molecule Src family inhibitors in solid tumors [reviewed by Kopetz and colleagues (7)].

Evidence from preclinical work suggests that Src alters sensitivity to various chemotherapeutics, including platinum-based chemotherapy (15–17). In an ovarian carcinoma cell line, treatment with the Src inhibitor PP2 reversed cisplatin resistance in a multidrug resistance cell line compared with its isogenic control (18). Expression of a dominant-negative, kinase-defective Src mutant resulted in increased sensitivity to oxaliplatin-mediated apoptosis in KM12-L4 human colon tumor cells *in vitro* (15). Predicting in which tumor cells Src inhibition would be a valuable addition to chemotherapeutic regimens using oxaliplatin and better understanding the mechanisms by which this occurs would lead to improved selection of patients that would benefit from Src inhibitors.

In the current study, we investigated the antitumor activity of dasatinib, an orally bioavailable, potent, multitargeted kinase inhibitor of Src (19), in combination with oxaliplatin using *in vitro* and *in vivo* models. We evaluated the effect of chronic exposure to oxaliplatin on Src activity both *in vitro* and *in vivo*. In colon tumors grown in the livers of nude mice, treatment with either agent alone resulted in nonsignificant reductions in tumor size, whereas combination therapy markedly diminished hepatic tumor volume. Using *in vitro* studies, the ability of oxaliplatin to induce both Src activity and ROS correlated with effectiveness of the combination

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

S. Kopetz and D.P. Lesslie contributed equally to this work.

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treatment. We show that Src inhibition in combination with oxaliplatin has efficacy in metastatic colon cancer and provide the first indication of a molecular phenotype that might be susceptible to such combinations.

## Materials and Methods

**Colon cancer cell lines and culture conditions.** HT29, LS174T, SW480, and HCT116 (American Tissue Culture Collection) and KM12-L4 and DiFi (gifts of Dr. I.J. Fidler, The University of Texas M. D. Anderson Cancer Center) cells, all derived from human colon adenocarcinomas, were maintained as a subconfluent monolayer in DMEM/F-12 nutrient mixture and 2 mmol/L glutamine (HT29, LS174T, SW480, and HCT116), MEM with sodium pyruvate, glutamine, and nonessential amino acids (KM12-L4), or complete McCoy's medium (DiFi) supplemented with 10% fetal bovine serum (Hyclone Laboratories) without antibiotics. All cells were incubated in 5% $CO_2$ at 37°C. Cells were routinely screened for *Mycoplasma* and found to be *Mycoplasma*-free. Oxaliplatin-resistant HT29-OxR and KM12-OxR cell lines were established and maintained as described previously (20).

**Cytotoxicity assays.** Oxaliplatin (Sanofi-aventis; purchased from the institutional pharmacy) was freshly prepared in deionized water for each experiment. Dasatinib (provided by Bristol-Myers Squibb), a multitargeted kinase inhibitor of Src family kinases and Abl, was prepared as a 10 mmol/L stock solution in DMSO. Proliferation was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay as described previously (21).

For combination treatments, 5,000 cells were plated overnight followed by treatment with increasing doses of dasatinib and oxaliplatin individually and in combination at a fixed ratio. Dasatinib was added 30 min before oxaliplatin unless stated otherwise. Combination indices were obtained using Calcusyn 2.0 (Biosoft) using methods of Chou and Talalay for formal synergy analyses (22). Synergy was defined based on the terminology of Chou (23).

For clonogenic assays, 200 or 500 cells were plated on 10 cm plates, allowed to adhere for 24 h, and then treated with specified doses of oxaliplatin and/or dasatinib for 48 h. After 14 days, plates were fixed with ethanol and stained with crystal violet (0.5% w/v). Colonies containing >50 cells were manually counted. Comparison of resulting colony counts was done with the two-tailed $t$ test.

**Transfection.** Subconfluent HT29 cells were transfected with two Src-targeted small interfering RNA expression plasmids and vector alone as published previously (24). Single colonies of stable transfectants were isolated and expanded for further analysis.

**Western blotting and immunoprecipitation.** Cells were lysed and clarified and proteins were separated via 8% SDS-PAGE followed by transfer onto polyvinylidene difluoride membranes (Amersham; ref. 24). The membranes were incubated with anti-Src monoclonal antibody (MAb327; Calbiochem-Novabiochem), anti-phospho-Src$^{Y416}$ and anti-β-actin polyclonal antibodies (both from Cell Signaling Technology), or anti-thioredoxin (BD Biosciences) followed by horseradish peroxidase-conjugated secondary antibodies (Bio-Rad). Proteins were visualized by incubation with enhanced chemiluminescence

