Molecular Cancer Therapeutics

JAK2 Inhibitor SAR302503 Abrogates PD-L1 Expression and Targets Therapy-Resistant Non–small Cell Lung Cancers

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Abstract

Lung cancer is the leading cause of cancer-related deaths worldwide. Approximately 85% of all lung cancers are non–small cell histology [non–small cell lung cancer (NSCLC)]. Modern treatment strategies for NSCLC target driver oncogenes and immune checkpoints. However, less than 15% of patients survive beyond 5 years. Here, we investigated the effects of SAR302503 (SAR), a selective JAK2 inhibitor, on NSCLC cell lines and tumors. We show that SAR is cytotoxic to NSCLC cells, which exhibit resistance to genotoxic therapies, such as ionizing radiation, cisplatin, and etoposide. We demonstrate that constitutive IFN-stimulated gene expression, including an IFN-related DNA damage resistance signature, predicts for sensitivity to SAR. Importantly, tumor cell–intrinsic expression of PD-L1 is IFN-inducible and abrogated by SAR. Taken together, these findings suggest potential dual roles for JAK2 inhibitors, both as a novel monotherapy in NSCLCs resistant to genotoxic therapies, and in tandem with immune checkpoint inhibition. *Mol Cancer Ther; 17(4); 732–9. ©2018 AACR.*

Introduction

Lung cancer is the leading cause of cancer-related deaths worldwide. In the United States alone, lung cancer accounts for more cancer-related deaths than prostate, breast, and colorectal cancers combined (1). Approximately 85% of lung cancers are non–small cell lung cancers (NSCLC) (2). Standard therapies for patients with locally advanced or metastatic lung cancer consist of radiotherapy and chemotherapy. However, most patients fail to achieve long-term survival with such treatments.

Analyses of NSCLC genomes have identified key driver mutations leading to advances in targeted therapies, which have improved survival (3, 4). Targeted therapies against EGFR and ALK have been approved as first-line treatments for NSCLC patients harboring corresponding genomic aberrations. More recently, immunotherapies targeting the immune checkpoints programmed death 1 (PD-1) and programmed death ligand 1 (PD-L1) have demonstrated improved survivals in metastatic NSCLC patients previously treated with genotoxic chemotherapy (5, 6). Nevertheless, most patients relapse after initial responses to targeted agents and immunotherapies, thus supporting the need for alternative lung cancer treatments.

Emerging evidence supports the existence of oncogenic JAK/STAT signaling in various human malignancies. The JAK/STAT pathway is triggered by extracellular signals, including IFN, through interactions of cell surface receptors and JAKs, which activate STAT proteins, allowing nuclear translocation and resulting in the expression of hundreds of downstream genes involved in growth, metastasis, and immunity (7–9). We and others have previously demonstrated that constitutively activated JAK/STAT signaling is associated with resistance to radiotherapy and genotoxic chemotherapies in human cancers (3, 8, 10–12). JAK2 inhibitors are widely used in various autoimmune and myeloproliferative disorders as well as hematologic malignancies due to the prevalence of activating somatic V617F JAK2 mutations in these diseases (13, 14). However, activating JAK2 mutations are rare or absent in most solid tumors, although some solid tumors demonstrate amplification of JAK/STAT effectors (3, 13). Therefore, identifying those solid tumors that exhibit sensitivity to JAK/STAT inhibition remains a challenge, and biomarkers are needed to optimize the potential use of JAK inhibitors in cancer patients.

PD-L1 is an IFN-inducible gene regulated by JAK/STAT signaling and is expressed in a wide array of solid malignancies (6, 15, 16). Importantly, the PD-1/PD-L1 axis is a central inhibitor of antitumor immune responses. Clinical studies indicate that increased tumor cell expression of PD-L1 correlates with poor outcomes (17, 18). In contrast, NSCLCs exhibiting elevated expression of PD-L1 have improved responses to nivolumab and pembrolizumab as compared with patients with undetectable or low expression of PD-L1 (19–21). Although a positive correlation between the level of PD-L1 protein expression and response to PD-1/PD-L1 immunotherapy has been described for NSCLC, additional biomarkers such as immune cell infiltration, T-cell clonality, somatic mutational burden, and other genomic

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Note: Supplementary data for this article are available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

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doi: 10.1158/1535-7163.MCT-17-0667

signatures are being investigated as potential predictors of immunotherapy response (22–24). In addition, there is significant interest in utilizing drugs to potentiate the effects of immunotherapies. Recently, it was demonstrated in preclinical models that ruxolitinib, a JAK1/JAK2 inhibitor, suppressed PD-L1 expression and improved responses to anti-CTLA4 antibody therapy when administered sequentially, but not concurrently, with immune checkpoint blockade therapy (25). Further investigations into potential combination therapies to enhance antitumor immune responses are essential.

