Targeted Blockade of JAK/STAT3 Signaling Inhibits Ovarian Carcinoma Growth

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Abstract

Ovarian carcinoma is the fifth leading cause of death among women in the United States. Persistent activation of STAT3 is frequently detected in ovarian carcinoma. STAT3 is activated by Janus family kinases (JAK) via cytokine receptors, growth factor receptor, and non–growth factor receptor tyrosine kinases. Activation of STAT3 mediates tumor cell proliferation, survival, motility, invasion, and angiogenesis, and recent work demonstrates that STAT3 activation suppresses antitumor immune responses and supports tumor-promoting inflammation. We hypothesized that therapeutic targeting of the JAK/STAT3 pathway would inhibit tumor growth by direct effects on ovarian carcinoma cells and by inhibition of cells in the tumor microenvironment (TME). To test this, we evaluated the effects of a small-molecule JAK inhibitor, AZD1480, on cell viability, apoptosis, proliferation, migration, and adhesion of ovarian carcinoma cells in vitro. We then evaluated the effects of AZD1480 on in vivo tumor growth and progression, gene expression, tumor-associated matrix metalloproteinase (MMP) activity, and immune cell populations in a transgenic mouse model of ovarian carcinoma. AZD1480 treatment inhibited STAT3 phosphorylation and DNA binding, and migration and adhesion of cultured ovarian carcinoma cells and ovarian tumor growth rate, volume, and ascites production in mice. In addition, drug treatment led to altered gene expression, decreased tumor-associated MMP activity, and fewer suppressor T cells in the peritoneal TME of tumor-bearing mice than control mice. Taken together, our results show pharmacologic inhibition of the JAK2/STAT3 pathway leads to disruption of functions essential for ovarian tumor growth and progression and represents a promising therapeutic strategy.

Introduction

Ovarian carcinoma is the leading cause of morbidity and mortality among gynecologic cancers, with more than 21,000 new cases and 14,270 deaths estimated in the United States in 2014 (1). Early-stage ovarian cancer presents with few, if any, symptoms resulting in delayed diagnosis, by which time the disease has spread beyond the ovary and is at late stage (stage III or IV). The standard treatment for women diagnosed with ovarian carcinoma is aggressive surgical debulking accompanied by cytotoxic chemotherapy consisting of carboplatin and paclitaxel. Disease that initially responds well to this treatment frequently relapses in a more aggressive form and in most cases becomes drug resistant. With the exception of antiangiogenic VEGF-targeted therapeutics or PARP inhibitors, few targeted therapies have shown efficacy, and drug-resistant recurrent ovarian cancer remains incurable. One of the reasons for the lack of success in identifying effective targeted agents is that ovarian carcinomas are a group of cancers that differ in histology, genetic alterations, and cellular signaling pathway activation. High-grade serous carcinomas (HGSC) are the most common, aggressive, and lethal subtype of ovarian carcinoma. Extensive genomic analyses showed that nearly all HGSC have p53 mutations but few common drug-targetable alterations (2). These cancers exhibit significant genetic instability and tumor heterogeneity and lack common actionable oncogenic driver mutations. Therefore, in order for targeted therapies to show efficacy, they will likely need to have wide ranging biologic effects (3).

An attractive therapeutic target, based on its common activation in ovarian carcinoma and its broad cellular activities, is STAT3. STAT3 is a cytoplasmic transcription factor that mediates cytokine and growth factor signaling upon activation by Janus family kinases (JAK), receptor tyrosine kinases, and non–receptor tyrosine kinases associated with cytokine receptors (4). In normal tissues, STAT3 activation is strictly controlled, but the phosphorylated activated form of STAT3 is constitutively expressed in more than 70% of solid and hematologic tumors (5–7). STAT3 activation contributes to tumorigenesis via multiple cellular functions and biologic processes, including proliferation, survival, angiogenesis, metastasis, inflammation, and immune evasion (8). Several studies have shown that STAT3 is constitutively activated in ovarian carcinoma cell lines and primary human tumors (7, 9, 10). Activation of STAT3 is more commonly correlated with clinically aggressive high-grade ovarian carcinoma than...
more indolent low-grade cancers (7). High levels of expression of IL6 are common in ovarian carcinoma cells, ascites, and patient sera, supporting prominent activation of an IL6/JAK/STAT3 signaling axis in ovarian carcinoma cells and the tumor microenvironment (TME; refs. 11, 12). Moreover, a number of recent studies show that STAT3 activation in inflammatory cells in the TME directly support tumor initiation and maintenance in some cancers (13–15).

The dual role of persistent STAT3 activation in tumor promotion through tumor-intrinsic and tumor-extrinsic contributions in the TME (6, 16, 17) suggests that targeted inhibition of this signaling pathway would be a promising therapeutic approach for ovarian carcinoma. However, direct therapeutic targeting of STAT3 is somewhat challenging due to the lack of drug-targetable intrinsic catalytic activity of the protein. Natural products, small-molecule and decoy oligonucleotide inhibitors have been developed (reviewed in ref. 18), with decoy oligonucleotides in early clinical development (19). An alternative, clinically relevant, and currently available strategy is inhibition of upstream JAKs (20).

The therapeutic potential of targeting the JAK2/STAT3 pathway with AZD1480, a small-molecule ATP-binding inhibitor of JAK1/2 (16), has been investigated in models of several non-ovarian solid tumors, including glioblastoma, breast, small cell lung, prostate, and gastrointestinal cancers (6, 20–25). On the basis of the importance of the IL6/JAK/STAT3 signaling axis in ovarian carcinoma, we sought to explore the therapeutic effects of AZD1480 in the context of ovarian carcinoma, using human ovarian carcinoma cell lines and a transgenic mouse model that develops spontaneous ovarian carcinoma similar to human HGSC with 100% penetrance (26, 27). Employment of an immunocompetent genetically engineered mouse (GEM) model of ovarian carcinoma enabled the analysis of AZD1480-mediated effects on primary tumor growth and on the immune and TME. In this study, we show that targeted blockade of the JAK/STAT3 pathway inhibited ascites production, tumor-associated protease activity, protumorigenic cancer-associated inflammatory mediators, and ovarian carcinoma growth in vivo. These observations identify the JAK/STAT3 pathway as a potential therapeutic target in ovarian carcinoma.

