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Antiangiogenic and Antitumor Effects of Src Inhibition in Ovarian Carcinoma

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Abstract

Src, a nonreceptor tyrosine kinase, is a key mediator for multiple signaling pathways that regulate critical cellular functions and is often aberrantly activated in a number of solid tumors, including ovarian carcinoma. The purpose of this study was to determine the role of activated Src inhibition on tumor growth in an orthotopic murine model of ovarian carcinoma. *In vitro* studies on HeyA8 and SKOV3ip1 cell lines revealed that Src inhibition by the Src-selective inhibitor, AP23846, occurred within 1 hour and responded in a dose-dependent manner. Furthermore, Src inhibition enhanced the cytotoxicity of docetaxel in both chemosensitive and chemoresistant ovarian cancer cell lines, HeyA8 and HeyA8-MDR, respectively. *In vivo*, Src inhibition by AP23994, an orally bioavailable analogue of AP23846, significantly decreased tumor burden in HeyA8 ($P = 0.02$), SKOV3ip1 ($P = 0.01$), as well as HeyA8-MDR ($P < 0.03$) relative to the untreated controls. However, the greatest effect on tumor reduction was observed in combination therapy with docetaxel ($P < 0.001$, $P = 0.002$, and $P = 0.01$, for the above models, respectively). Proliferating cell nuclear antigen staining showed that Src inhibition alone ($P = 0.02$) and in combination with docetaxel ($P = 0.007$) significantly reduced tumor proliferation. In addition, Src inhibition alone and in combination with docetaxel significantly down-regulated tumoral production of vascular endothelial growth factor and interleukin 8, whereas combination therapy decreased the microvessel density ($P = 0.02$) and significantly affected vascular permeability ($P < 0.05$). In summary, Src inhibition with AP23994 has potent antiangiogenic effects and significantly reduces tumor burden in preclinical ovarian cancer models. Thus, Src inhibition may be an attractive therapeutic approach for patients with ovarian carcinoma.

Introduction

Due to the incongruity between symptomatology and early stage disease, 75% of women with ovarian cancer are diagnosed with advanced stage disease with spread beyond the

pelvis (1, 2). Because the 5-year survival for such late-stage disease is only 20% to 25% (3), ovarian cancer remains the most deadly of all gynecologic malignancies. Furthermore, despite initial response to surgical debulking of tumor and front-line chemotherapy with carboplatin and paclitaxel, most tumors eventually develop drug resistance, causing patients to succumb to their disease (4). Given this bleak clinical scenario, the development of new therapeutic strategies to combat ovarian cancer is needed.

Biological therapies based on vascular endothelial growth factor (VEGF) targeting are beginning to show promise in ovarian and other solid tumors (5–7). However, improvements in response have not translated to increased cure rates, necessitating the consideration for additional targets. Src, a nonreceptor tyrosine kinase of 60 kDa, is a particularly attractive target because it is activated in a majority of ovarian cancers and regulates a myriad of intracellular signal cascades responsible for critical tumor cell functions through extracellular stimulation by growth factors, growth hormones, and integrins (8). For example, Src affects proliferation through control of platelet-derived growth factor–stimulated increase in *myc* mRNA (9), and it also influences cellular motility and invasion when complexed with focal adhesion kinase to recruit vital regulators of extracellular signal-regulated kinases, *c-Jun* NH₂-terminal kinase and Rho signaling pathways (8). Increased cell survival is mediated through the Stat proteins with changes in the transcription of Stat-modulated gene, such as *c-fos*, *c-Jun*, *c-myc*, and cyclin D (10). Moreover, the emerging role of Src in angiogenesis has recently been noted because of its up-regulation of proangiogenic cytokines such as VEGF and interleukin 8 (IL-8; refs. 11–13).

Src has been found to be overexpressed in a majority of late stage ovarian tumors as well as a panel of ovarian cancer cell lines (14, 15). In addition, Src promotes tumor survival and resistance to chemotherapy in the ID8 mouse ovarian cancer cell line via increased Ras and Akt activations (16). Conversely, Src inhibition enhances paclitaxel-mediated cytotoxicity in ovarian cancer cell lines through caspase-9 independent activation of caspase-3 (16, 17). Furthermore, an antisense c-Src construct in human ovarian cancer cells curtailed tumor growth in a xenograft mouse model when compared with the parental cell line (18), suggesting its potential antitumor effects *in vivo*. However, the roles of Src inhibition using a clinically adaptable approach and its mechanisms of action have not been addressed in ovarian carcinoma.