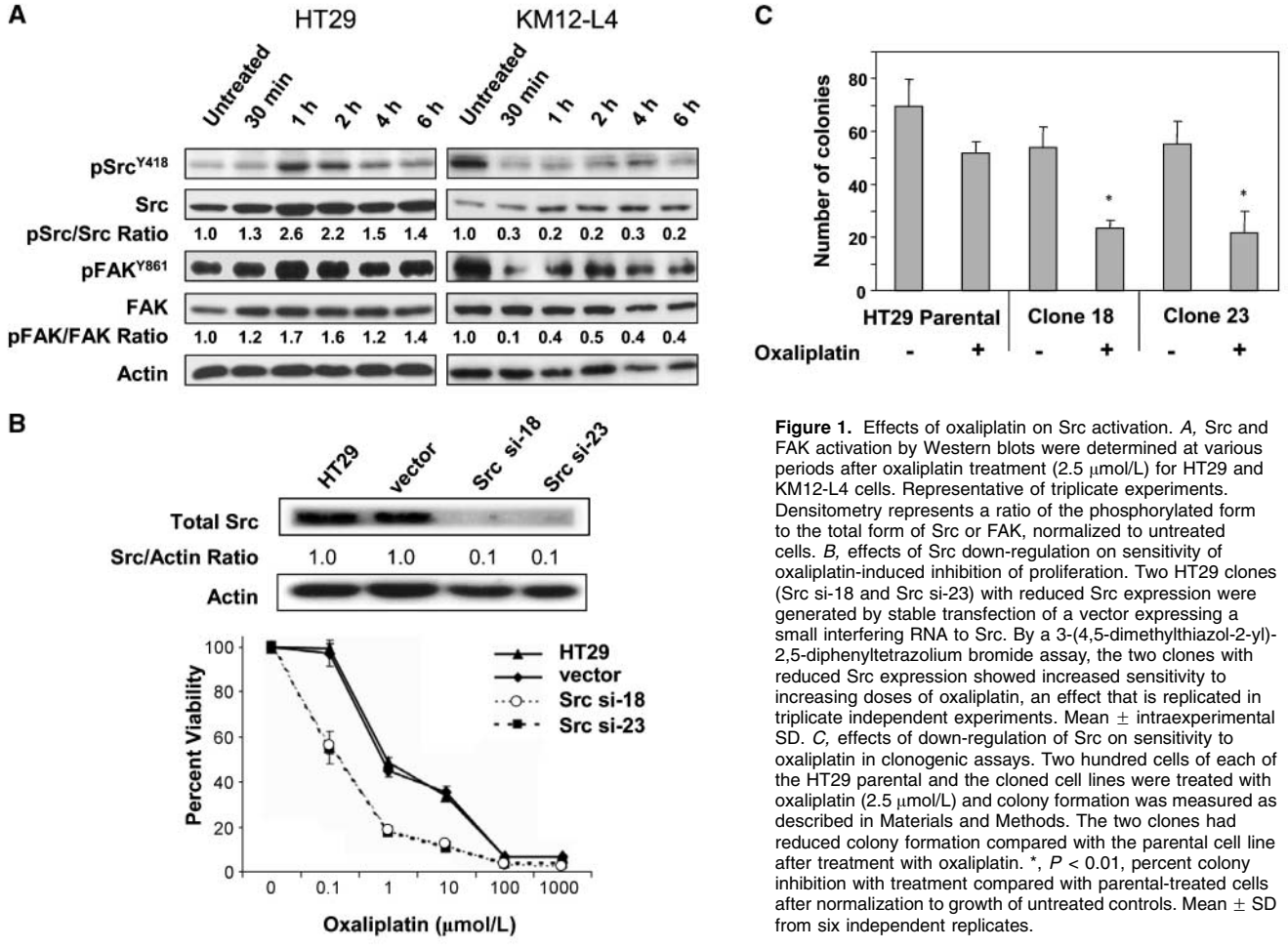


**Figure 1.** Effects of oxaliplatin on Src activation. *A,* Src and FAK activation by Western blots were determined at various periods after oxaliplatin treatment (2.5 μmol/L) for HT29 and KM12-L4 cells. Representative of triplicate experiments. Densitometry represents a ratio of the phosphorylated form to the total form of Src or FAK, normalized to untreated cells. *B,* effects of Src down-regulation on sensitivity of oxaliplatin-induced inhibition of proliferation. Two HT29 clones (Src si-18 and Src si-23) with reduced Src expression were generated by stable transfection of a vector expressing a small interfering RNA to Src. By a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, the two clones with reduced Src expression showed increased sensitivity to increasing doses of oxaliplatin, an effect that is replicated in triplicate independent experiments. Mean ± intraexperimental SD. *C,* effects of down-regulation of Src on sensitivity to oxaliplatin in clonogenic assays. Two hundred cells of each of the HT29 parental and the cloned cell lines were treated with oxaliplatin (2.5 μmol/L) and colony formation was measured as described in Materials and Methods. The two clones had reduced colony formation compared with the parental cell line after treatment with oxaliplatin. *, $P < 0.01$, percent colony inhibition with treatment compared with parental-treated cells after normalization to growth of untreated controls. Mean ± SD from six independent replicates.

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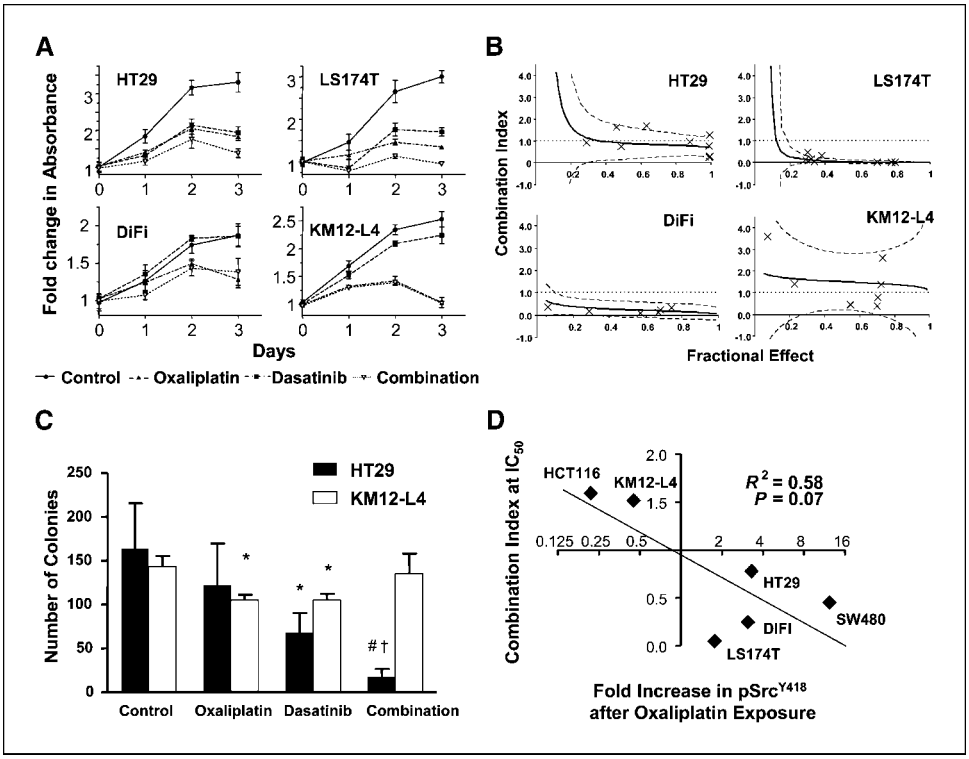