Here, we demonstrate the JAK2 inhibitor SAR302503 (SAR; formerly known as TG101348) (26) suppresses activation of STAT1 and STAT3 in the context of type II IFN signaling in NSCLC cell lines. We show that SAR decreases tumor cell growth and survival in a cell-autonomous context. In addition, we identify a subgroup of NSCLCs that is resistant to genotoxic lung cancer therapies, including ionizing radiation, cisplatin, and etoposide, which exhibit sensitivity to SAR. We characterize constitutive IFN-stimulated gene (ISG) expression as predictive of tumor response to SAR therapy. Finally, we demonstrate that SAR suppresses PD-L1 expression in NSCLC cells following type II IFN stimulation. Taken together, these data highlight dual actions of and novel applications for JAK2 inhibition in the treatment of therapy-resistant NSCLCs.

Materials and Methods

Reagents

SAR302503 [TG101348, see (26) for chemical structure] was acquired from Sanofi-Aventis and diluted in DMSO per the manufacturer's instructions. Ruxolitinib was purchased from Selleck Chemicals. Cisplatin and etoposide were manufactured by PCH Pharmachemie. Stock solutions were diluted in DMSO (Sigma-Aldrich) prior to further dilution in cell culture media. Human and mouse IFNγ was purchased from R&D Systems.

Cell lines

All lung cancer cell lines were previously authenticated and kindly provided by Dr. Matthew Meyerson (Dana-Farber Cancer Institute, Harvard Medical School received in 2013). Cell lines were maintained in DMEM supplemented with 10% heat-inactivated FBS, 100 U/mL penicillin, and 0.1 mg/mL streptomycin (GIBCO). Cell lines were tested for mycoplasma every 6 months using the Mycosensor PCR Assay (Agilent Technologies; last tested in June 2017).

Clonogenic survival assays

Cell lines were plated in triplicate at densities ranging from 2×10^2 to 2×10^3 per 100 mm culture dish. Twenty-four hours after plating, the cell lines were treated as follows: (i) ionizing radiation (2 or 5 Gy); (ii) cisplatin (0.5 μg/mL); (ii) etoposide (0.1 μg/mL); and (iv) SAR203502 (500 or 1,000 nmol/L). Each cell culture plate was washed with 0.85% NaCl solution and stained with 2% crystal violet (Thermo Fisher Scientific). Colonies comprised of at least 50 cells were then manually counted. *In vitro* experiments based on clonogenic survival assays were performed in triplicate.

Western blot analysis

All cell lines were grown in 75 cm^2 cell culture flasks to 80% to 100% confluency. Western blot analysis was performed using conventional techniques with anti-pStat1 (sc-16570), anti-Stat1 (sc-464), anti-Stat2 (sc-476), anti-pStat3 (sc-8059), anti-Stat3 (sc-8019), anti-Stat5 (sc-377069), anti-Jak2 (sc-278), anti-IRF9 (14167-1-AP), and anti–β-actin (sc-47778HRP) antibodies. Antibodies were obtained from Santa Cruz Biotechnology and Proteintech Group.

Flow cytometry

Cell lines were plated in triplicate in 6-well plates and treated with increasing doses of SAR (1, 2, or 5 μmol/L). Twenty-four hours following SAR treatment, cells were treated with IFNγ (20 ng/mL). Forty-eight hours later, the cells were harvested for flow cytometry. Cells were then stained with antibodies against PD-L1 (BioLegend). Samples were collected on FACSCalibur Flow Cytometer (BD Biosciences) and data were analyzed using FlowJo software (Tree Star Inc.).

Animal models

To examine the effects of SAR on *in vivo* tumor growth, 7- to 8-week-old athymic nude mice were purchased from Harlan Laboratories. NCIH1944 cells were selected on the basis of sensitivity to SAR *in vitro*. Athymic nude mice ($n = 5$/group) were subcutaneously injected with 1×10^7 cells at one hind limb. Tumor volume was measured twice weekly with calipers, and tumor volume was approximated using the equation for an ellipsoid: abc/2. Mice were sacrificed when tumors reach 2,000 mm^3. Mice treated with SAR received 120 mg/kg twice a day via oral gavage. Mice treated with ionizing radiation received 10 Gy × 2 fractions using the RadSource Technologies X-ray RS-2000 Biological Irradiator operating at 160 kVp and 25 mA at a dose rate of 2.20 Gy/minute. Animal experiments were performed in duplicate in a confirmatory manner. All studies performed on mice were approved by the IACUC of the University of Chicago (Chicago, IL).

Analysis of cell line and clinical cancer datasets

NSCLC genomic and expression datasets were collected from the Broad-Novartis Cancer Cell Line Encyclopedia (CCLE). Clinical cancer datasets and corresponding microarray gene expression were downloaded from Gene Expression Omnibus (GEO) using the identifiers GSE31210, GSE37745, and GSE14814. Probe set intensities were preprocessed, normalized, and quantified as indicated for each respective study. Ingenuity Pathway Analysis (IPA) was used to identify top-ranked gene networks and cellular functions. TSP-IRDS scores were calculated as described previously (10).

Statistical analysis

All analyses were performed with JMP 9.0 (SAS Institute Inc.). Student's *t* tests were used to calculate differences between normally distributed continuous variables. ANOVA was used to calculate differences among samples when the total number of groups was greater than two. Mann–Whitney *U* tests were used to calculate differences between nonnormally distributed continuous variables when sample sizes were small. Log-rank tests or Cox proportional hazards models were used to assess differences in overall survival between patient groups. A P value ≤ 0.05 was considered statistically significant.