The Src-selective pyrazolopyrimidines inhibitors (PP1 or PP2) have been previously used for Src inhibition in both *in vitro* and *in vivo* studies (12, 19–21); however, these agents have not advanced to clinical use due, in part, to their intrinsic lack of potency (20, 22). Recently, a novel class of ATP-based inhibitors of Src, including AP23846 and AP23994 (ARIAD Pharmaceuticals, Cambridge, MA) have become noteworthy, offering a 10-fold greater potency when compared with PP2 (12). Furthermore, AP23994, an orally available analogue of AP23846, is ideal for *in vivo* investigation of Src inhibition given its superior bioavailability (23). Based on the critical role of Src in ovarian cancer progression, we considered that these novel inhibitors would have both direct and indirect effects on ovarian carcinoma. To examine this hypothesis, we carried out a series of *in vitro* and *in vivo* experiments using both chemosensitive and chemoresistant cell lines.

Materials and Methods

Src inhibition

AP23846 and AP23994 (ARIAD Pharmaceuticals) were used for *in vitro* and *in vivo* inhibition, respectively (Fig. 1). The compounds were designed and synthesized using previously described methods (23). The Src kinase selectivity profiles of AP23846 and AP23994 revealed nearly identical properties of both compounds relative to Src family

kinases (i.e., Src, Fgr, Hck, Lck, and Yes) and other kinases, including Abl, Flt1, Flt3, KDR, and epidermal growth factor receptor (Fig. 1). AP23846 was diluted in DMSO (Sigma, St. Louis, MO) to the desired concentrations. AP23994 was prepared for oral gavage, and therefore dissolved in 15% *N,N*-dimethylacetamide, 14% TPGS (vitamin E), 5% Tween 80, 26% polyethylene glycol 400, and 40% water.

Cell lines and culture

The source and derivation of the established human ovarian cancer cell lines have been described previously (24). The ovarian cancer cell lines HeyA8 and SKOV3ip1 were maintained and propagated in RPMI 1640 supplemented with 15% fetal bovine serum and 0.1% gentamicin sulfate (Gemini Bioproducts, Calabasas, CA). The HeyA8-MDR cell line was generated through sequential exposure to increasing sublethal concentrations of paclitaxel and found to have cross-resistance to cisplatin, adriamycin, dactinomycin, vinblastine, and vincristine, which was made available as a generous gift from Dr. Isaiah J. Fidler (The University of Texas M.D. Anderson Cancer Center, Houston, TX). HeyA8-MDR was maintained and propagated in the same media as the parental cells with the addition of 300 Ag/mL of paclitaxel. All *in vitro* and *in vivo* experiments were conducted using cell lines at 70% to 80% confluence.

For *in vivo* i.p. injection, cells were harvested with either EDTA or Trypsin-EDTA (Life Technologies, Carlsbad, CA) and centrifuged at 1,000 rpm for 7 minutes at 4°C, then washed twice with PBS, and reconstituted in the appropriate volume of HBSS (Life Technologies) for a final concentration of 1.25×10^6 cells/mL for HeyA8 or 5×10^6 cells/mL for SKOV3ip1 and HeyA8-MDR in 200 μ L for i.p. injections.

Western blot and immunoprecipitation

Cultured cell lysates were prepared by washing in PBS then incubating in modified radioimmuno-precipitation assay (RIPA) lysis buffer with 1 $\times$ protease inhibitor (Roche, Mannheim, Germany) for 10 minutes in 4°C. The cells were then scraped from the plates and collected for centrifuge at 13,000 rpm for 20 minutes at 4°C. The supernatant was stored in -80°C. Tumor lysates were prepared from resected *in vivo* tumors with the confirmed presence of malignancy as indicated by H&E staining from the very same tumor block. The *in vivo* tumors were collected at time of animal sacrifice, and snap-frozen in liquid nitrogen, then stored in -80°C. A small pellet of frozen tumor was immersed in modified RIPA lysis buffer with 5 $\times$ protease inhibitor on ice for 10 minutes and then homogenized with an electric homogenizer. The admixture was centrifuged at 12,000 rpm in 4°C for 15 minutes and supernatant was extracted for protein analysis.