Materials and Methods

Cell lines, culture conditions, and reagents

Cell lines used in this study were chosen based on high levels of phosphorylated, constitutively activated STAT3 as detected by pSTAT3(Y705) (ref. 3 and Xiao and Connolly, unpublished data). Human ovarian carcinoma cell lines OVCAR-5, OVCAR-8, and A1847 were obtained from the Fox Chase Cancer Center (FCCC) Cell Culture Facility (Philadelphia, PA; deposited by Dr. Thomas Hamilton). The identity of the cells was authenticated by short tandem repeat (STR) analysis and comparison to early-passage stocks of the parental cells donated by Dr. Hamilton. Human ovarian carcinoma cells were cultured in RPMI-1640 media (Life Technologies) supplemented with 10% FBS (Atlanta Biologicals), penicillin/streptomycin (100 units/mL and 100 μg/mL, respectively; Life Technologies/Invitrogen), 0.25 units/mL insulin (Novo Nordisk). MOVCAr-5009 and MOVCAr-5447 were derived in our laboratory from the ascites of ovarian tumor–bearing female TgMsiir-Tag (27). MOVCAr-5009 cells stably transduced with retroviral STAT3 targeting shRNA or shMLP vector (28) were cultured in DMEM supplemented with 4% FBS, penicillin/streptomycin, and 1× insulin/transferin/sele-nium (supplied as a 100× stock by Life Technologies/Invitrogen). The structure of AZD1480 is published (16), and drug was provided by AstraZeneca (D. Huszar) and dissolved in DMSO (Sigma) for in vitro experiments. For in vivo drug dosing, AZD1480 was formulated in 0.5% hyaluronic acid/0.1% Tween 80 (Sigma). Recombinant human IL6 (PeproTech), 50 ng/mL, was administered to cells for 3 hours. Primary antibodies used were: α-pJAK2Y1007/1008, α-pSTAT3Y705, α-STAT3, and α-JAK2 (all from Cell Signaling Technology); α-β actin and α-Tag (Santa Cruz Biotechnology); and α-cleaved PARP (Millipore).

Cell viability, proliferation, and apoptosis assays

The effects of AZD1480 on ovarian carcinoma cell viability were evaluated using CellTiter-Blue Cell Viability Assay (Promega) according to manufacturer’s instructions. Cells (3 × 10^4 cells/mL) were plated in triplicate on 96-well plate, allowed to adhere for 24 hours, and then treated with AZD1480 (0–10 μmol/L) for 72 hours before analysis. To evaluate the effect of drug treatment on proliferation, 1.7 × 10^4 cells were plated in 24-well plates, incubated for 24 hours, then treated with AZD1480 (0–10 μmol/L). After 6 to 72 hours of drug treatment, plates were fixed in 4% paraformaldehyde, stained with 0.1% crystal violet, and absorbance measured at 590 nm. Induction of apoptosis was evaluated by Annexin V staining (Guava Nexin Reagent, Millipore) of cells treated with 0 to 5 μmol/L AZD1480 for 48 hours. Cytosoft 3.6.1 software (Merck Millipore). Etoposide (100 nmol/L). Sigma-Aldrich) was used as a positive control for induction of apoptosis.

Migration and adhesion assays

Migration was assayed and quantified as described (29). Briefly, 4 × 10^4 cells were suspended in serum-free media and seeded in duplicate in 24-well culture plates containing 8-μm pore inserts. Complete media were added to the bottom chamber and the plate was incubated for 24 hours at 37°C in 5% CO2. Cells were fixed with 4% paraformaldehyde, stained with 1% crystal violet in 25% methanol, and 5 bright-field images per insert (10× magnification) were taken with a CCD camera coupled to a Nikon Eclipse E800 microscope. Cellular adhesion was assayed by suspending cells in serum-free media and plating in triplicate on 96-well plates precoated with 10 μg/mL type I collagen (BD Biosciences), 2 μg/mL fibronectin (Sigma-Aldrich), or 3% bovine serum albumin (control). After 1-hour incubation, adherent cells were fixed with 4% PFA, stained with crystal violet and counted. Migration and adhesion experiments were repeated three times and the mean number of cells/tissue field (migration) or mean number of cells/well ± SEM calculated.

Immunoblot and ELISA analysis

Cells and tissue were lysed with Mammalian Protein Extraction Reagent (MPER) or Tissue Protein Extraction Reagent (TPER), respectively (Thermo Scientific). Lysis buffers were supplemented with Halt Phosphatase Inhibitor Cocktail (Thermo Scientific) and Complete Mini Protease Inhibitor Cocktail (Roche Diagnostics), and protein concentration was determined using the BCA (bicinchoninic acid) assay (Thermo Scientific). Immunoblotting

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was performed as described (3, 29): Protein extracts were subjected to SDS-PAGE on 4% to 12% gradient PAGE (Life Technologies) and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore). Membranes were blocked in 5% non-fat milk in PBST, incubated overnight at 4°C with primary antibody, followed by horseradish peroxidase–conjugated secondary antibody (GE Healthcare) and signal detected with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific). To determine the effects of AZD1480 treatment on STAT3 activation, vehicle- and drug-treated tumors were lysed in cold TPER containing protease and phosphatase inhibitors, homogenized in a Precellys 24-Dual homogenizer (Bertin Technologies) and pSTAT3Y705 determined by immunoblot analysis with a p-STAT3Y705 antibodies and by electrochemiluminescent ELISA pSTAT3Y705 assay (MesoScale Discovery) according to the manufacturer's instructions.