Results

SAR302503 suppresses STAT1 and STAT3 signaling in NSCLC cells

We initially compared the *in vitro* cytotoxic actions of SAR302503 (SAR; JAK2 inhibitor) and ruxolitinib (JAK1/JAK2

inhibitor) on a panel of human and murine cancer cell lines. We found SAR was cytotoxic at concentrations between 0.1 and 1 μmol/L, whereas ruxolitinib had minimal to no cytotoxicity below 1 μmol/L concentrations (Supplementary Fig. S1). On the basis of these results, we further evaluated the effects of SAR on JAK/STAT–dependent signaling in NSCLC cell lines. A549 and NCIH460 NSCLC cells were stimulated with type II IFNγ in combination with increasing doses of SAR and assessed for STAT1 (Y701) and STAT3 (Y705) protein phosphorylation using Western blot analysis. The data demonstrate that SAR suppressed IFNγ-induced STAT1 and STAT3 phosphorylation in a dose-dependent manner (Fig. 1A and B). In contrast, SAR had no measurable effects on total STAT1 or STAT3 protein levels. These results confirmed that SAR suppresses type II IFN-inducible activation of STAT1 and STAT3 in NSCLC tumor cells.

NSCLCs exhibit differential sensitivities to oncologic therapies

We characterized the sensitivities of sixteen NSCLC cell lines to chemotherapies [etoposide (0.1 μg/mL) or cisplatin (0.5 μg/mL)] and ionizing radiation (5 Gy), which are utilized in the treatment of clinical NSCLCs, as well as to SAR (1 μmol/L) using clonogenic survival assays (Supplementary Table S1). K-means clustering indicated the presence of four subgroups of NSCLC cell lines with varying sensitivities to individual treatments (Fig. 2A; Supplementary Table S1). Cluster 4 was comprised of four cell lines (NCIH2228, NCIH23, NCIH460, and HCC78) that exhibited relative sensitivity to chemotherapies and ionizing radiation, whereas Cluster 2 included five cell lines (A549, COLO699, NCIH1437, NCIH2030, and MORCPR) that exhibited relative resistance to all treatments. Both Cluster 2 and Cluster 4 cell lines were also resistant to SAR. In contrast, 50% of NSCLC cell lines belonged to Clusters 1 (NCIH1755, HCC1833, and HCC44) and 3 (NCIH1944, NCIH2077, NCIH358, and NCIH520). Interestingly, these cell lines demonstrated relative resistance to the tested chemotherapies and ionizing radiation, but sensitivity to SAR (Fig. 2B). Notably, KRAS driver mutation did not associate with chemotherapy, radiation, or SAR sensitivity (Supplementary Fig. S2). All cell lines tested had wild-type EGFR. In corroboration with cell line data, athymic nude mice bearing NCIH1944 (Cluster 3) tumor xenografts and receiving oral administration of SAR demonstrated a significant decrease in tumor growth as compared with control treated mice (Fig. 2C). As a positive control, NCIH1944 tumor xenografts exhibited sensitivity to treatment with ionizing radiation (Supplementary Fig. S3). These data raised the possibility that subgroups of NSCLCs that are resistant to genotoxic therapies are potentially sensitive to SAR.

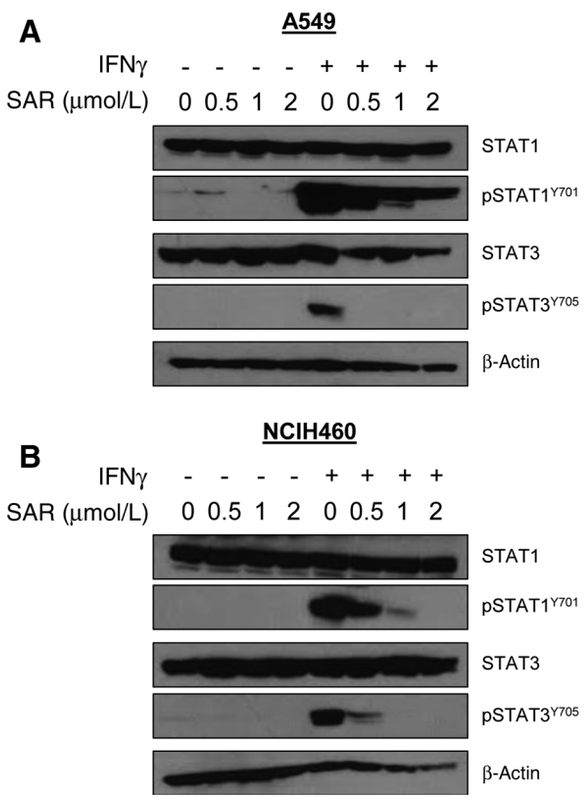

Figure 1.

JAK2 inhibitor SAR302503 (SAR) suppresses inducible STAT1/STAT3 signaling in NSCLC cell lines. Western blot analysis of total and phosphorylated STAT1 and STAT3 proteins in human A549 (**A**) and NCIH460 (**B**) tumor cells that were either control treated or stimulated by IFNγ (20 ng/mL) in the presence of increasing doses of SAR (0, 0.5, 1, and 2 μmol/L). β-Actin served as a loading control.