All Western blot probing for phospho-Src^{Y419} (pSrc^{Y419}) was completed after immunoprecipitation with total-Src. Briefly, 500 μ g of protein in a total volume of 650 μ L of modified RIPA lysis buffer with protease inhibitor was allowed to incubate with 6 μ L of total-Src antibody (monoclonal antibody 327; Oncogene Sciences, Manhasset, NY) overnight in 4°C. Subsequently, 50 μ L of purified 1:1 protein G agarose slurry (Upstate Cell Signaling Solutions, Lake Placid, NY) was added to the samples and rotated for 2 hours in 4°C. This was followed by three sequential washings with RIPA lysis buffer. Finally, an appropriate amount of 3 $\times$ Laemmli's sample buffer was added and boiled for 5 minutes. Anti-phospho-Src^{Y419} antibody was used at a 1:1,000 dilution (Cell Signaling Technology, Danvers, MA), and visualized using anti-rabbit horseradish peroxidase at 1:1,000 dilution (Amersham Biosciences UK Limited, Buckinghamshire, England). We then stripped the blot and reprobbed for total-Src, which served as loading controls.

Cell viability assay

In order to determine the *in vitro* effects of AP23846 on the growth of ovarian cancer cells alone and in combination with docetaxel, 2,000 cells per well were plated in a 96-well plate with experimental conditions set in triplicate. Cells were then subjected to sequentially diluted concentrations of AP23846 alone, or in combination with docetaxel, and then incubated for 72 hours in 37°C. To assess for cell viability, 50 μ L of 0.15% 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma) was added to each well, and incubated for 2 hours at 37°C. The medium/3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide preparation was then removed from each well, and 100 μ L of DMSO (Sigma) was added, and read at 570 nm absorbance (Ceres UV 900C, Bio-Tek Instrument, Inc., Winooski, VT) within 30 minutes.

Caspase-3 activity

Ovarian cancer cells (1×10^6) were plated in each well of a six-well plate for the following conditions: vehicle control, docetaxel at IC₉₀, 1 μ mol/L of AP23846, combination of docetaxel and AP23846, and 10 μ mol/L of cisplatin served as a positive control. Caspase-3 activity was measured using substrate DEVD-AFC from an apoptosis detection kit (BD Bioscience/Clontech, Palo Alto, CA).

Murine orthotopic *in vivo* ovarian carcinoma model

Female athymic nude mice (NCR-*nu*) were purchased from the National Cancer Institute-Frederick Cancer Research and Development Center (Frederick, MD) and housed in specific pathogen-free conditions. They were cared for in accordance with guidelines set forth by the American Association for Accreditation of Laboratory Animal Care and the U.S. Public Health Service Policy on Human Care and Use of Laboratory Animals, and all studies were supervised by our home institutional Animal Care and Use Committee.

The therapy experiments were done in three separate ovarian cancer cell lines: HeyA8, SKOV3ip1, and HeyA8-MDR. To assess tumor growth, therapy was started 1 week after i.p. injections. There were four treatment groups with 10 mice in each group: (a) control (vehicle only); (b) docetaxel, 50 μ g, injected i.p. every week; (c) AP23994, 50 mg/kg, given by daily gavage; and (d) combination group with both weekly i.p. docetaxel and daily AP23994. The mice were monitored for adverse effects, and sacrificed by cervical dislocation 3 to 4 weeks after the initiation of treatment. In addition, we assessed the early changes in VEGF and microvessel density (MVD) in the tumor microenvironment by treating formed tumors at 18 days after cell line injection when tumor size was 0.5 to 0.75 cm for 5 days. This short-term therapy experiment was completed using the HeyA8 ovarian cancer cell line.

At the time of sacrifice, mouse weights, tumor weights, number of tumor nodules, location, and ascites volume were measured and recorded. Plain tumor and specimens immersed in optimum cutting temperature medium were snap-frozen for lysate and frozen slide preparations, respectively. Tumor specimens were also fixed in formalin for paraffin preparation.