**Electrophoretic molecular shift assay**

The effects of AZD1480 treatment on STAT3 DNA-binding activity was performed by electrophoretic molecular shift assay (EMSA) analysis as described (30). Briefly, cells were grown in the presence or absence of AZD1480 (0, 0.1 or 1.0 μmol/mL) for 24 hours, and nuclear extracts prepared by lysis at 4°C in buffer containing 10 mmol/L HEPES, 10 mmol/L KCl, 1.5 mmol/L MgCl2, and 1 mmol/L dithiothreitol (DTT) supplemented with protease and phosphatase inhibitors (as described for immunoblotting analysis) followed by addition of 10% IGEPAL CA-630 (octylphenoxypolyethoxyethanol). Nuclei were isolated by centrifugation and resuspended in nuclear lysis buffer containing 20 mmol/L HEPES, 400 mmol/L NaCl, 1.5 mmol/L MgCl2, 0.2 mmol/L EDTA, 1 mmol/L DTT, and 5% glycerol and supplemented with protease and phosphatase inhibitors. MOVCA02099 cells stably transduced with a STAT3-targeted shRNA were used as a negative control for DNA binding, and cells treated for 3 hours with 50 ng/mL recombinant IL6 were used as a positive control. Protein concentration was determined with Bradford assay (Bio-Rad). Nuclear extracts were incubated with [32P]-conjugated oligonucleotide probe containing STAT3 consensus-binding sequence (5’-AGC TTC ATT TCC CGT AAA TCA C-3’; Invitrogen) or a mutant oligonucleotide (5’-CAT CCT TCT GGT CCC TCC TAG ATC-3’; Santa Cruz Biotechnology). DNA-binding specificity was determined by supershifting DNA–STAT3 complexes following preincubation of samples with an e-STAT3 antibody. Samples were subjected to 5% nondenaturing PAGE and gels were vacuum dried and exposed to radiography film.

**Transgenic mice and drug treatment**

All procedures involving mice were approved by the FCCC Institutional Animal Care and Use Committee (IACUC). C57BL/6 TgMISIR-TAg mice have been described (26) and are maintained under pathogen-free conditions on a standard rodent diet (2018SX Teklad Global, Harlan Laboratories; ref. 26). Female TgMISIR-TAg mice were generated by breeding male TgMISIR-TAg mice to C57BL/6J females. Transgenic female offspring were monitored by baseline MRI, as described (refs. 27, 31 and below) to confirm the presence of ovarian tumor, defined as enlargement of one ovary to 50 mm3, at which time mice were treated with vehicle (0.5% hypermellose/0.1% Tween-80) or AZD1480 (30 mg/kg in 0.5% hypermellose/0.1% Tween-80) by oral gavage (p.o.) twice daily (BID). Three separate cohorts of mice were randomized into 2 groups and treated as follows: For the in vivo drug efficacy study (cohort 1), mice (n = 17 per group) were treated with vehicle or AZD1480 on a 5 days on/2 days off schedule for 8 to 9 weeks or until mice met humane criteria for euthanasia. For analysis of AZD1480-mediated alterations in gene expression (cohort 2), mice (n = 4 per group) with about 500 mm3 tumors were treated with vehicle or 30 mg/kg AZD1480 and euthanized 6 hours later for tumor tissue collection. For detection of tumor-associated protease activity (cohort 3), mice (n = 5 per group) were treated with vehicle or AZD1480 on a 5 days on/2 days off schedule for 3 weeks.

**In vivo MRI and fluorescent molecular tomography**

Tumor growth was monitored and quantified by weekly imaging using a 7-Tesla vertical wide-bore magnet, equipped with a Bruker DRX 300 spectrometer as described (27, 31). The tumor volume was determined using 3-dimensional MRI data sets and volumetric analysis to calculate tumor volume (27). Volumetric analysis was performed on datasets that were blinded with regard to treatment group. Weekly baseline images were initiated when mice were 8 to 10 weeks old. When the volume of one ovary reached 50 mm3, drug or vehicle treatment was initiated. Once treatment commenced, mice were imaged weekly and the tumor volumes calculated. Tumor volume data were submitted to the biostatistician (S. Litwin) for analysis and determination of tumor growth rates. Prior work in our laboratory showed that ovarian tumors in TgMISIR-TAg mice express integrin αvß3 and activated tumor-associated matrix metalloproteases (MMP) that are predicted to contribute to tumor dissemination (31). To determine the effects of AZD1480 treatment on integrin αvß3 expression and tumor-associated MMP activity, mice were subjected to fluorescent molecular tomography (FMT) imaging for detection of the integrin αvß3–binding imaging probe IntegriSense and activation of the MMP-degradable fluorescent imaging probe MMPSense as described (31). IntegriSense and MMPSense fluorescent imaging probes were purchased from PerkinElmer. IntegriSense is an integrin-targeted imaging agent that binds to integrin αvß3 receptors and MMPSense is an optically silent molecular imaging probe that is activated in the presence of proteolytically active MMPs, including MMP2, 3, 7, 9, 12, and 13 (PerkinElmer, Inc.). Tumor-bearing mice (n = 5 per group) treated with vehicle or AZD1480 were given intravenous (retroorbital) injections of MMPSense and IntegriSense (PerkinElmer, Inc.) and subjected to combined FMT-MRI imaging as described (31).