Constitutive ISG expression predicts SAR sensitivity

We examined the relationship between SAR sensitivity and molecular features in NSCLC cell lines. Mutation data were available for 15 of 16 NSCLC cell lines through the CCLE database (27). Given that JAK2 V617F mutation predicts response to JAK2 inhibitor therapy in hematologic malignancies, we examined for this mutation in NSCLC cell lines. Overall, we found that 27 of 643 (4.2%) carcinoma cell lines in the CCLE harbored JAK2 mutations, including 4 of 184 (2.2%) NSCLC cell lines. However, no V617F JAK2 mutations were identified in our panel. Further analysis of JAK/STAT mutations identified a JAK2 R1117M missense mutation in NCIH358 cells as well as a TYK2 G943C missense mutation in HCC1833 cells. Interestingly, these two cell lines exhibited a 50-fold relative hypersensitivity to SAR (SF = 0.005) when compared with the mean survival fraction (SF = 0.25) for the remaining 13 cell lines, suggesting that rare mutations in JAK/STAT signaling effectors may be associated with SAR sensitivity.

In contrast, using gene expression data obtained from the CCLE, we identified 199 genes whose expression values significantly correlated with survival fraction after SAR treatment and were differentially expressed between SAR-sensitive and SAR-resistant cell lines (Fig. 3A; Supplementary Table S2). We found overexpression of multiple ISGs in SAR-sensitive cell lines, including IRF9, IRF7, and ISG15. Elevated expression of IRF9 (Pearson correlation $r = -0.51$, $P = 0.013$), IRF7 ($r = -0.46$, $P = 0.014$), and ISG15 ($r = -0.55$, $P = 0.010$) correlated with reduced survival fraction after SAR treatment (Fig. 3B). IPA of genes overexpressed in SAR-sensitive cell lines confirmed a significant enrichment by ISG pathways mediating an inflammatory tumor phenotype (Fig. 3C; Supplementary Fig. S4), as well as gene networks supporting cellular growth and proliferation (Supplementary Fig. S5). Consistent with these data, overexpression of IRF9 protein, but not JAK2 or STAT proteins, was also associated with SAR sensitivity in NSCLC cell lines (Fig. 3D and E).

Previously, we identified an overlapping pattern of ISG expression, which we termed the IFN-related DNA damage resistance

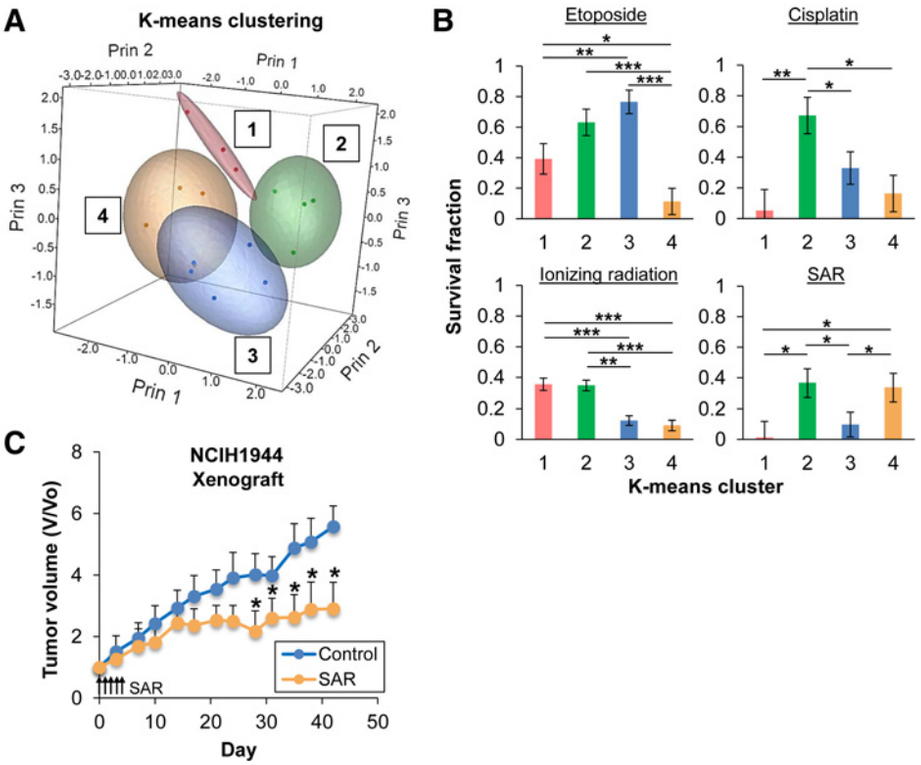

Figure 2.