Immunohistochemistry staining of proliferating cell nuclear antigen, IL-8, VEGF and CD31

In general, paraffin-embedded tissue slides of mouse tumors collected at the time of necropsy were dewaxed by setting on heating blocks at 60°C for 30 minutes, followed by immersion in xylene, and successively diluted solutions of ethanol. Antigen retrieval was accomplished in 0.1 mol/L of sodium citrate buffer, (pH6.0), and cut sections were microwaved. Blocking of endogenous peroxidase activity was completed using 0.3% hydrogen peroxide in methanol solution for 15 minutes at room temperature and nonspecific

tissue binding was blocked with 1 hour incubation in 5% normal horse serum and 1% normal goat serum in PBS. Respective primary antibodies were then applied and set overnight in 4°C as follows: anti-proliferating cell nuclear antigen (PCNA) antibody (mouse IgG; Dako, Carpinteria, CA) was used after initial overnight incubation in mouse F(ab)₂ fragment-specific antibody in 4°C to block endogenous mouse IgG antibodies; anti-IL-8 antibody (rabbit IgG; Biosource International, Camarillo, CA); anti-VEGF antibody (rabbit IgG; Santa Cruz Biotechnology Inc., Santa Cruz, CA); and anti-CD31 antibody (rat IgG; PharMingen, BD Biosciences, San Diego, CA). Appropriate secondary horseradish peroxidase-conjugated antibodies were applied accordingly, and signal was visualized after incubation in 3,3'-diaminobenzidine (Phoenix Biotechnologies, Huntsville, AL) and counterstained with Gill's no. 3 hematoxylin (Sigma). CD31 staining was done on freshly cut frozen slides and fixed in cold acetone with no antigen retrieval necessary.

Determination of IL-8 level by ELISA

Blood was collected from mice from all treatment arms during the HeyA8 therapy experiment on a weekly basis via the tail vein. The blood from the respective treatment arms was centrifuged at 1,000 rpm for serum collection in order to determine IL-8 levels by ELISA using a commercially available kit (R&D Systems Inc., Minneapolis, MN). Absorbance on the plate was read at 450 nm within the next 30 minutes (Ceres UV 900C, Bio-Tek Instrument).

Intradermal Miles assay

Treatment ($n = 3$ mice per group, four groups total) as described above was started on healthy female nude mice for a duration of 5 days. On day 6, 200 μ L of 0.5% Evan's blue dye was injected into the tail vein of all mice, and allowed for even systemic distribution. Next, intradermal injections with 50 μ L of both VEGF (10 ng/mL, R&D Systems) and PBS into the flanks were done. The mice were sacrificed after 20 minutes, and the skin unsheathed to determine the extent of intradermal dye spread as a measure of vascular permeability (25). The PBS side served as control. The dye area was measured, and the area calculated by the formula of a shape that can best describe the affected region (circle versus square versus rectangle). Interpretation was based on positive blue area induced by VEGF as a number of fold increase relative to the positive blue area induced by PBS.

Statistical analysis

Continuous variables were compared using Student's *t* test or ANOVA. For non-normally distributed data sets, the Mann-Whitney rank sum test was used. Survival curves were plotted by Kaplan-Meier method, and differences determined by log-rank test. A $P < 0.05$ on two-tail testing was considered statistically significant.

Results

In vitro effects of Src inhibition

Prior to initiating *in vivo* therapy experiments, we first determined the dose- and time-dependent effects of AP23846 on Src phosphorylation *in vitro* using the ovarian cancer cell lines, HeyA8 and SKOV3ip1. Both cell lines were exposed to increasing drug concentrations (250 nmol/L, 500 nmol/L, and 1 μ mol/L) for 24 hours and cell lysates were then collected. The expression of pSrc^{Y419} was analyzed by Western blot (Fig. 2A). AP23846 treatment resulted in a dose-dependent down-regulation of pSrc^{Y419}, the active form of Src. Because the 1 μ mol/L dose resulted in the greatest inhibition of pSrc^{Y419}, we treated HeyA8 and SKOV3ip1 with this dose of AP23846, and collected cell lysates at various time points. Western blot analysis of cell lysates revealed that pSrc^{Y419} inhibition

begins within 1 hour in both of the ovarian cancer cell lines, and this inhibition lasts for at least 24 hours (Fig. 2B).