**Tissue preparation and analysis**

Mice were euthanized, necropsied, and examined for tumors and the presence of ascites (defined as the obvious presence of bloody, frequently cellular fluid upon midline incision through the abdominal wall). Reproductive tracts were removed and ovarian tumors length (l) and width (w) were measured with calipers and tumor volume calculated (l x w² / 2.5; ref. 31). Individual portions of tumors were fixed in 10% neutral-buffered formalin, paraffin-embedded, and sectioned for hematoxylin and eosin (H&E) staining and immunohistochemical detection of TAg as described (26). Additional portions of tumor tissue were snap-frozen in liquid nitrogen for protein analysis and RNA extraction. To evaluate immune cell populations in peritoneal washes, spleen, and primary tumors, specimens were collected and

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processed at necropsy. Peritoneal cavities were washed with PBS supplemented with 2% FBS, spleens and portions of tumors were harvested. Spleens were dissociated between sterile glass slides; tumors were cut into small pieces and incubated in a dissociation mixture (0.09% collagenase, 0.09% dispase, 0.9% FBS in DMEM). Red blood cells were removed by incubation with 0.84% NH₄Cl. For staining of intracellular markers, cell samples were prepared with the BD Cytofix/Cytoperm Kit (BD Biosciences) according to manufacturer’s instructions.

Flow cytometry
Cells were isolated from peritoneal washes and from similar size spleen or primary tumor tissue, and cell number was determined and normalized to ensure comparable analysis was performed. Cells were preincubated with anti-FcγRII/IIB antibody (provided by L.J. Sigal) to block nonspecific binding and then stained with a mixture of fluorophore-labeled antibodies: CD45 eFlour450, CD19 PE, Foxp3 APC (eBioscience, Inc.); F4/80 APC, CD11b PE, CD4 FITC, Ly-6G/Ly-6C (Gr-1) PE-Cy7 (BioLegend). Samples of cell suspensions were analyzed with a BD LSRII flow cytometer (BD Biosciences), and data were analyzed with FlowJo software (Tree Star Inc).

Microarray analysis and quantitative RT-PCR
Mouse tissue RNA samples were prepared using the RNAeasy Mini Kit (QIAGEN) according to manufacturer’s instructions and submitted to the Fox Chase Cancer Center (FCCC, Philadelphia, PA) Genomics Facility for gene expression analyses by microarray and quantitative RT-PCR. The RNA concentrations were determined with a NanoDrop spectrophotometer (Thermo Fisher Scientific) and quality was determined by Agilent Bioanalyzer RNA kits (Agilent), and the RNA integrity (RIN) numbers were between 9.5 and 10. RNA was subsequently amplified and labeled using the Low RNA Input Linear Amplification Kit (Agilent) and labeled cRNA targets hybridized onto Agilent 44K microarrays. Raw expression data obtained from Agilent microarrays were background corrected and quantile normalized across experimental conditions (32). The LIMMA (Linear Models for Microarray Data; ref. 33) methodology was applied to the log-transformed expression data to identify differentially expressed genes across conditions. Gene Ontology (GO) enrichment analysis of the expression data to identify differentially expressed genes across conditions. Statistical analysis for all in vitro assays was conducted with Prism 5.0 (GraphPad Software). All imaging data (MRI and FMT) and endpoint analyses (tumor volume, presence or absence of ascites, flow cytometric data) were submitted to the Bioinformatics and Bioinformatics Facility for analysis. Specific analyses performed for each assessment are described in Results and figure legends. In all cases, P < 0.05 was considered significant.

Results

In vitro effects of AZD1480 treatment on ovarian carcinoma cell lines
Constitutive activation of STAT3 is common in ovarian carcinoma cell lines and tumors (7, 9, 10, 36, 37). While RNA interference strategies can be used for in vitro studies, there are currently no STAT3-specific small-molecule inhibitors that can be used for in vitro studies. As JAKs are key upstream mediators of STAT3 activation in solid tumors (16), we sought to determine the effects of targeted inhibition of JAKs on STAT3 activation in cell culture and mouse models of ovarian carcinoma. To evaluate the effects of JAK/STAT3 pathway inhibition on viability, apoptosis, and proliferation of ovarian carcinoma cells, the small-molecule inhibitor AZD1480 was chosen based on its potent JAK1/2-selective activity and inhibition of STAT3 signaling in several solid tumor models and successful use in models of other solid tumors (6, 20–25). Exposure of human (A1847, OVCAR-5, and OVCAR-8) and murine (MOVCAR-5009 and MOVCAR-5447) ovarian carcinoma cells to increasing concentrations of AZD1480 (0, 0.05, 0.1, 1, 5, and 10 μmol/L) for 24 hours resulted in dose-dependent inhibition of STAT3 activation, measured by evaluating pSTAT3 levels by immunoblot analysis (Fig. 1A) and binding to a specific radiolabeled DNA element by EMSA (Supplemental Fig. S1). Substantial reductions of pSTAT3 protein and DNA binding were observed at 0.05 or 0.1 μmol/L AZD1480, with little or no detectable levels remaining in cells treated with 1 μmol/L. Consistent with previous studies on cell lines from several solid tumors (6, 16, 20), low concentrations (0, 0.05, 0.1, and 1 μmol/L) of AZD1480 that significantly depleted pSTAT3 had no discernible effect on cell viability, although it was significantly reduced in the presence of 5 μmol/L AZD1480 (Fig. 1B and Supplementary Fig. S1A). In agreement, accumulation of Annexin V+ apoptotic cells was seen in the presence of 5.0 μmol/L AZD1480, with cleaved PARP levels observed at lower concentrations of drug (0.5–1.0 μmol/L; Fig. 1C). Similarly, cell proliferation was inhibited only in the presence of high concentration (5 μmol/L) AZD1480 (Fig. 1D). Similar results were observed in cultured murine ovarian carcinoma (MOVCAr) cells; AZD1480 treatment or inhibition of STAT3 by STAT3-targeting shRNA or the small-molecule STAT3IC had little effect on cell
AZD1480 treatment reduces phosphorylated STAT3 levels and inhibits ovarian carcinoma cell migration and adhesion. A, human (A1847, OVCAR-5, and OVCAR-8) and murine (MOVCAR-5447 and MOVCAR-5009) ovarian carcinoma cells were treated with DMSO (vehicle) or increasing concentrations of AZD1480 (0.05, 0.1, 1, 5, 10 μmol/L) for 24 hours and protein lysates subjected to immunoblot analysis with antibodies recognizing pSTAT3TY705 and total STAT3. B, A1847, OVCAR-5, and OVCAR-8 cells were grown in the presence of DMSO (vehicle) or increasing concentrations of AZD1480 (0.05, 0.1, 0.5, 1, 5, 10 μmol/L) for 72 hours and cell viability was determined by CellTiter Blue Viability Assay. Data indicate the mean percentage viability calculated from triplicate samples from 3 independent experiments (±SEM). C, OVCAR-5 and OVCAR-8 cells were treated with 0, 0.5, 1, or 5 μmol/L AZD1480 for 48 hours and analyzed for the presence of Annexin V-PE+ cells and PARP cleavage. Data shown are the mean values (±SEM) from 3 independent experiments. Cleaved PARP levels were detected by immunoblot analysis. D, the effect of increasing concentrations of AZD1480 on cell proliferation was determined by exposure of A1847, OVCAR-5, and OVCAR-8 cells to 0, 0.1, 1, or 5 μmol/L AZD1480 for 6, 24, 48, and 72 hours. Cells were fixed and stained with crystal violet, and plates read on a spectrophotometer to determine the optical density (OD590 nm). Data are presented as the mean OD590 nm ± SEM (n = 3). E, chemotactic migration assays were performed to determine the effects of 1.0 μmol/L AZD1480 treatment on migration of A1847, OVCAR-5, and OVCAR-8 cells. F, the effects of 1.0 μmol/L AZD1480 treatment on ovarian carcinoma cell adhesion to fibronectin and type I collagen was determined. The bars depict the mean number of migrated or adherent cells ± SEM (n = 3).

