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 deaths worldwide. Approximately 85% of all lung cancers are non-small-cell histology (NSCLC). Modern treatment strategies for NSCLC target driver oncogenes and immune checkpoints. However, less than fifteen percent of patients survive beyond five 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 interferon-stimulated gene expression, including an Interferon-Related DNA Damage Resistance Signature (IRDS), predicts for sensitivity to SAR. Importantly, tumor cell-intrinsic expression of PD-L1 is interferon-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.
INTRODUCTION

Lung cancer is the leading cause of cancer 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 (NSCLCs) (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 PD-1 and 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 and immunotherapies, thus supporting the need for alternative lung cancer therapies.

Emerging evidence supports the existence of oncogenic JAK/STAT signaling in various human malignancies. The JAK/STAT pathway is triggered by extracellular signals, including interferons (IFN), through interactions of cell surface receptors and Janus kinases (JAKs) which activate Signal Transducer and Activator of Transcription (STAT) proteins allowing nuclear translocation and resulting in the expression of hundreds of down-stream 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 which exhibit sensitivity to JAK/STAT inhibition remains a challenge and biomarkers are needed to optimize the potential use of JAK inhibitors in cancer patients.

Programmed death ligand 1 (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-L1/PD-L1 axis is a central inhibitor of anti-tumor immune responses. Clinical studies indicate that increased tumor cell expression of PD-L1 correlates with poor outcomes (17, 18). By contrast, NSCLCs exhibiting elevated expression of PD-L1 have improved responses to nivolumab and pembrolizumab as compared to patients with undetectable or low expression of PD-L1 (19-21). While 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 signatures are being investigated as potentially superior 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 pre-clinical 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 anti-tumor 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 are 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 manufacturer’s instructions. Ruxolitinib was purchased from Selleck Chemicals (Houston, USA). Cisplatin and etoposide were manufactured by PCH Pharmachemie (Haarlem, Netherlands). Stock solutions were diluted in DMSO (Sigma-Aldrich) prior to further dilution in cell culture media. Human and mouse IFN-gamma was purchased from R&D laboratories.

**Cell Lines**

All lung cancer cell lines were previously authenticated and kindly provided by Dr. Matthew Meyerson (received in 2013). Cell lines were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, 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 6/2017).
Clonogenic Survival Assays

Cell lines were plated in triplicate at densities ranging from $2 \times 10^2$ to $2 \times 10^3$ per 100 mm culture dish. Twenty-four hours after plating, the cell lines were treated as follows: (1) ionizing radiation (2 or 5 Gy); (2) cisplatin (0.5 µg/ml); (3) etoposide (0.1 µg/ml); (4) SAR203502 (500 or 1000 nM). Each cell culture plate was washed with 0.85% NaCl solution and stained with 2% crystal violet (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 80-100% 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 µM). Twenty-four hours following SAR treatment, cells were treated with interferon-gamma (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) and data were analyzed using FlowJo software (Tree Star Inc.).
Animal models

To examine the effects of SAR on in vivo tumor growth, 7-8 week-old athymic nude mice were purchased from Harlan Laboratories. NCIH1944 cells were selected based on sensitivity to SAR in vitro. Athymic nude mice (n=5 per group) were subcutaneously injected with 1 x 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 x 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/min. 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.

Analysis of cell line and clinical cancer datasets

Non-small cell lung cancer genomic and expression data sets were collected from the Broad-Novartis Cancer Cell Line Encyclopedia (CCLE). Clinical cancer data sets and corresponding microarray gene expression were downloaded from Gene Expression Omnibus (GEO) using the identifiers GSE31210, GSE37745 and GSE14814. Probe set intensities were pre-processed, normalized and quantified as indicated for each respective study. Ingenuity Pathway Analysis (IPA) as used to identify top-ranked gene networks and cellular functions. TSP-IRDS scores were calculated as previously described (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 non-normally distributed continuous variables when sample sizes were small. Log-rank tests or Cox proportional hazard models were used to assess differences in overall survival between patient groups. A $P$-value of $\leq 0.05$ 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 (Rux; JAK1/JAK2 inhibitor) on a panel of human and murine cancer cell lines. We found SAR was cytotoxic at concentrations between 0.1-1 $\mu$M whereas Rux had minimal to no cytotoxicity below 1 $\mu$M concentrations (*Supplemental Figure S1*). Based on 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 IFN-gamma 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 IFN-gamma-induced STAT1 and STAT3 phosphorylation in a dose-dependent manner (*Figures 1A and 1B*). 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 $\mu$g/mL] or cisplatin [0.5 $\mu$g/mL]) and ionizing radiation (5 Gy), which are utilized
in the treatment of clinical NSCLCs, as well as to SAR (1 μM) using clonogenic survival assays (Supplemental Table S1). K-means clustering indicated the presence of four subgroups of NSCLC cell lines with varying sensitivities to individual treatments (Figure 2A and Supplemental Table S1). Cluster 4 was comprised of four cell lines (NCIH2228, NCIH23, NCIH460 and HCC78) which exhibited relative sensitivity to chemotherapies and ionizing radiation, while Cluster 2 included five cell lines (A549, COLO699, NCIH1437, NCIH2030 and MORCPR) which 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 (Figure 2B). Notably, KRAS driver mutation did not associate with chemotherapy, radiation, or SAR sensitivity (Supplemental Figure 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 to control treated mice (Figure 2C). As a positive control, NCIH1944 tumor xenografts exhibited sensitivity to treatment with ionizing radiation (Supplemental Figure S3). These data raised the possibility that subgroups of NSCLCs which are resistant to genotoxic therapies are potentially sensitive to SAR.