Based on the suggested role of Src inhibition in sensitizing cancer cells to cytotoxic chemotherapy, we examined the effects of inhibitory doses of AP23846 on docetaxel sensitivity. AP23846 at 500 nmol/L enhanced the growth inhibition of docetaxel by 50-fold in the HeyA8 cells (Fig. 2C). Remarkably, this enhancement was even more profound in the HeyA8-MDR cells with a 250-fold improvement with respect to docetaxel alone (Fig. 2C). In order to test whether mediators of apoptosis are activated by Src inhibition, we measured caspase-3 levels following treatment with AP23846 alone and in combination with docetaxel. There was a 1.7-fold increase in caspase-3 after treatment with AP23846 in the HeyA8 cells compared with the controls ($P < 0.05$) and >3-fold increase in the SKOV3ip1 cells ($P < 0.05$; Fig. 2D). These results are similar to docetaxel monotherapy. The combination of AP23846 and docetaxel resulted in a 4.6-fold and 7.3-fold increase in caspase-3 activity in the HeyA8 and SKOV3ip1 cell lines, respectively (both $P < 0.05$; Fig. 2D), suggesting an additive effect.

***In vivo* Src inhibition**

For *in vivo* experiments, we used AP23994, an oral analogue of AP23846 (Fig. 1) with similar structural and *in vitro* profiles but with better bioavailability when compared with the original compound (23). To conduct *in vivo* kinetics experiments, female nude mice bearing palpable HeyA8 abdominal tumors at 17 to 20 days postinjection were treated with a single dose of AP23994 at either 10, 30, or 50 mg/kg by oral gavage. The animals were then sacrificed at 24 or 48 hours posttreatment ($n = 3$ per time point). Tumors were harvested and analyzed by Western blot to determine the duration of drug action. Western blot revealed that AP23994 achieved down-regulation of pSrc^{Y419} consistently at the 50 mg/kg dose (data not shown). A subsequent *in vivo* kinetic experiment was then initiated to determine the frequency of drug dosing. Using the same model as described above, we found that Western blot of tumor lysate collected after single treatment at 50 mg/kg revealed pSrc^{Y419} down-regulation at 12 hours with re-expression by 24 hours, although even at 24 hours, expression of activated Src did not return to pretreatment levels (Fig. 3A). Therefore, for all subsequent *in vivo* experiments, the 50 mg/kg dose was administered daily.

Therapy experiments using AP23994 in orthotopic murine models

To determine the effects of AP23994 on *in vivo* ovarian cancer growth, multiple experiments were done. HeyA8 and SKOV3ip cell lines were used because they were derived from women with advanced, therapy-refractory ovarian cancers, and represent the extremes of growth seen in clinical disease. To simulate advanced disease, we started therapy 1 week after tumor cell injection, with animals divided into the following four treatment groups ($n = 10$ per group): (a) control, in which animals received vehicle only (i.p. and gavage); (b) docetaxel, 50 μ g once per week; (c) AP23994, 50 mg/kg every day; (d) docetaxel 50 μ g once a week plus AP23994 50 mg/kg every day. We terminated the therapy experiments when the control animals exhibited signs of significant tumor burden and seemed to be moribund (3–4 weeks of therapy depending on the cell line). AP23994 alone consistently decreased tumor burden (Fig. 3B–D): there was a 70% reduction in HeyA8 ($P = 0.02$; Fig. 3B), and a 72% reduction in SKOV3ip1 ($P = 0.01$; Fig. 3C). The efficacy of AP23994 proved to be comparable to that of docetaxel in these two cell lines. Combination therapy resulted in the greatest tumor reduction compared with either docetaxel or AP23994 alone, with 95% reduction in the HeyA8 model ($P < 0.001$) and 98% in the SKOV3ip1 model ($P = 0.002$) when compared with controls.

Because chemotherapy resistance is a common clinical problem in ovarian carcinoma, we next examined the HeyA8-MDR cell line, which was derived from the parental HeyA8 cell line and is resistant to multiple agents such as paclitaxel, adriamycin, dactinomycin, vinblastine, and vincristine. Remarkably, AP23994 alone produced a 75% reduction in tumor weight in this chemoresistant cell line ($P < 0.03$; Fig. 3D). As expected, treatment with docetaxel did not significantly reduce tumor burden; however, combination therapy resulted in 88% tumor reduction in the HeyA8-MDR model ($P = 0.01$).