Statistical analysis for data collected from viability and apoptosis assays was performed using a one-way ANOVA test followed by a multiple comparison test; proliferation assay data were analyzed with 2-way ANOVA followed by the multiple comparison test; migration and adhesion data were analyzed with an unpaired t test. P < 0.05 was considered significant (*, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001).
viability or proliferation (Supplementary Fig. S1B–S1D). Collectively, these results show that low doses of AZD1480 potently inhibited STAT3 activation in cultured ovarian carcinoma cells without significantly affecting the viability or proliferative capacity of these cells. Although higher doses of AZD1480 induced significant apoptosis, these findings suggest that additional factors (s) besides direct induction of apoptosis in cultured ovarian carcinoma cells (e.g., effects on the tumor microenvironment and/or cytokine or chemokine signaling) may contribute to the full effect of the drug in vivo.

We therefore sought to evaluate the effect of AZD on other aspects of ovarian carcinoma cell biology in cultured cells. Dissemination of ovarian carcinoma occurs primarily through local spread in the abdominal cavity, requiring the capacity of tumor cells to shed from the primary tumor, migrate, and adhere to secondary sites in the peritoneum (38). Previous work showed that activated STAT3 is a key mediator of ovarian carcinoma motility (7, 39); therefore, the effects of AZD1480 on cell migration were evaluated using Transwell chemotactic migration assays. Migration was significantly reduced in A1847, OVCAR-5, and OVCAR-8 cells treated with 1 μmol/L AZD1480 (Fig. 1E). Similarly, pretreatment with 1 μmol/L AZD1480 inhibited the capacity of A1847, OVCAR-5, and OVCAR-8 cells to adhere to fibronectin or type I collagen (Fig. 1F). Together, these results show that AZD1480 inhibits STAT3 activation and ovarian carcinoma cell migration and adhesion, suggesting the potential for blockade of JAK/STAT3 signaling to inhibit ovarian carcinoma growth and dissemination in vivo.

AZD1480 inhibits ovarian carcinoma growth and ascites production in transgenic mice

We used a transgenic mouse model of ovarian carcinoma to evaluate the therapeutic potential of AZD1480 in vivo. Transgenic MISIR-TAg mice develop spontaneous ovarian carcinoma that resembles high-grade serous ovarian carcinoma with 100% penetrance (26, 27). This model is particularly appropriate for this analysis, as full transformation by SV40 large TAg requires STAT3 (40), and tumors and cell lines derived from tumor-bearing mice exhibit high levels of constitutively activated STAT3 (Fig. 1A and data not shown). Moreover, the presence of a functional immune system enables evaluation of the effects of JAK/STAT3 inhibition on the immune TME. To assess the impact of AZD1480 on tumor growth, mice were dosed twice daily with 30 mg/kg by oral gavage, based on previous studies (16, 20) and on pilot pharmacodynamic studies; in tumor-bearing MISIR-TAg mice, treatment with 30 mg/kg AZD1480 resulted in drastically reduced pSTAT3Y705 levels at 2 and 6 hours posttreatment, with recovery at 24 hours (Supplementary Fig. S2). The presence of tumors in mice was confirmed by MRI, once ovary volume reached 50 mm³ (Fig. 2A–B), mice were randomized for treatment with AZD1480 or vehicle (n = 17 mice per group). Mice were treated for 8 to 9 weeks, and tumor growth was monitored and quantified by weekly MRI. Longitudinal growth data demonstrated a highly significant (P < 0.0001) delay in tumor growth rate and smaller ovarian tumors (P < 0.0014) in the AZD1480-treated mice compared with vehicle-treated controls (Fig. 2C and D). Caliper measurements confirmed the tumor volumes calculated using MRI data, showing significantly smaller tumors in AZD1480-treated mice (Figs. 2E and 3A). Moreover, significantly fewer mice had detectable ascites at necropsy; 2 of 17 (12%) AZD1480-treated mice compared with 12 of 17 (70%) vehicle-treated mice (Fig. 2F, P = 0.0013). This study was designed to evaluate the effect of a limited period of AZD1480 treatment on the growth of radiologically confirmed, actively growing tumors. As such, the study did not address prevention of tumor formation or survival after drug withdrawal. In many cases, vehicle-treated mice met humane criteria for euthanasia before the study endpoint; on average, mice were vehicle-treated for 39 days and lived to 158 days. Most mice in the AZD1480 group remained on drug for the duration of the study (on average, AZD1480-treated 53 days) and lived to 181 days. Although the mice in the AZD1480-treated group lived longer (P = 0.014), tumors grew while mice were on drug (compare Fig. 2B with C–E), suggesting tumors would continue to progress if drug were withdrawn.