**Figure 2.** Effects of combination treatment with the Src inhibitor dasatinib and oxaliplatin on growth are cell line dependent. *A,* cells were treated with 100 nmol/L (HT29) or 350 nmol/L (LS174T, DiFi, and KM12-L4) dasatinib, 5 μmol/L (DiFi), 13 μmol/L (HT29 and LS174T), or 19 μmol/L (KM12-L4) oxaliplatin, or the combination. Using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, absorbance at 570 nmol/L was measured at baseline and every 24 h for 3 days. Cell growth of HT29 and LS174T was less after combination treatment than after treatment with either agent alone by day 3 ($P < 0.01$ for each comparison). Mean ± SD of representative of three similar experiments, normalized to baseline (day 0) absorbance. *B,* formal synergy analysis by median-effects method shows cell line–dependent response. Cytotoxicity by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide of the combination of dasatinib and oxaliplatin using median-effects method shows varying patterns of interaction for each of the four primary cell lines. Combination indices <1 are increasingly supraadditive, whereas values >1 are increasingly less than additive. A fractional effect of 1 represents complete cytotoxicity for the combination, where 0 is no effect. The combination indices for LS174T and DiFi remain <1 for all fractional effects. Representative of experiments done in triplicate. *Dotted lines,* modeled 95% confidence intervals. *C,* clonogenic assay following combination treatment. Five hundred HT29 and KM12-L4 cells were treated with 2.5 μmol/L oxaliplatin, 100 nmol/L dasatinib, or the combination, and colony formation was measured after 14 days. There were fewer colonies after combination treatment than treatment with either agent alone for HT29 but not KM12-L4. *, $P < 0.05$ versus control; #, $P < 0.001$ versus control; †, $P < 0.01$ versus both single-agent dasatinib and oxaliplatin. Mean ± SD from six independent replicates. *D,* combination indices representing degree of Src activation after oxaliplatin exposure in six colon tumor cell lines. The fold increase in phospho-$Src^{Y418}$ by densitometry of Western blot after 1 h of 2.5 μmol/L oxaliplatin exposure on a log scale is plotted against the combination index of oxaliplatin and dasatinib from the median-effects analysis at the 50% fractional effect ($IC_{50}$ for the combination). Combination indices <1 are increasingly supraadditive, whereas values >1 are increasingly less than additive. Cell lines with Src activation after oxaliplatin exposure have a trend toward increasing additivity of the combination of a Src inhibitor and oxaliplatin. *Diamond,* cell line.

detection reagents (Perkin-Elmer) and exposure to film. For immunoprecipitation, cell lysates (500 μg protein) were incubated 12 h at 4°C with 10 μL of the total Src monoclonal antibody as described previously (24).

**Oxidative stress assays.** Cells (70% confluent) were trypsinized, washed, and exposed to ROS-reactive 10 μmol/L 2′,7′-dichlorofluorescein diacetate (Molecular Probes) or a ROS-insensitive analogue 5-(and-6)-carboxyfluorescein diacetate for 30 min in the dark at 37°C (25). Cells were washed and analyzed by flow cytometry using the FL1 channel (FACSCalibur; Becton Dickinson). Data were analyzed using CellQuest software (BD Bioscience). The antioxidants *N*-acetylcysteine (NAC; Sigma) and Tiron (Sigma) were used as negative controls. Thioredoxin reductase activity was evaluated using a colorimetric assay (Cayman Chemical) as described previously (26).

**Murine hepatic tumor model.** Male athymic nude mice (NCI-*nu/nu*; Animal Production Area, National Cancer Institute-Frederick Cancer Research & Development Center) were maintained under specific pathogen-free conditions in American Association for Accreditation of Laboratory Animal Care-approved facilities. To produce hepatic tumors, $1 \times 10^6$ viable HT29 cells were injected directly into the left lobe of the liver as described previously (27).

**Treatment of established hepatic tumors.** Fourteen days after hepatic injection, mice were randomized into four treatment groups: control, oxaliplatin, dasatinib, or oxaliplatin and dasatinib in combination. Oxaliplatin was dissolved in 5% dextrose, diluted in HBSS, and injected intraperitoneally at a concentration of 5 mg/kg twice weekly. Dasatinib (15 mg/kg) was solubilized in a citrate/citric acid buffer and administered by daily oral gavage. Control animals received citrate buffer daily and HBSS twice weekly. Mice were weighed weekly and monitored daily.

**Necropsy procedures.** All mice were sacrificed on day 42 after tumor cell injection, weighed, and necropsied. Tumors were excised and measured, with volume calculated by standard techniques (28). Statistical comparison between groups was done by *t* test with Welch correction for unequal variances. Processing for immunohistochemistry was done as described previously (14).