To further evaluate the effects of AP23994 therapy on other variables of tumor growth, we examined tumor incidence as well as number of nodules (Table 1). Again, the greatest effect was observed with combination therapy. Combination therapy consistently produced fewer tumor nodules with 75.3% reduction in HeyA8 ($P = 0.008$), 85.7% in SKOV3ip1 ($P = 0.002$), and 52.4% in HeyA8-MDR models ($P = 0.034$). The decrease in the number of nodules occurred despite having comparable tumor incidence. Furthermore, AP23994 was also effective in blocking ascites in the SKOV3ip1 model. This particular cell line is known to produce ascites and large quantities of tumor nodules. Specifically, the controls had a mean 1.65 ± 0.54 mL of ascites compared with none in the docetaxel, AP23994, or combination groups ($P < 0.001$).

Antiproliferative effects of AP23994 therapy

To determine potential mechanisms by which AP23994 inhibits tumor growth, we examined its effects on cell proliferation by performing PCNA stains on tumors collected from the therapy experiments. Representative slides, and the mean percentage of PCNA-positive nuclei of each experimental group for the HeyA8 cell line are presented in Fig. 4A. The proliferation index for the control group was 69.5%, and this was significantly reduced to 54.3% in the docetaxel alone group ($P = 0.03$), 49.9% in the AP23994 group ($P = 0.02$), and 37.3% in the combination therapy group ($P = 0.007$). Similar antiproliferative effects of AP23994 were also seen in the SKOV3ip1 cell line (data not shown). In the HeyA8-MDR cell line, as expected, treatment with docetaxel had a negligible effect on tumor cell proliferation (52.3% versus 60.1% for control, $P = 0.22$). However, the trend of proliferation index reduction remained in the AP23994 and combination therapy arms [45.7% ($P = 0.004$) and 34.1% ($P < 0.001$), respectively].

Antiangiogenic effects of AP23994

Based on studies in other tumor systems demonstrating a role for Src in angiogenesis (26), we next determined whether angiogenesis and associated markers were affected by Src inhibition. MVD was calculated following CD31 staining of tumors from each of the therapy groups (Fig. 4B). The decrease in MVD of the AP23994 group approached significance ($P = 0.059$), and MVD of the combination treatment group using both docetaxel and AP23994 was significantly reduced when compared with that of the control group (7.37 versus 18.87, $P = 0.02$).

To determine potential mediators of antiangiogenic effects of Src inhibition, we next examined the effects of AP23994 on known angiogenic cytokines such as IL-8 and VEGF. Figure 4C depicts representative staining pattern of IL-8 from all therapy groups revealing high-intensity staining in the control group, and markedly reduced expression in the combination group. We collected mice sera via tail vein phlebotomy every treatment week and determined the IL-8 levels by ELISA in the pooled blood of mice from their respective groups. The greatest decrease in IL-8 levels were noted in the combination group (36% lower after 1 week of therapy, and 92% lower after 4 weeks). These findings suggest that serum IL-8 might be a useful biomarker for Src inhibitor-based therapy. VEGF levels were

also decreased by AP23994 with the greatest reduction in the combination treatment of docetaxel and AP23994 (Fig. 4D).

Based on the decrease in angiogenic cytokines following AP23994 treatment, we sought to determine whether vessel permeability was altered as well (27). The intradermal Miles assay was used to examine the effects of AP23994 on vascular endothelia of normal, non-tumor-bearing nude female mice after a short-term therapy of 5 days. With the PBS injections serving as positive controls, we found that only mice treated with AP23994 alone and the combination of docetaxel and AP23994 exhibited significant reduction (both $P < 0.05$; Fig. 5) in vascular permeability as represented by the spread of the blue dye in the murine dorsal skin. These results provide *in vivo* functional evidence to support Src inhibition as a modality to decrease endothelial permeability mediated through VEGF.

Discussion

The key findings from the present study are that Src inhibition through a novel small-molecule inhibitor, AP23994, alone and in combination with cytotoxic chemotherapy, significantly reduced tumor growth in both chemosensitive and chemoresistant ovarian cancer models. These effects were achieved, at least in part, through decreased tumor proliferation, and a combination of down-regulation of angiogenic cytokines such as VEGF and IL-8 as well as reduced MVD.