To confirm AZD1480-mediated inhibition of STAT3 activation, snap-frozen tumor tissue specimens were evaluated by immunoblot and ELISA for detection of pSTAT3Y705 levels. Immunoblot analysis showed reduced pSTAT3Y705 levels (Fig. 3B). An ELISA was used to quantify pSTAT3Y705 levels, further validating the significant (P < 0.0001) AZD1480-mediated reduction of STAT3 activation in tumors (Fig. 3C). Taken together, these results show that AZD1480 results in significant tumor growth inhibition and that this effect may be due, at least in part, to reduction of STAT3 activity in primary tumors.

Gene expression is altered in tumors from AZD1480-treated mice

The primary functional consequences of STAT3 activation are related to its role as a transcription factor (41). To define the effects of AZD1480 treatment on STAT3-mediated transcription, RNA was isolated from ovarian tumors of mice that were treated with AZD1480 or vehicle (n = 4 per group), and global changes in gene expression were analyzed by genome-wide microarray analysis (Geo accession number GSE63092: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE63092). Unsupervised clustering analysis allowed separation into 2 groups: drug-treated tumors clustered separately from vehicle-treated controls (data not shown). Using 2-fold change and P < 0.01 cutoff, we identified a list of 10 upregulated and 87 downregulated genes in AZD1480-treated tumors (Fig. 4A and Supplementary Table S1). Notably, the list of differentially expressed genes included a large number of genes previously shown to be regulated by STAT3 (42), including genes involved in cancer and wound healing and inflammation and immune response (e.g., Ccl12, Ccl11, Cxcl10, Cxcl12, Cxcl9, Icam1, Cd59, Fas, Oas1, Oas2, Usp18). Differentially expressed genes were found to be significantly enriched for ribonucleoside metabolic process and IFN and cytokine-mediated signaling pathways with GO enrichment analysis (Supplementary Table S2). A subset of genes was selected for individual validation by qRT-PCR. Differential expression was shown for Ccl12, Cxcl11, Cxcl10, Cxcl12, Stat3, and Rasd1 (Fig. 4B). These results confirm the direct inhibitory effects of AZD1480 on STAT3 transcriptional activity in vivo and strongly suggest that the observed tumor growth inhibition result was due to inhibition of STAT3.

AZD1480 treatment inhibits tumor-associated integrin αvβ3 expression and MMP activity

Expression of integrin αvβ3 and activation of tumor-associated MMPs in tumor and stromal cells is an essential component of ovarian carcinoma growth and dissemination (43–46). As MMP
expression can be induced by activated STAT3 (42, 47–49), we hypothesized that the AZD1480-mediated tumor growth inhibitory effects were due, in part, to inhibition of tumor-associated integrin αvβ3 signaling and MMP activation. To investigate this directly, we used methods developed in our laboratory for combined anatomic and FMT to detect and quantify in vivo integrin αvβ3 expression and MMP activation (31). Mice with ovarian tumors were treated with vehicle or AZD1480 (n = 5 per group) for 4 weeks and imaged weekly by MRI and FMT to determine tumor volume and to quantify integrin αvβ3 and MMP probe binding and activation. Once again, AZD1480-treated mice exhibited significantly reduced tumor growth as evidenced by MRI (Fig. 5A and B). In addition, there was a significant reduction in integrin αvβ3 probe binding and in MMP probe activation (Fig. 5A, C, and D). These results confirm the in vivo tumor growth inhibitory effects of AZD1480 treatment and suggest that the tumor-inhibitory effects of this agent are mediated, in part, by decreased tumor-associated integrin αvβ3 expression and MMP activity.

Effects of JAK/STAT3 inhibition on immune cell populations

The well-established feed-forward signaling between STAT3 in tumors and in the microenvironment (50, 51) and the lack of effect of AZD1480 treatment on cell growth, viability, and survival in cultured ovarian carcinoma cells suggested that the tumor growth inhibitory effects of targeted disruption of the JAK/STAT3 pathway may not be confined to the activity of the drug only in tumor cells. Current thinking in ovarian carcinoma research...
emphasizes the potential importance of targeting the protumorigenic inflammatory response as an adjunct to conventional and tumor-targeted therapies (52). At the outset of this study, we hypothesized that targeted inhibition of JAK/STAT3 with AZD1480 would exert effects on both tumor cells and on immune cells in the TME, for example, by inhibiting protumorigenic, immunosuppressive tumor-associated macrophages, myeloid-derived suppressor cells (MDSC), or regulatory T (Treg) cells. By using an immunocompetent GEM model of ovarian carcinoma, we were able to assess the effects of AZD1480 on immune cell subpopulations in the spleen, tumors, and in the peritoneal microenvironment, the principal site of ovarian carcinoma dissemination. Immune cell populations were stained and analyzed by flow cytometry for detection of CD45\(^+\)CD11b\(^+\)CD4\(^+\)FoxP3\(^+\) T cells, CD45\(^+\)F4/80\(^+\)CD11b\(^+\) Gr1\(^-\) MDSCs, and CD45\(^+\)CD4\(^+\)FoxP3\(^+\) T cells, in a subset of the original cohort of 34 mice evaluated for drug efficacy. Analysis of tumors showed that the number of infiltrating leukocytes was small and that the majority of these cells were CD45\(^+\)F4/80\(^+\)CD11b\(^+\) macrophages (Supplementary Fig. S3A). Notably, analysis of leukocyte populations present in the peritoneal cavity showed that while there were no significant differences in CD45\(^+\)F4/80\(^+\)CD11b\(^+\) macrophages or CD45\(^+\)CD11b\(^+\)GR-1\(^-\) MDSCs (Supplementary Fig. S3B), there was a significant reduction in both the number and percentage of CD45\(^+\)CD4\(^+\) and CD45\(^+\)CD4\(^+\)FoxP3\(^+\) T cells in AZD1480-treated mice compared with controls (Fig. 6A and B). There were no significant differences in either of these populations detected in the spleens of vehicle- and drug-treated mice (Fig. 6A and B). These observations show that AZD1480 treatment is accompanied by reduction of protumorigenic T-cell subpopulations in the peritoneal cavity, whereas splenic immune cell subpopulations remain unchanged.