NSCLC cell lines exhibit differential sensitivity to ionizing radiation, genotoxic chemotherapy, and SAR. **A,** K-means clustering of NSCLC cell lines by survival fraction after treatment with ionizing radiation (5 Gy), cisplatin (0.5 μg/mL), etoposide (0.1 μg/mL), or SAR (1 μmol/L) demonstrating the existence of four unique subgroups of cell lines. Cluster 1: NCIH1755, HCC1833, and HCC44; Cluster 2: COLO699, NCIH1437, NCIH2030, and MORCPR; Cluster 3: A549, NCIH1944, NCIH2077, NCIH358, and NCIH520; Cluster 4: NCIH2228, NCIH23, NCIH460, and HCC78. **B,** Clonogenic survival fraction of NSCLC cell lines grouped by K-means cluster. Data represent mean ± SEM. **C,** *In vivo* growth of NCIH1944 tumor xenografts in athymic nude mice treated with SAR (120 mg/kg twice a day via oral gavage) on days 0 to 4 after tumors reached an average volume of 150 mm^3 as compared with control treated tumors. $n = 5$ mice per group. Tumor volume, V. Initial tumor volume, V0. *P* values determined using Student's *t* test. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.

signature (IRDS) (10, 28). On the basis of these findings, we previously developed a patient-level top-scoring pair (TSP)-IRDS gene classifier predictive of breast cancer outcomes after postoperative radiation and/or chemotherapy (10, 29). The TSP-IRDS classifier comprises seven pairs of ISGs and reference genes. The expression value for each ISG is compared with its respective reference gene on a per-sample basis. Each gene-pair is provided a score of 0 or 1 based on whether the ISG exhibits smaller or larger expression when compared with its reference gene (30, 31). The sum of the seven gene-pair values determines the TSP-IRDS score, which can range from zero to seven, where larger values indicate a greater number of constitutively overexpressed ISGs. High TSP-IRDS scores were associated with poor clinical outcomes after radiation and/or chemotherapy in clinical breast cancers. We determined TSP-IRDS values for each NSCLC cell line and performed ROC analysis to determine whether the TSP-IRDS score is predictive of SAR sensitivity. We found that a TSP-IRDS score of 2 discriminated SAR-resistant (score $\leq$ 2) and SAR-sensitive (score > 2) NSCLC cell lines with a sensitivity of 83% and specificity of 88% (AUC = 0.91, $P = 0.042$; Supplementary Fig. S6A–S6C). Collectively, these data indicate that sensitivity to SAR is associated with constitutive activation of JAK/STAT signaling and ISG expression profiles.

IFN-stimulated gene expression correlates with chemotherapy resistance and poor survival in NSCLC patients

We hypothesized that patients whose tumors exhibited high TSP-IRDS scores would have poor prognoses and derive less benefit from genotoxic chemotherapy as compared with low TSP-IRDS tumors. In a large clinical dataset of NSCLC ($n = 246$), we identified variable expression of ISGs and TSP-IRDS scores across patient samples (Fig. 4A and B; Supplementary Fig. S7). ISG expression was independent of clinical and molecular features commonly used to classify clinical NSCLCs (Fig. 4A). We found that high TSP-IRDS tumors exhibited 1.3-fold elevated risks for relapse or death after surgery when compared with low TSP-IRDS tumors in early-stage NSCLC (Supplementary Fig. S8A and S8B). In patients with locally advanced NSCLC, high TSP-IRDS scores associated with a significantly inferior 10-year overall survival as compared with patients with low TSP-IRDS scores (26% vs. 45%), resulting in an HR of 1.9 (95% CI, 1.0–3.8; $P = 0.05$) for risk of death after surgery. Moreover, in a clinical dataset derived from NSCLC patients treated on the JBR.10 clinical trial with surgical lung resection and randomized to observation or adjuvant cisplatin-based chemotherapy (Supplementary Fig. S9A), high TSP-IRDS scores predicted a lack of survival benefit after cisplatin chemotherapy. In contrast, patients with low TSP-IRDS tumors experienced a nearly 45% absolute benefit in disease-specific survival at 10 years (Supplementary Fig. S9B and S9C). Taken together, high TSP-IRDS NSCLCs exhibit adverse patient outcomes after surgery and derive less benefit from postoperative cisplatin-based genotoxic chemotherapy as compared with low TSP-IRDS NSCLCs.

JAK2 inhibition abrogates tumor cell–intrinsic expression of PD-L1

PD-L1 (*CD274*) is an IFNγ/JAK2-inducible gene that is often expressed on tumor cells and plays a major role in antitumor immune suppression. Importantly, immunotherapies targeting the PD-1/PD-L1 axis have prolonged survival in NSCLC patients with locally advanced or metastatic disease. We found that PD-L1 gene expression significantly correlated with the expression of