**Constitutive interferon-stimulated gene 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 Cancer Cell Line Encyclopedia (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 to 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.

By 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 (Figure 3A and Supplemental Table S2). We found overexpression of multiple IFN-stimulated genes (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 (Figure 3B). Ingenuity Pathway Analysis (IPA) of genes overexpressed in SAR-sensitive cell lines confirmed a significant enrichment by ISG pathways mediating an inflammatory tumor phenotype (Figure 3C and Supplemental Figure S4), as well as gene networks supporting cellular growth and proliferation (Supplemental Figure 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 (Figures 3D and 3E).

Previously, we identified an overlapping pattern of ISG expression which we termed the IFN-Related DNA Damage Resistance Signature (IRDS) (10, 28). Based on these findings, we previously developed a patient-level top-scoring pair (TSP)-IRDS gene classifier predictive of
breast cancer outcomes after post-operative 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 to 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 to 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 over-expressed 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 receiver operator characteristic (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 ≤2) and SAR-sensitive (score >2) NSCLC cell lines with a sensitivity of 83% and specificity of 88% (AUC = 0.91, \( P = 0.042 \)) (Supplemental Figure S6A-C). Collectively, these data indicate that sensitivity to SAR is associated with constitutive activation of JAK/STAT signaling and ISG expression profiles.

**Interferon-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 to 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 (Figures 4A and 4B and Supplemental Figure S7). ISG expression was independent of clinical and molecular features commonly used to classify clinical NSCLCs (Figure 4A). We found that high TSP-IRDS tumors exhibited 1.3-fold elevated risks for relapse or death after surgery when compared to low TSP-IRDS tumors in early stage...
NSCLC (Supplemental Figures S8A and S8B). In patients with locally advanced NSCLC, high TSP-IRDS scores associated with a significantly inferior 10-year overall survival as compared to patients with low TSP-IRDS scores (26% vs. 45%) resulting in a hazard ratio 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 (Supplemental Figure 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 (Supplemental Figures S9B and S9C). Taken together, high TSP-IRDS NSCLCs exhibit adverse patient outcomes after surgery and derive less benefit from post-operative cisplatin-based genotoxic chemotherapy as compared to low TSP-IRDS NSCLCs.

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

Programmed death ligand 1 (PD-L1, CD274) is an IFN-gamma/JAK2-inducible gene that is often expressed on tumors cells and plays a major role in anti-tumor 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 ISGs associated with resistance to radiation and/or genotoxic chemotherapy (Pearson r=0.43, P<0.001) (Figure 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 to TSP-IRDS scores and found a significant stepwise increase in PD-L1 expression with increasing TSP-IRDS scores (P = 0.022, analysis of variance (ANOVA); Figure 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 to 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 to patients with tumors demonstrating low PD-L1 expression (Figure 4E). These findings suggested that PD-L1 is co-expressed with ISGs associated with cytotoxic therapy resistance and contributes to poor clinical outcomes after NSCLC treatment.

Given that SAR suppresses IFN-gamma/JAK2 signaling, we examined whether SAR also suppresses IFN-gamma-dependent expression of PD-L1 in NSCLC cell lines. Using flow cytometric analysis we found that IFN-gamma stimulation increased PD-L1 expression in all cell lines investigated (Figure 4F). The addition of SAR significantly suppressed IFN-inducible PD-L1 expression in all NSCLC cell lines (Figure 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 IFN-gamma-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 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 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 interferon-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 to 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 IC50 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 IC50 values for cell growth inhibition of greater than 10 μM 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 Interferon-Stimulated Genes (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 which 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. Programmed death 1 (PD-1) is a receptor that downregulates T-cell function, while programmed death ligand 1 (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 anti-tumor 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 (46-48). 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 (43). 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, 44, 45).
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, co-amplification of PD-L1 and JAK2 has been implicated in the development of Hodgkin lymphoma, large B-cell lymphoma, and triple negative breast cancer (46). 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 interferon-stimulated genes associated with treatment resistance and immune evasion. As a direct anti-tumor agent, SAR induces cytotoxicity in a subgroup of therapy resistant NSCLCs that can be predicted based on 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.
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Therapy in HNSCC. J Cancer 2017; 8:332-344.