**Discussion**

A significant majority of patients with advanced stage ovarian carcinoma experience disease recurrence and eventually develop resistance to front-line chemotherapy. With the exception of antiangiogenic therapies (e.g., bevacizumab) or PARP inhibitors, few promising agents have emerged for patients with recurrent and/or drug-resistant disease that meaningfully or durably extend survival. In cancers like ovarian carcinoma that lack common, specific, drug-targetable oncogenic driver mutations (2), therapeutic targeting of proteins that have broad-spectrum protumorigenic activities may be the most effective strategy. We have already explored this approach, showing that inhibition of HSP90 was highly effective in preclinical models of ovarian carcinoma (3), and establishing a clinical trial to evaluate ganetespib, a small-molecule HSP90 inhibitor, in combination with paclitaxel in ovarian carcinoma in patients (NCT01962948). Like HSP90, the importance of persistent activation of STAT3 in a variety of human cancers, including ovarian carcinoma, is well-established (5, 7, 9, 10). STAT3 activation may occur via multiple upstream signals, including activation via IL6/IL6R-mediated activation of JAK2, which is a prominent mechanism present in ovarian carcinomas (11, 12). Activated growth factor receptor and non–growth factor tyrosine kinases (e.g., Src family kinases) may also play a role in STAT3 activation, with prior work suggesting that JAK family kinases may cooperate with these proteins to activate STAT3 (16). Constitutive STAT3 activation has tumor cell intrinsic consequences as well as effects within the extracellular matrix (ECM) and stromal cells of the tumor microenvironment, resulting in increased tumor cell proliferation, survival, motility, and invasiveness, as well as tumor-promoting angiogenesis and evasion of tumor-suppressing immunity. While these broad ranging
activities make STAT3 an attractive therapeutic target, druggability has been challenging due to the lack of intrinsic catalytic activity of the protein. In the absence of direct pharmacologic inhibitors of STAT3 that are suitable for in vivo use, an alternate strategy for inhibiting STAT3 function is to target direct upstream activators or other mediators of its function and/or stability. Our prior work showed significant ganetespib-mediated inhibition of STAT3 activation in cultured ovarian carcinoma cells and xenografts, and synergistic inhibition of ovarian carcinoma cell viability when ganetespib was combined with a JAK inhibitor (3).

In this study, we investigated the potential efficacy of directly targeting JAK/STAT3 activation in ovarian carcinoma using the small-molecule JAK1/2 inhibitor AZD1480. Consistent with previous studies in other solid tumors (6, 16, 20), AZD1480 treatment resulted in significant inhibition of STAT3 phosphorylation (pSTAT3Y705) and DNA binding at low drug concentrations;
However, similar concentrations of drug had little effect on cell viability, proliferation, or survival of cultured ovarian carcinoma cells. It is now becoming clear that cytotoxic effects of JAK inhibitors are largely limited to cells of hematologic origin, particularly JAK-mutant MPN lines, and rarely seen in solid tumor cell lines in culture. The work describing AZD1480 (16) evaluated the effects of this compound and similarly showed low or no cytotoxicity in several cultured solid tumor cell lines but tumor growth inhibition in xenografts. Similar observations of significant tumor growth inhibition of cell line xenografts that exhibited growth inhibition in xenografts. The work describing AZD1480 (16) evaluated particularly JAK-mutant MPN lines, and rarely seen in solid tumor inhibitors are largely limited to cells of hematologic origin, particularly JAK-mutant MPN lines, and rarely seen in solid tumor cell lines in culture. The work describing AZD1480 (16) evaluated the effects of this compound and similarly showed low or no cytotoxicity in several cultured solid tumor cell lines but tumor growth inhibition in xenografts. Similar observations of significant tumor growth inhibition of cell line xenografts that exhibited low in vitro cytotoxicity have been made in several solid tumor models (6, 20–25, 53). The underlying reasons for these seemingly discordant results are unclear but may be attributed to possible effects specific to conditions of 2D cell culture or alternatively effects of the tumor microenvironment and/or disruption of a critical cytokine-mediated signaling axis, for example, IL6 (6, 21). The capacity of cells to migrate and adhere to type I collagen or fibronectin was also significantly reduced in cells treated with AZD1480. This finding is consistent with prior work demonstrating a direct role for STAT3 in mediating migration in cultured ovarian carcinoma cells (7). Therefore, although AZD1480 exhibited low cytotoxicity in cultured ovarian carcinoma cells, it did suppress STAT3 activity via decreased phosphorylation and DNA binding and by inhibiting STAT3-related functional properties required for ovarian carcinoma dissemination. Maximal effects of JAK-targeted inhibition may require the complex interaction of the TME, which is supported by the "feed forward loop" described in mammary and renal carcinomas and by the protumorigenic effects of JAK/STAT3 signaling in immune cells in the TME (13–15).