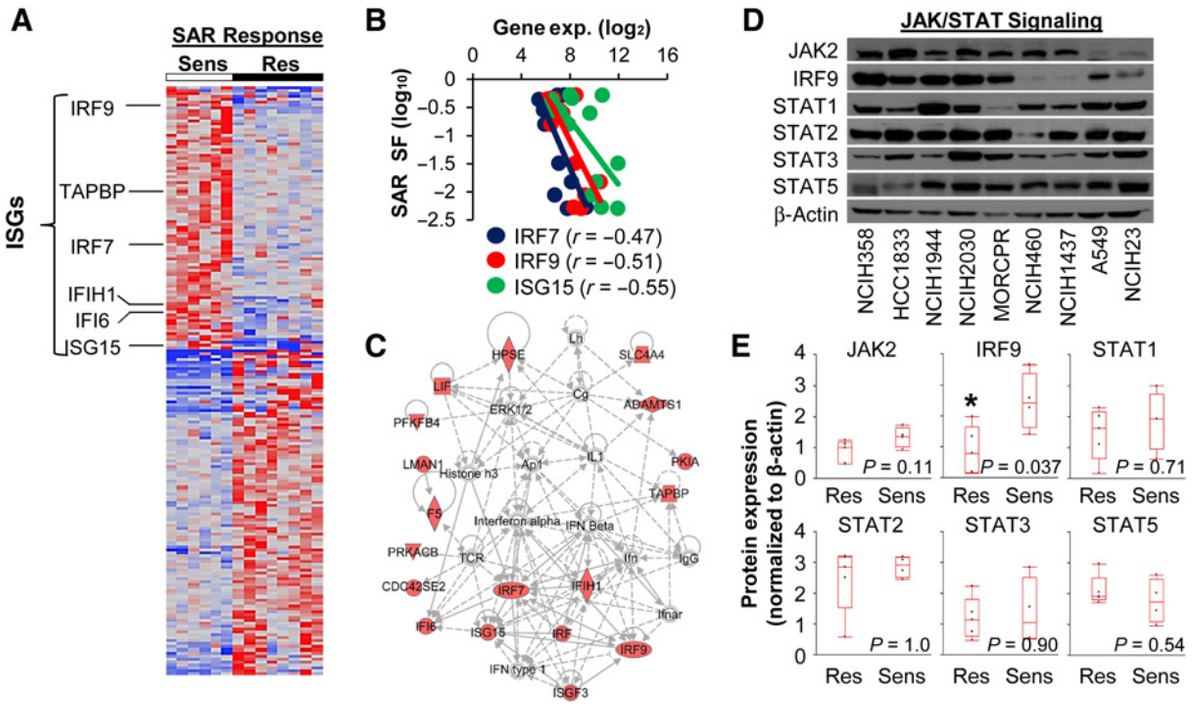

Figure 3.

Correlation of ISG expression and SAR sensitivity in NSCLC cell lines. **A,** Differentially expressed genes between SAR-sensitive (survival fraction < 0.2) and SAR-resistant (survival fraction $\geq$ 0.2) lung cancer cell lines. SAR-sensitive and SAR-resistant cell lines were distinguished on the basis of a cut-off survival fraction of 0.2, the mean SAR survival fraction across all cell lines. Gene expression values were determined from the CCLE. Upregulated genes are denoted in red, whereas downregulated genes are denoted in blue. **B,** Inverse relationship between expression of ISGs and survival fraction after treatment with SAR (1 μmol/L). **C,** IPA demonstrating a top-ranked IFN-regulated inflammatory gene network overexpressed in SAR-sensitive cells. **D,** Western blot analysis demonstrating basal expression of JAK/STAT signaling proteins as a function of SAR sensitivity. β-Actin served as a loading control. **E,** Quantification of Western blot analysis bands after normalization to β-actin. Data are shown as quantile plots for cell lines distinguished by survival fraction (SF) after treatment with 1 μmol/L SAR [resistant (Res, $n = 5$): SF $\geq$ 0.2; sensitive (Sens, $n = 4$): SF < 0.2]. *P* values determined using Mann–Whitney *U* test. *, $P \leq 0.05$.

ISGs associated with resistance to radiation and/or genotoxic chemotherapy (Pearson $r = 0.43$, $P < 0.001$; Fig. 4C). Emerging evidence has demonstrated that elevated TSP-IRDS scores also predict sensitivity to anti–PD-1 therapy in human cancers (32). We compared PD-L1 expression with TSP-IRDS scores and found a significant stepwise increase in PD-L1 expression with increasing TSP-IRDS scores ($P = 0.022$, ANOVA; Fig. 4D), suggesting that elevated PD-L1 expression is found in tumors that are most resistant to ionizing radiation and/or genotoxic chemotherapy. This result is consistent with recent clinical trial data demonstrating that PD-L1–overexpressing NSCLCs exhibit higher response rates and overall survival after anti–PD-L1 immunotherapy when compared with responses after standard first-line cisplatin-based chemotherapy in the upfront setting for metastatic NSCLC (32). In addition, we found that high PD-L1 expression was associated with adverse clinical outcomes in both early-stage and locally advanced NSCLCs treated with definitive surgery. Patients harboring tumors with elevated PD-L1 gene expression (defined as the top 50th percentile) demonstrated inferior overall survival as compared with patients with tumors demonstrating low PD-L1 expression (Fig. 4E). These findings suggested that PD-L1 is coexpressed with ISGs associated with cytotoxic therapy resistance and contributes to poor clinical outcomes after NSCLC treatment.

Given that SAR suppresses IFNγ/JAK2 signaling, we examined whether SAR also suppresses IFNγ-dependent expression of PD-L1 in NSCLC cell lines. Using flow cytometric analysis, we found that IFNγ stimulation increased PD-L1 expression in all cell lines investigated (Fig. 4F). The addition of SAR significantly suppressed IFN-inducible PD-L1 expression in all NSCLC cell lines (Fig. 4F). SAR also decreased basal PD-L1 expression, which was measurable in 2 of 6 cell lines. Taken together, these data demonstrated that SAR decreases basal and IFNγ-inducible PD-L1 expression in tumor cells in human and murine models of lung carcinoma.