**Antibodies for immunohistochemical analysis.** Primary antibodies were purchased from the following manufacturers: rabbit anti-vascular endothelial growth factor (VEGF; A20; Santa Cruz Biotechnology), rabbit anti-cleaved caspase-3 (Biocare Medical), rat anti-mouse CD31 antibody (BD Bioscience), and mouse anti-proliferating cell nuclear antigen clone PC10 (DAKO). Secondary antibodies used for immunohistochemistry were peroxidase-conjugated goat anti-rabbit IgG, $F(ab')_2$ (Jackson ImmunoResearch Laboratories), biotinylated goat anti-rabbit (Biocare Medical), streptavidin horseradish peroxidase (DAKO), rat anti-mouse IgG2a horseradish peroxidase (Serotec, Harlan Bioproducts for Science), and goat anti-rat horseradish peroxidase (Jackson ImmunoResearch Laboratories). Fluorescent secondary antibodies used were Alexa 488-conjugated goat anti-rabbit IgG (Molecular Probes) and Alexa 594-conjugated goat anti-rat IgG (Molecular Probes).

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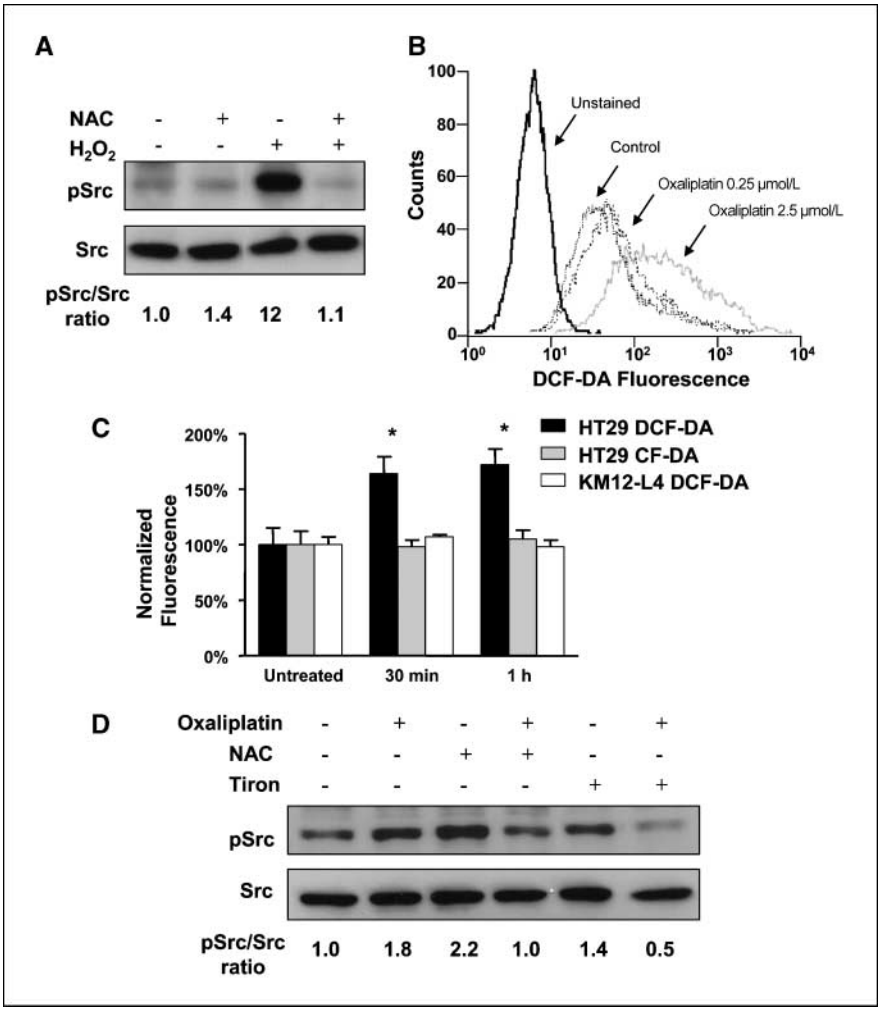


**Figure 3.** Induction of ROS following Src activation. *A,* immunoblotting was done for phospho-Src and total Src after 1 h incubation with $H_2O_2$ (1 mmol/L) alone or with a 30 min pretreatment with 24 mmol/L NAC, showing a robust Src activation by ROS. *B,* generation of ROS by increasing concentrations of oxaliplatin. HT29 cells were treated for 60 min with specified oxaliplatin doses. Cells were stained with the fluorescent dye 2′,7′-dichlorofluorescein diacetate (*DCF-DA*), which becomes fluorescent in the presence of intracellular ROS. Flow cytometry was done as described in Materials and Methods. *C,* oxaliplatin-induced intracellular ROS is cell line dependent. Median fluorescence, normalized to untreated cells, is shown for 30 and 60 min after exposure to oxaliplatin (0.25 μmol/L) in HT29 and KM12-L4 cells treated with the ROS-sensitive fluorescent probe 2′,7′-dichlorofluorescein diacetate. As a negative control, HT29 cells were treated with oxaliplatin (0.25 μmol/L) and the ROS-insensitive fluorescent probe 5-(and-6)-carboxyfluorescein diacetate (*CF-DA*). *Columns,* mean of three experiments; *bars,* SD. *D,* effect of oxaliplatin on Src activation. HT29 cells were incubated 60 min with oxaliplatin (0.25 μmol/L) with or without 30 min pretreatment with antioxidants NAC (1 mmol/L) or Tiron (50 μmol/L), and phospho-Src and total Src were immunoblotted. Fold increases of phospho-Src relative to total Src by densitometry are shown, normalized to untreated cells. Densitometry values and blots are representative of triplicate assays.

Immunohistochemical procedures for proliferating cell nuclear antigen, cleaved caspase-3, and VEGF were done and quantitated as described previously (29, 30). Control samples exposed to a secondary antibody alone showed no specific staining. Frozen sections embedded in OCT compound were used for CD31 (PECAM-1) staining and quantification was done as described previously (13).