Src has been found to be overexpressed in a panel of human ovarian cancer cell lines as well as late stage and metastatic ovarian carcinoma (15). In fact, Src activation has been correlated with tumor prognosis in colorectal and breast carcinomas (28, 29). Furthermore, SKOV3 cells transfected with antisense c-Src constructs had reduced tumor growth in a xenograft murine model and decreased VEGF production *in vitro* (18). Because Src is a therapeutic target that resides at the nexus of multiple cellular pathways, its culpable role in ovarian cancer biology has been characterized in tumor invasion (30–32). However, to the best of our knowledge, the current study provides the first *in vivo* data in an orthotopic murine model on the direct and indirect effects of Src inhibition.

There is growing evidence that Src plays a critical role in tumor angiogenesis. Src seems to be a key regulator of IL-8, an important angiogenic cytokine (33–35). IL-8 regulation is accomplished in an Src-dependent manner via decreased phosphorylation of extracellular signal-regulated kinases-1/2 and p38 (13). Src is also essential for the hypoxia-mediated induction of VEGF (36), and Src knockdown, through antisense technology, can reduce both the constitutive and hypoxia-induced VEGF and produce tumors with decreased vascularity (37). Moreover, VEGF-mediated vascular permeability has been shown to be an Src-dependent process (38), suggesting an entry for tumor cell extravasation beyond the primary microenvironment. Recently, Weis and colleagues have shown that restoration of endothelial barrier function via Src inhibition can suppress tumor cell extravasation *in vivo* (27). The integrity of endothelial barrier may be important in tumor extravasation as a mechanism of metastasis and in ascites production. Indeed, Src inhibition was highly effective in blocking the development of ascites in our models. However, Src inhibition alone in our series of ovarian cancer models did not consistently achieve a significant reduction in the number of tumor nodules. This is not surprising, given that ovarian cancer dissemination occurs through direct tumor shedding, and not by hematogenous spread. Furthermore, as shown through the intradermal Miles assay, VEGF-mediated vascular permeability was significantly decreased in mice treated with AP23994, and this may have important therapeutic implications for patients afflicted with ovarian carcinoma. Ascites and pleural effusions are serious sequelae of the disease that pose a medical threat. In this study,

Src inhibition eliminated ascites, and this is due, in part, to the decreased activity of VEGF-mediated vascular permeability.

Our data regarding the effects of Src inhibition in reducing tumor growth is commiserate with the emerging role of Src in ovarian and other cancers. VEGF, responsible not only for tumor angiogenesis but also endothelial survival (39, 40), was down-regulated *in vivo*. It is also known that inhibition of VEGF receptor phosphorylation, which may be achieved through Src inhibition, sensitizes tumor endothelial cells to cytotoxic agents leading to apoptosis (41), thus providing a mechanism behind the additive advantage of combination therapy with AP23994 and docetaxel.

Following initial response to chemotherapy, most patients with ovarian cancer will develop chemoresistant tumors and eventually succumb to the disease (2). Src is known to promote chemoresistance, and its inhibition restores sensitivity through the activation of caspase-3-mediated apoptosis (14, 17, 21, 42). Furthermore, we have shown that *in vitro* Src inhibition by AP23846 significantly up-regulates caspase-3 activity, which may be an additional explanation for decreased tumor growth in chemoresistant models. Specifically, *in vitro* cytotoxicity was enhanced following Src inhibition even in the HeyA8-MDR cell line, rendering similar IC₅₀ levels to the chemosensitive parental cell line. These effects were also shown in the HeyA8-MDR *in vivo* model in which tumor growth was unimpeded by docetaxel monotherapy; however, AP23994 alone resulted in decreased tumor growth, which was even greater in combination with docetaxel. Because this compound was able to elicit such a favorable response across multiple ovarian cancer models, it may be efficacious in both front-line and refractory settings.

In summary, we have shown that AP23994, a purine-derived Src inhibitor, is highly effective in reducing tumor burden *in vivo*, alone and in combination therapy with a conventional cytotoxic agent in both chemosensitive and chemoresistant ovarian cancer models. Therefore, treatment with potent Src inhibitors merits further investigation, especially in ovarian carcinoma, in which chemoresistance after repeated therapy is almost inevitable.

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Figure 1. AP23846 and AP23994. Chemical structures and kinase selectivities of the two ATP-based Src kinase inhibitors.

Figure 2.