Discussion

Despite advances in treatment options for NSCLC patients, including targeted therapies and immune checkpoint inhibitors, lung cancer remains the leading cause of cancer-related deaths worldwide. JAK inhibitors have demonstrated promise for use in benign diseases and hematologic malignancies; however, little is known regarding their potential use in solid malignancies. In the current report, we investigated the use of a JAK2 inhibitor SAR302503 as an adjuvant therapeutic agent for NSCLC. Numerous studies have shown that activation of the JAK/STAT pathway is a critical mediator of NSCLC chemotherapy resistance and

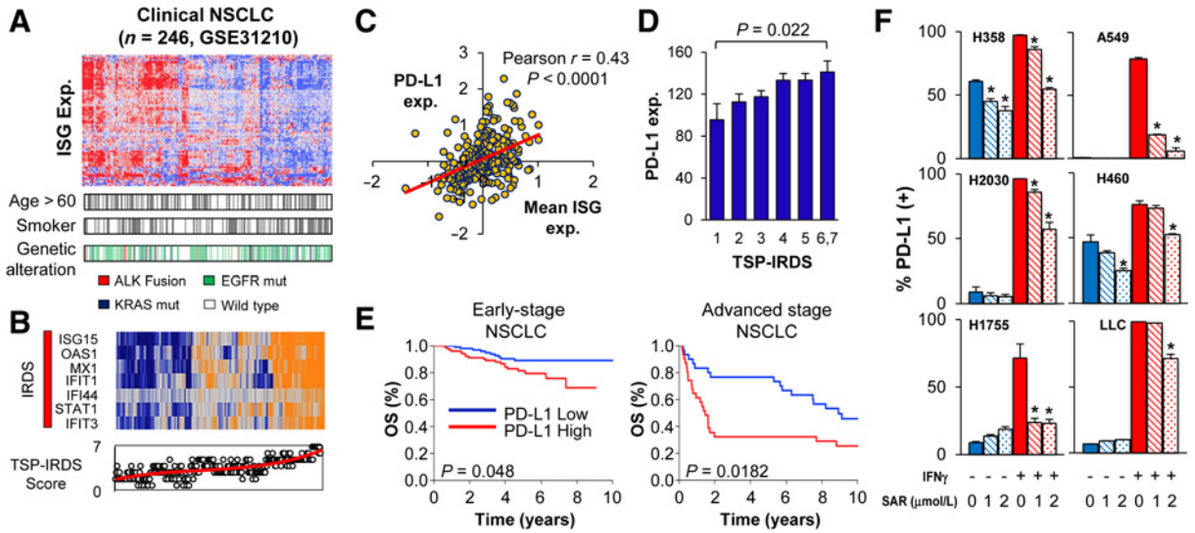

Figure 4.

PD-L1 is coexpressed with IFN genes and suppressed by JAK2 inhibition. **A,** Variable expression of ISGs (58) across a clinical dataset of NSCLC patient samples (GSE31210). Red indicates high expression, whereas blue denotes low expression. **B,** TSP-IRDS expression scores in GSE31210 patients. Orange indicates high expression, whereas blue denotes low expression. **C,** Correlation of PD-L1 gene expression with mean ISG expression in GSE31210 NSCLC patients. Mean ISG expression was determined using values from **A**. Correlation coefficient determined using Pearson correlation analysis. **D,** PD-L1 gene expression as a function of TSP-IRDS score. *P* value determined using ANOVA. **E,** Kaplan–Meier survival curves of overall survival for early stage (GSE31210, $n = 246$) and locally advanced (GSE37745, $n = 61$) NSCLC patients treated with surgical resection for curative intent and categorized by PD-L1 expression. Low and high PD-L1 expressions were distinguished by the median value across each cohort. Statistical significance was determined using log-rank tests. **F,** Suppression of basal and IFNγ-inducible (20 ng/mL) PD-L1 expression in lung cancer cell lines. PD-L1 protein expression was determined using flow cytometry. *P* values were determined using Student's *t* test. *, $P \leq 0.05$.

oncogenesis through enhanced proliferation, angiogenesis, and immune escape (14, 33, 34). In addition, aberrant JAK/STAT signaling plays an important role in resistance to other targeted therapies, such as EGFR inhibitors (35, 36). Here, we demonstrated that SAR suppresses IFN-inducible activation of STAT1 and STAT3 in NSCLC cell lines, which is consistent with recent reports regarding the potential therapeutic effects of JAK/STAT inhibitors in solid tumors (15, 33, 37, 38).