Statistical differences for continuous variables were examined using the two-tailed Student's *t* test, with Pearson's correlation coefficients used to describe the relationship between two continuous variables. $P < 0.05$ was considered statistically significant. SDs represent interexperimental variability unless otherwise stated.

## Results

**Effect of oxaliplatin on Src activity.** Numerous forms of stress including cisplatin treatment lead to Src activation (31); however, the effect of oxaliplatin was not determined previously. For these studies, HT29, LS174T, KM12-L4, and DiFi colon cancer cells were examined for molecular and biological differences among these cells (Supplementary Fig. S1; ref. 32). Cells were treated with 2.5 μmol/L oxaliplatin and expression of total Src and phospho-Src (recognizing phospho-$Src^{Y418}$, the activated form of Src) was examined at various times thereafter. Increases in phospho-Src but not total Src as well as phospho-$FAK^{861}$ but not T-FAK were observed at 1h after oxaliplatin treatment in HT29 (Fig. 1*A*) and LS174T and DiFi (Supplementary Fig. S2) cell lines, with return to the baseline activation by 6 h (Fig. 1*A*). Kinetics and magnitude of activations were similar to that induced by growth factors and other chemotherapeutic agents (33, 34). Conversely, activated Src decreased in the KM12-L4 cell line. These results show that oxaliplatin induces Src activation in some but not all colon cancer cells.

**Src down-regulation sensitizes HT29 cells to oxaliplatin.** To specifically examine the role of Src in regulating oxaliplatin sensitivity, HT29 cells were stably transfected with small interfering RNA constructs targeting the *c-src* gene (90% reduction of Src; Fig. 1*B*) as described in Materials and Methods. Reduced expression of Src led to increased cytotoxicity in response to oxaliplatin (Fig. 1*B*) and reduced colony formation (Fig. 1*C*). Thus, Src activity mediates oxaliplatin sensitivity/resistance in some colon cancer cell lines.

**Pharmacologic inhibitor of Src is synergistic with oxaliplatin.** To further explore the effect of Src inhibition on oxaliplatin sensitivity, growth of the abovementioned cells at subconfluency was determined after exposure to fixed doses of oxaliplatin, dasatinib, or the combination. The combination of dasatinib and oxaliplatin was significantly more effective in inhibiting cell growth than either agent alone in HT29 and LS174T cells (the lines in which oxaliplatin had the greatest ability to induce Src activation) but not in DiFi and KM12-L4 cells (Fig. 2*A*). Formal synergy calculations were done as described in Materials and Methods (Fig. 2*B*). At the $IC_{50}$ for the combination, the summary

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combination indices were 0.05, 0.25, 0.78, and 1.52 for LS174T, DiFi, HT29, and KM12-L4, respectively, showing synergy for LS174T and DiFi. At the $IC_{50}$, the combination index suggested a modest supra-additive effect for HT29. The above results were confirmed with a clonogenic assay (Fig. 2*C*).

There were no clear distinctions in histology, molecular phenotypes, or single-agent chemotherapy sensitivity of the cell lines that might account for synergy with the combination of oxaliplatin and the Src inhibitor. However, in the cell lines evaluated, the extent of Src activation following oxaliplatin treatment appeared to correlate with the degree of additivity of combination treatment, suggesting that dasatinib provides the most synergy in cells with a robust Src activation after oxaliplatin administration. To further explore this hypothesis, two additional cell lines (HCT116 and SW480) were evaluated. The SW480 cell line robustly activated Src after oxaliplatin treatment (combination index of 0.45 at the $IC_{50}$), whereas HCT116 cells were inhibited in Src activity after oxaliplatin and failed to show synergy with the combination (combination index of 1.59 at the $IC_{50}$). The ability to activate Src in different cells after oxaliplatin suggests a trend toward a significant correlation (Fig. 2*D*).

**Oxaliplatin-induced Src activation is mediated by ROS.** As oxaliplatin has been implicated in generation of ROS, and oxidative stress is known to activate Src, we determined if there were a relationship between ROS production and Src activation. As shown in Fig. 3*A*, hydrogen peroxide (as a positive control) activated Src and increased FAK phosphorylation, which was inhibited with the ROS inhibitor NAC in HT29 cells. Therefore, HT29 cells were treated with oxaliplatin and ROS levels were examined as described in Materials and Methods. A dose-dependent increase in intracellular ROS was evident 30 min after oxaliplatin treatment in the HT29 cell line when the redox-sensitive fluorescent probe 2′,7′-dichlorofluorescein diacetate was used (Fig. 3*B* and *white columns* in Fig. 3*C*) but not when the redox-insensitive probe 5-(and-6)-carboxyfluorescein diacetate was used (Fig. 3*C, gray columns*). In contrast, increased intracellular ROS was not observed in KM12-L4 cells (Fig. 3*C, black columns*), suggesting that cell-dependent differences in Src activation may correlate with ROS generation after oxaliplatin administration. Pretreatment with NAC at concentrations that showed no cellular toxicity within 24 h abolished the ROS increase after oxaliplatin treatment (data not shown). Pretreatment with NAC and the antioxidant vitamin E analogue Tiron inhibited Src and FAK phosphorylation (Fig. 3*D*), showing that oxaliplatin activation of Src in HT29 cells is ROS-dependent. Src activation is also observed after NAC or Tiron treatment likely due to activation of proliferation pathways (3, 35).

However, at the concentrations used in this study, oxaliplatin did not affect thioredoxin reductase activity required for maintenance of the intracellular antioxidant thioredoxin (Supplementary Fig. S3*B*; ref. 36).