In vivo, we showed significant inhibition of tumor growth rate, final tumor volume, and production of malignant ascites in ovarian tumor-bearing transgenic mice treated with AZD1480 as a single agent. Tumor growth inhibition was accompanied by reduced levels of pSTAT3 
\[\text{S}^{\text{Tyr705}}\], significant inhibition of tumor-associated MMP activity and altered gene expression in the AZD1480-treated mice. Many of the differentially expressed genes identified by microarray analysis are well-established STAT3 transcriptional targets (42). Direct AZD1480-mediated effects on tumor cell proliferation and survival are suggested by significant downregulation of Cyclin D1 (Ccd1), a key mediator of STAT3-induced proliferation, and upregulation of Rasd1, a Ras-related protein associated with induction of apoptosis (10, 54). Expression and activation of tumor-associated MMPs and signaling through integrins is central to ECM remodeling and ovarian carcinoma tumor progression in human ovarian carcinoma and in Msisir-TAg transgenic mice (31, 43–48). However, tumor-associated MMPs are largely produced by stromal cells, and detection of MMP protein levels by immunohistochemical or immunoblotting analyses does not provide information about tumor-associated protease activity (55). Using a combined FMT/MRI approach developed in our laboratory (31), we showed JAK inhibitor treatment resulted in significant inhibition of integrin \(\alpha v \beta 3\) probe binding and MMP probe activation, strongly supporting a central role for JAK/STAT3 in regulation of tumor-associated MMP activity. The significant reduction in ascites formation in AZD1480-treated mice may also be related to inhibition of tumor-associated MMPs in the TME.

Interestingly, analysis of differentially expressed genes in tumors from vehicle- and AZD1480-treated mice revealed subsets of genes related to interferon and cytokine responses as well as genes related to innate immune response, host defense, and immune system processes. These changes strongly support the importance of JAK/STAT3 signaling in interactions of tumor cells and tumor-associated inflammation in ovarian carcinoma development. Prior work demonstrated alterations in infiltrating immune cell populations in immunocompetent mouse models of mammary carcinoma (6). Specifically, mice treated with AZD1480 exhibited significant reduction of tumor-infiltrating CD45\(^{+}\)CD11b\(^{+}\)Gr-1\(^{-}\) MDSC. In our analysis of infiltrating immune cells present in tumors and the peritoneal TME, no significant differences in the number or percentage of immunosuppressive CD45\(^{+}\)CD11b\(^{+}\)Gr-1\(^{-}\) MDSCs or CD45\(^{+}\)F4/80\(^{+}\)CD11b\(^{+}\)macrophages were shown. The reasons for the lack of effect on MDSCs are unclear but suggest that the effects of JAK inhibitors may be tissue-specific. Interestingly, there was a significant reduction in both CD45\(^{+}\)CD14\(^{+}\) T cells and CD45\(^{+}\)CD4\(^{+}\)Foxp3\(^{+}\) T cells present in the peritoneal tumor microenvironment. It is possible that the reduction in Foxp3\(^{+}\) cells may be a reflection of the reduction in total number of CD45\(^{+}\)CD14\(^{+}\) cells,
but this appears not to be a general effect of the drug, as there is no difference in these subpopulations in the spleens of drug- or vehicle-treated mice. The reduction of CD45⁺CD4⁺Foxp3⁺ cells is noteworthy, as Treg cells are key mediators of immunosuppression and angiogenesis, and the presence of high levels of Treg cells is inversely correlated with survival in patients with ovarian carcinoma (56, 57). The significant depletion of CD45⁺CD4⁺Foxp3⁺ cells in the peritoneal TME of AZD1480-treated mice suggests that tumor growth inhibition is mediated, at least in part, by effects of the drug on tumor-associated immunosuppressive inflammatory cells. Unlike the inhibition of Treg cells in the peritoneal cavity, we observed a relatively low number of tumor-infiltrating leukocytes in primary tumors from vehicle- or drug-treated mice. Therefore, while AZD1480-mediated inhibition may block ascites formation via effects on protumorigenic inflammation, primary tumor growth may be affected to a lesser extent by drug-mediated effects on inflammation. While AZD1480 exhibited little toxicity in monolayer cultured cells, we cannot rule out the possibilities that it may inhibit growth or survival cells in tumors in vivo or may influence other elements of the microenvironment including the protumorigenic activities of tumor-associated fibroblasts or angiogenesis or cytokine and/or chemokine signaling networks (6, 21).

Further studies of the effects of targeted inhibition of JAK/STAT3 inhibition on Treg cell production and function are warranted, as these cells are believed to be central to ovarian carcinoma tumor development and progression, and depletion of these cells has been proposed as a therapeutic strategy for treatment of patients with ovarian carcinoma.
Consistent with observations in preclinical models of other solid tumors \cite{6,16,21,24,25}, our data show that targeted inhibition of the JAK/STAT3 pathway with AZD1480 significantly inhibits ovarian tumor growth in a transgenic mouse model of ovarian carcinoma. Our data suggest that the pharmacologic inhibition of JAK1/2 with AZD1480 has direct effects on tumor cells as well as tumor-associated MMPS in the ECM and infiltrating immune cells in the peritoneal microenvironment. However, the absence of tumor regression suggests that the most effective strategy for incorporation of JAK/STAT3 pathway inhibitors will be by identifying combination therapies that maximize antitumor response. A recent study investigating the combination of a JAK inhibitor (ruxolitinib) and cisplatin in NSCLC showed the combination was additive or antagonistic in cisplatin sensitive cells with “low” JAK2 expression but synergistic or strongly synergistic in cisplatin-resistant cells with “high” JAK2 levels \cite{58}. This suggests that combination of a JAK/STAT3 inhibitor with platinum therapy may be effective, particularly in cancers with high levels of JAK/STAT3 activation. The significant and broad effects of JAK/STAT3 pathway inhibition support further investigation of this class of inhibitors in combination with standard cytotoxic agents or other targeted therapeutics for treatment of ovarian carcinoma.

Disclosure of Potential Conflicts of Interest

D. Huszar has ownership interest (including patents) in AstaZeneca. No potential conflicts of interest were disclosed by the other authors.

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References


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