Dose and time kinetics of AP23846 *in vitro* and its cytotoxic properties. **A**, HeyA8 and SKOV3ip1 ovarian cancer cell lines were exposed to increasing concentrations of AP23846 *in vitro*. The cell lysates were then collected after 24 hours of treatment and subjected for Western analysis for pSrc^{Y419} with immunoprecipitation for total Src. **B**, subsequently, a time-kinetic experiment was completed *in vitro* for HeyA8 and SKOV3ip1. Cells were treated with 1 Amol/L of AP23846 and collected at 1, 2, 4, 8, 12, 24, and 48 hours and analyzed by Western blot for compound onset and duration of actions in pSrc^{Y419} down-regulation. **C**, cell viability assay for HeyA8 and HeyA8-MDR cells treated with docetaxel alone and in combination with AP23846. **D**, caspase-3 activity was measured for HeyA8 and SKOV3ip1 cell lines following treatment with: vehicle control, docetaxel, AP23846, or combination of docetaxel and AP23846 (*Doc + AP*), and cisplatin, a positive control (*, $P < 0.05$ compared with controls).

Figure 3.

Mean tumor weights and distribution with treatment of AP23994 alone or in combination with docetaxel. *A*, *in vivo* time kinetic experiment completed with AP23994 in which HeyA8 tumors were collected at 12 and 24 hours from animals after single treatment at 50 mg/kg. Each lane represents a different animal. Mice injected with HeyA8 (*B*), SKOV3ip1 (*C*), or HeyA8-MDR (*D*) were initiated in control (vehicle alone), docetaxel (50 Ag) once a week, AP23994 (50 mg/kg) every day, and combination of docetaxel and AP23994. When control mice were moribund (~3–4 weeks after start of therapy), animals from all groups were sacrificed, tumors excised, and weight recorded. Columns, mean of tumor weights and individual weights from these trials; bars, SD (*, $P < 0.05$ compared with controls).

Figure 4. Immunohistochemistry analysis of tumors. *A*, representative PCNA staining from tumors collected at time of necropsy in the HeyA8 therapy experiment. Graph depicts proliferation index in the four treatment arms in order of control, docetaxel, AP23994, and docetaxel + AP23994. *B*, representative CD31 staining for MVD from tumors collected after 5 days of treatment in order to assess angiogenic activity in the tumor microenvironment as an early event. Graph depicts mean MVD counts from the four treatment groups in the same order as described in (*A*). *C*, representative IL-8 staining from tumors collected at the time of necropsy in the HeyA8 therapy experiment. *D*, representative VEGF staining from tumors collected after therapy for 5 days (*, $P < 0.05$ compared with controls).

Figure 5.

Intradermal Miles assay. Female nude mice ($n = 3$), were treated with the four treatment arms for 5 days. Evan's blue dye was then injected systemically via the tail vein with subsequent intradermal injection of both PBS and VEGF (10 ng/mL). The mice skin was then unsheathed to reveal the blue area as a result of VEGF injection. PBS injection site served as positive control. Graph depicts fold increase in affected area as a ratio of VEGF over PBS (*, $P < 0.05$ compared with controls).

Table 1

Tumor characteristics in the therapy experiments

Cell line	Group	Tumor incidence (%)	No. of nodules (mean $\pm$ SD)	P values for no. of nodules
HeyA8	Control	100	5.38 $\pm$ 2.83	
	Docetaxel	100	2.67 $\pm$ 1.32	0.036
	AP23994	100	2.75 $\pm$ 1.83	0.049
	Docetaxel + AP23994	100	1.33 $\pm$ 0.52	0.008
SKOV3ip1	Control	100	11.67 $\pm$ 9.8	
	Docetaxel	100	2.8 $\pm$ 0.84	0.004
	AP23994	100	8.7 $\pm$ 5.8	Not significant
	Docetaxel + AP23994	100	1.67 $\pm$ 0.52	0.002
HeyA8-MDR	Control	100	5.0 $\pm$ 2.79	
	Docetaxel	100	6.57 $\pm$ 2.37	Not significant
	AP23994	50	1.9 $\pm$ 2.28	0.018
	Docetaxel + AP23994	62.5 [*]	2.38 $\pm$ 2.26	0.034

* Tumor incidence was five out of a total of eight mice in this group.