**Intrahepatic tumor growth is inhibited by combination therapy.** We determined if the above-described effects also occurred on growth of colon tumor cells in the liver, best approximating the stage of disease in which treatment would commence for metastatic colon cancer patients. We therefore examined the effects of dasatinib, alone or in combination with oxaliplatin, on established colorectal tumors in the liver using HT29 cells. The results (Fig. 4) show similar rates of tumor formation with incidences of 80% to 89% for all groups. Treatment with either dasatinib or oxaliplatin as monotherapies led to no statistically significant reductions in tumor size at the concentrations used. In contrast, combination therapy resulted in a significant 92% reduction in tumor volume relative to untreated controls ($P < 0.01$). None of the treatment schema affected mouse weight nor were signs of toxicity evident.

**Effect of combination therapy on tumor proliferation and apoptosis.** Treatment with oxaliplatin and dasatinib in combination reduced proliferating cell nuclear antigen-positive cells by 90% versus controls ($P < 0.001$) and an additional 32% to 34% compared with either dasatinib or oxaliplatin alone ($P < 0.001$; Fig. 5). Cleaved caspase-3-positive cells (Fig. 5) and TUNEL-positive cells (data not shown) increased after treatment with oxaliplatin and combination therapy compared with control. Thus, in this model, oxaliplatin and dasatinib in combination are effective in inhibiting tumor cell growth *in vivo*.

**Dasatinib inhibited VEGF and reduced microvessel count.** As also shown in Fig. 5, oxaliplatin monotherapy resulted in VEGF staining equivalent to that of untreated control cells. However, treatment with dasatinib, alone and in combination with oxaliplatin, significantly reduced VEGF expression by tumor cells. Dasatinib and oxaliplatin reduced vessel count by 53% and 55% ($P < 0.005$), respectively, and by 89% ($P < 0.001$) in combination

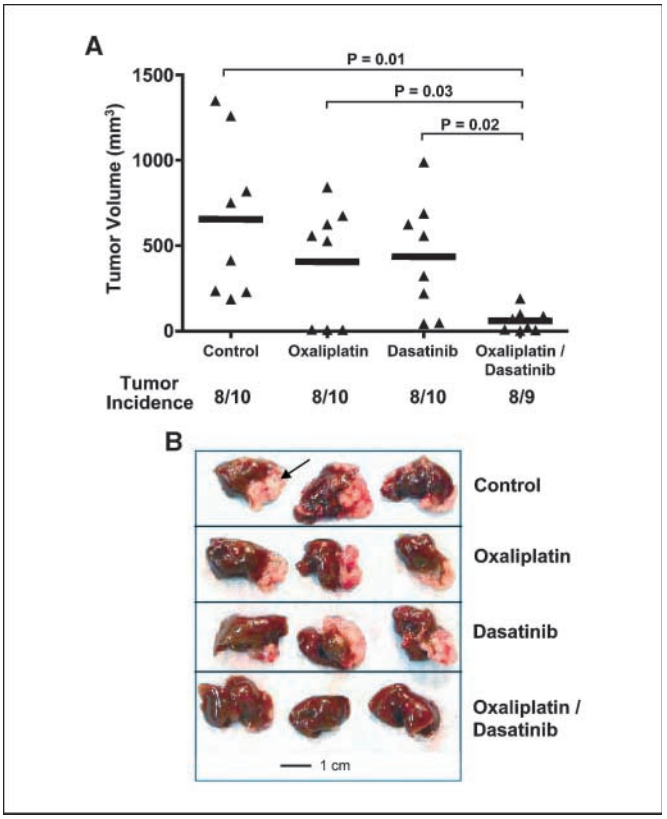


**Figure 4.** Effect of combination treatment with oxaliplatin and dasatinib in a murine model of metastatic colorectal cancer in the liver. Mice underwent intrahepatic injection of HT29 cells as described in Materials and Methods. After 14 days, mice were treated with oxaliplatin (5 mg/kg intraperitoneally, twice weekly) and dasatinib (15 mg/kg orally, daily). *A,* effects of combination therapy on intrahepatic growth of HT29 cells. Tumor volumes and incidence of tumor formation as a proportion. *Bars,* average. *P* values for comparison with combination treatment group. Tumor incidence is defined as the number of mice with visible tumor at the time of necropsy divided by the total number of mice successfully injected. *B,* representative livers from each group. Whole liver specimen is shown, with visible tumor (*arrow*).

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relative to untreated controls, an additional 33% reduction versus either agent alone ($P < 0.001$), suggesting that oxaliplatin has additional effects on vessels that are independent of VEGF.

**Chronic oxaliplatin exposure is associated with stable Src activation.** To assess the effect of oxaliplatin on Src activity *in vivo*, we subjected whole tumor lysates from each group in the murine experiment to Western blot analysis. Oxaliplatin monotherapy (5 mg/kg) resulted in ~3-fold increase in $Src^{Y418}$ phosphorylation compared with untreated controls, whereas, as expected, dasatinib (15 mg/kg) resulted in marked reduction in $Src^{Y418}$ phosphorylation but not Src expression (Fig. 6*A*).

Finally, we examined stable established oxaliplatin-resistant cell lines derived from HT29 and KM12-L4 (20). After growth in oxaliplatin-free serum for 48 h, the HT29/OxR and KM12-L4/OxR cell lines showed a 2- to 3-fold increase in phospho-$Src^{Y418}$, with little effect on total Src, compared with the cognate parental oxaliplatin-sensitive cell lines (Fig. 6*B*). These data show that stable Src activation results from chronic exposure to oxaliplatin and is associated with an oxaliplatin-resistant phenotype.