We investigated the cytotoxic effects of SAR on a panel of NSCLC cell lines in comparison with other established lung cancer therapies. We found that a large number of NSCLC cell lines were resistant to genotoxic therapies but sensitive to SAR. Our data suggest a potential application of SAR and similar compounds as a second-line therapy for lung cancer patients who failed radiation and/or genotoxic chemotherapies. It is important to note that the growth-inhibitory effects of SAR required drug concentrations greater than the IC_{50} value for JAK2 kinase inhibition, which can lead to suppression of JAK2 as well as other kinases. These findings are consistent with recent data demonstrating median IC_{50} values for cell growth inhibition of greater than 10 μmol/L for lung and aerodigestive cancers (39). As such, we cannot rule out the possibility that in addition to JAK2, other kinases could also be suppressed at the SAR concentrations we utilized.

We also explored potential biomarkers for SAR sensitivity in lung cancer cells as a step toward the design of companion diagnostics for JAK2 inhibitor therapy in lung cancer patients. We found that constitutive expression of ISGs is associated with sensitivity of NSCLC cells to SAR. Combining transcriptomic and protein-level analysis we found that IRF9 gene and protein expression correlated with sensitivity to SAR, thus promoting IRF9 as a promising biomarker for SAR sensitivity. Previously, based on emerging evidence demonstrating elevated TSP-IRDS scores predict resistance to ionizing radiation and genotoxic chemotherapies in human breast cancers, as well as concomitant sensitivity to anti–PD-1 therapy in human melanoma, we developed a TSP-IRDS gene signature for NSCLCs treated with genotoxic therapies (8, 10). The TSP-IRDS is a simple patient-level gene classifier based on the comparison of seven IRDS and non-IRDS gene pairs. Here, we report that the TSP-IRDS classifier successfully predicts lung cancer cell sensitivity to SAR with a specificity of 88% and a sensitivity of 83%. These data identify TSP-IRDS and/or IRF9 as potential predictors of SAR sensitivity and potentially other drugs that suppress JAK/STAT signaling (13, 26).

Immune checkpoint blockade is changing the face of cancer treatment and has been recently approved in several malignancies, including lung cancer, bladder cancer, melanoma, and renal cell cancer, with other approvals in the pipeline. PD-1 is a receptor that downregulates T-cell function, whereas PD-L1 is the principal ligand for PD-1. PD-L1 is expressed on tumor and some host cells and activates the PD-1 receptor, thereby suppressing antitumor T-cell immune responses (40–42). Importantly, PD-L1 is regulated through IFN/JAK signaling, which can potentially induce different outcomes depending on the timing and intensity of exposure to IFN (43–46). Recent data show that the PD-L1 promoter region contains IRF1, STAT1/STAT3, and STAT2/STAT5–binding sites, which control the expression of PD-L1 mRNA (47). Our data indicate that basal overexpression of PD-L1 correlates with poor prognosis in clinical lung cancers. This is consistent with previous observations noting an association between poor prognosis and constitutive IFN signaling in tumor cells with resistance to genotoxic stress (8, 9, 29, 48).

However, the impact of PD-L1 expression in predicting response to immune checkpoint therapy is incompletely understood. Recent observations indicate that NSCLC patients with high PD-L1 levels in tumor cells exhibit greater responses to nivolumab and pembrolizumab than patients with undetectable or low expression of PD-L1 (19–21). However, in patients with squamous histology, response rates were similar regardless of PD-L1 expression (20). In addition, objective response rates to anti–PD-L1 immunotherapy can be as high as 40% in patients with low PD-L1 expression (23), suggesting that additional biomarkers to predict immunotherapeutic responses are needed. In addition, coamplification of PD-L1 and JAK2 has been implicated in the development of Hodgkin lymphoma, large B-cell lymphoma, and triple-negative breast cancer (43). In this regard, our results indicate that JAK2 inhibitors may allow for pharmacologic modulation of PD-L1 expression. The potential therapeutic implications of these interactions warrant further investigation (9, 42).

In conclusion, our data demonstrate that SAR has dual functions by acting both on cell-autonomous properties of tumor cells and at the level of tumor-immune interactions through suppression of ISGs associated with treatment resistance and immune evasion. As a direct antitumor agent, SAR induces cytotoxicity in a subgroup of therapy-resistant NSCLCs that can be predicted on the basis of ISG biomarkers, such as the TSP-IRDS score and IRF9 expression. In addition, our results demonstrate that SAR attenuates tumor PD-L1 expression, thus potentially modulating the therapeutic effects of immune checkpoint blockade therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: S.P. Pitroda, G.-F. Liu, H. Liang, R.R. Weichselbaum, N.N. Khodarev

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Acknowledgments

The authors would like to thank Dr. Rebecca Bagley (Sanofi) who kindly provided the JAK2 inhibitor SAR302503. The authors are grateful to Drs. George R. Stark and Hyeon Joo Cheon at the Cleveland Clinic for their critical review of the manuscript. This work was supported by the Lung Cancer Research Foundation (to R.R. Weichselbaum), Ludwig Center for Metastasis Research (to R.R. Weichselbaum), the Ludwig Foundation for Cancer Research (to R.R. Weichselbaum), the Prostate Cancer Research Foundation (to R.R. Weichselbaum), and a generous gift from Mr. and Mrs. Vincent Foglia (to R.R. Weichselbaum).

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Received July 12, 2017; revised November 27, 2017; accepted January 17, 2018; published first February 21, 2018.

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