## Discussion

Despite advances in the development of new chemotherapeutic agents, colorectal cancers eventually develop chemoresistance, resulting in disease progression. Thus, therapeutic strategies that would resensitize tumors to these agents would improve outcome. In this report, we show that one mediator of oxaliplatin sensitivity/resistance in some colon tumor cells is the nonreceptor tyrosine kinase Src. Progressive increases in Src activity are a hallmark of colorectal cancer (9, 37). Numerous physiologic stresses lead to increased Src activity, including tumor hypoxia and oxidative stress. In turn, increased Src activity increases cellular migration, invasion, and expression of proangiogenic factors such as VEGF and interleukin-8 (24). Thus, Src inhibitors have generated interest

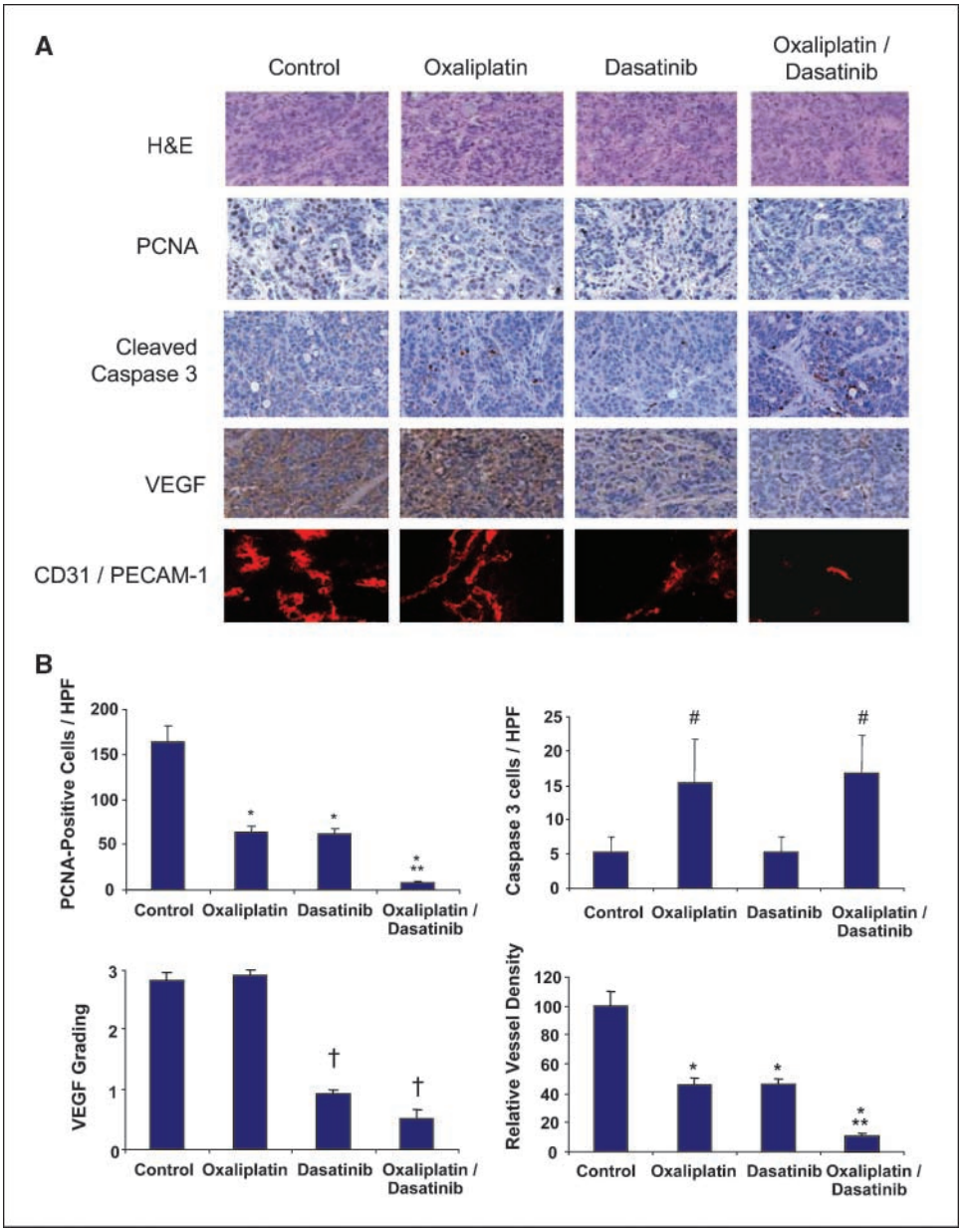


**Figure 5.** Effects of treatment with oxaliplatin and dasatinib on proliferation and angiogenesis. *A,* immunohistochemistry from resected murine HT29-derived hepatic tumors. Representative sections from untreated, oxaliplatin-treated, dasatinib-treated, and combination-treated hepatic tumors. *B,* quantitation of immunohistochemistry. Immunohistochemistry was quantified as described in Materials and Methods. For cleaved caspase-3 and proliferating cell nuclear antigen (*PCNA*), counts represent the number of cells positive by immunohistochemistry per high-power field (*HPF*). For VEGF, this represents the average of an ordinal scale of expression. For relative vessel density, vessel count is normalized to untreated tumor. *Columns,* mean; *bars,* SD. *, $P < 0.001$ versus control; **, $P < 0.001$ versus oxaliplatin or dasatinib alone; #, $P < 0.05$ versus control; †, $P < 0.001$ versus control or oxaliplatin alone.

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for treatment of patients with colorectal carcinomas (7). Understanding mechanisms by which Src activation affects current therapeutic regimens is critical if Src family kinase inhibitors are to become part of the standard therapeutic arsenal in some advanced colorectal cancer patients.

Herein, we show novel relationships between Src activity and oxaliplatin administration. Src is activated after oxaliplatin administration through a ROS-dependent mechanism with a strong trend toward a correlation between the degree of Src activation after oxaliplatin administration and the degree of synergy with dasatinib and oxaliplatin. In a murine model of colorectal cancer liver metastases, the combination reduces the size of the liver tumors with associated antiangiogenic and proapoptotic effects. These suggest a novel mechanism for Src activation following oxaliplatin administration.

Platinum agents affect redox status through generation of ROS and through formation of covalent adducts with intracellular thiols. Cisplatin treatment directly results in generation of ROS possibly through electrons liberated as a direct byproduct of DNA/platinum adduct formation or through electron leakage from the mitochondrial respiratory chain (5, 6). Thioredoxin reductase, in particular, readily forms inactivating platinum-thiol adducts, depleting the reduced form of thioredoxin (36), although no change in thioreductase activity was observed in this study, consistent with a prior report that showed inhibition only at oxaliplatin concentrations above what are used therapeutically (36). Platinum agents may affect signal transduction pathways in addition to the canonical effects on DNA synthesis resulting from adduct formation (2, 38, 39), which may explain our results.

Previous work showed that reactive oxygen and nitrogen species induce Src activation (40, 41). We show that antioxidants prevent this, although we cannot preclude additional cellular effects. The mechanisms underlying the oxidative stress-induced Src activation are not fully elucidated. Intriguingly, a previous study has shown that oxidation of the cysteine residues of Src after integrin ligation results in increased Src activation due to a conformational change in the enzyme (41, 42). Oxidation may also inactivate the phosphatase PTPB1B, implicated in activating Src by catalyzing dephosphorylation of the negative regulatory Y530 (35).

Colon cancer cell fate after chemotherapy-induced oxidative stress is variable, with some studies showing an additive effect of antioxidants and chemotherapy (43), whereas other studies show antagonism when oxaliplatin is combined with NAC or a superoxide dismutase mimic (3, 44). These studies suggest that altering the oxidative balance in cells is dependent on multiple factors and may be difficult to apply to clinical care (3, 45).

Our data imply that the combination of dasatinib and oxaliplatin will not affect all colon tumor cells, as also shown by others (15). However, we show that, for the majority of colon tumor cells, synergistic effects are observed. Understanding what governs these cell-dependent responses may provide guidance for selection of appropriate patients for treatment with Src inhibitors.

Another unexpected finding in this study was the increased ability of the combination to reduce mean microvessel density. Although Src inhibition decreases VEGF expression, our results suggest that Src inhibitors in combination therapies may have additional clinically relevant antiangiogenic properties when combined with chemotherapeutics. Therefore, the potential benefit of Src inhibitors may derive not only from the modulation of intrinsic cellular resistance but also from enhanced effects on the tumor-associated vasculature.

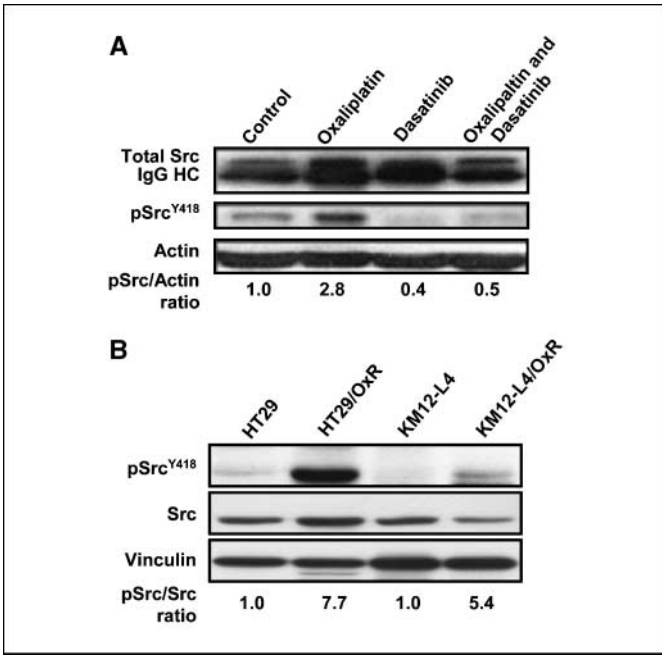


**Figure 6.** Chronic Src activation is associated with oxaliplatin exposure and resistance. *A,* murine hepatic tumors treated with oxaliplatin have increased Src activity. HT29 hepatic tumors were harvested 3 days after last oxaliplatin treatment. Tumor lysates were immunoprecipitated for total Src and then immunoblotted for total and phospho-Src. Total Src (*top band*) has a molecular weight close to the IgG heavy chain (*HC*; *bottom band*), resulting in two bands on Western blot. Phospho-Src is increased in hepatic tumors from mice treated with oxaliplatin alone. Densitometry indicates ratio of phospho-Src to actin, normalized to untreated tumors. *B,* activation of Src in oxaliplatin-resistant cell lines. Expression by Western blot of total Src and phospho-Src in stable oxaliplatin-resistant (*OxR*) cell lines relative to sensitive parental cells. Cells were removed from oxaliplatin for 48 h before lysis. Densitometry indicates ratio of phospho-Src to total Src relative to the same ratio in the respective parental cell line and is representative of replicated blots.

In murine models, Src inhibitors most commonly affect properties associated with metastasis, without significant effect on proliferation. In a clinical trial in colorectal cancer from our institution, the Src inhibitor AZD0530 failed to show efficacy as a single agent (46). However, preliminary results from an ongoing trial in refractory metastatic colorectal cancer suggest activity when a Src inhibitor is combined with an oxaliplatin-containing regimen (47). Given preclinical studies showing the ability of Src inhibitors to overcome chemoresistance as well as resistance to "targeted" agents, such as the epidermal growth factor receptor monoclonal antibody cetuximab (16–18, 48), this same approach may be broadly applicable to other combinations in alternate tumor types.

## Disclosure of Potential Conflicts of Interest

S. Kopetz: Consultant/advisory board, Sanofi-aventis. L.M. Ellis: Commercial research grants, ImClone, Sanofi-aventis, and Amgen; honoraria from speakers' bureau, Genentech; consultant/advisory board, Sanofi-aventis. The other authors disclosed no potential conflicts of interest.

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