FIGURE LEGENDS:

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 interferon-gamma (IFNγ, 20 ng/mL) in the presence of increasing doses of SAR (0, 0.5, 1 and 2 μM). β-actin served as a loading control.

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 μM) 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 ± standard error of mean. (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 0-4 after tumors reached an average volume of 150 mm^3 as compared to control treated tumors. n=5 mice per group. Tumor volume, V. Initial tumor volume, V0. P-values determined using Student’s t-test. *P≤0.05; **P≤0.01; ***P≤0.001.

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 >=0.2) lung cancer cell lines. SAR-sensitive and SAR-resistant cell lines were
distinguished based on a cutoff survival fraction of 0.2, the mean SAR survival fraction across all cell lines. Gene expression values were determined from the Cancer Cell Line Encyclopedia (CCLE). Upregulated genes are denoted in red, while downregulated genes are denoted in blue.

(B) Inverse relationship between expression of ISGs and survival fraction after treatment with SAR (1 μM). (C) Ingenuity Pathway Analysis (IPA) demonstrating a top-ranked interferon-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 bands after normalization to β-actin. Data are shown as quantile plots for cell lines distinguished by survival fraction (SF) after treatment with 1 μM SAR (resistant (Res, n=5): SF >= 0.2; sensitive (Sens, n=4): SF < 0.2). P-values determined using Mann-Whitney U test. *P≤0.05.

**Figure 4. PD-L1 is co-expressed with interferon genes and suppressed by JAK2 inhibition.**

(A) Variable expression of interferon-stimulated genes (58) across a clinical dataset of NSCLC patient samples (GSE31210). Red indicates high expression, while blue denotes low expression.

(B) TSP-IRDS expression scores in GSE31210 patients. Orange indicates high expression, while 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 used in (A). Correlation coefficient determined using Pearson correlation analysis. (D) PD-L1 gene expression as a function of TSP-IRDS score. P-value determined using Analysis of Variance (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 expression were distinguished by the median value across each cohort. Statistical significance
was determined using log-rank tests. (F) Suppression of basal and IFN-gamma-inducible (20 ng/mL) PD-L1 expression in lung cancer cell lines. PD-L1 protein expression was determined using flow cytometry. $P$-values determined using Student’s $t$-test. *$P \leq 0.05$. 
Figure 1

A549

| IFN\(\gamma\) | - | - | - | - | + | + | + | + |
| SAR (\(\mu\)M) | 0 | 0.5 | 1 | 2 | 0 | 0.5 | 1 | 2 |

STAT1

pSTAT1\(^{Y701}\)

STAT3

pSTAT3\(^{Y705}\)

\(\beta\)-actin

NCIH460

| IFN\(\gamma\) | - | - | - | - | + | + | + | + |
| SAR (\(\mu\)M) | 0 | 0.5 | 1 | 2 | 0 | 0.5 | 1 | 2 |

STAT1

pSTAT1\(^{Y701}\)

STAT3

pSTAT3\(^{Y705}\)

\(\beta\)-actin
Figure 2

A. K-means clustering

B. Survival fraction

C. NCIH1944 xenograft

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Figure 3

A

SAR Response

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<td>IFI6</td>
<td></td>
</tr>
<tr>
<td>ISG15</td>
<td></td>
</tr>
</tbody>
</table>

B

Gene Exp. (log2)

<table>
<thead>
<tr>
<th>SAR SF (log10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
</tr>
<tr>
<td>4</td>
</tr>
<tr>
<td>8</td>
</tr>
<tr>
<td>12</td>
</tr>
<tr>
<td>16</td>
</tr>
</tbody>
</table>

IRF7 (r = -0.47)
IRF9 (r = -0.51)
ISG15 (r = -0.55)

C

D

JAK/STAT signaling

- JAK2
- IRF9
- STAT1
- STAT2
- STAT3
- STAT5

\(\beta\)-actin

NCI-H358
NCI-H1944
NCI-H2030
NCI-H460
NCI-H437
A549
NCI-H23

E

Protein expression (normalized to \(\beta\)-actin)

- JAK2
- IRF9
- STAT1

Res | Sens
--- | ---
P = 0.11

Res | Sens
--- | ---
P = 0.037

Res | Sens
--- | ---
P = 0.71

Res | Sens
--- | ---
P = 1.0

Res | Sens
--- | ---
P = 0.90

Res | Sens
--- | ---
P = 0.54
Figure 4

A. Clinical NSCLC (n = 246, GSE31210)

B. ISG Exp.

Age >60
Smoker
Genetic Alteration
- ALK fusion
- EGFR mut
- KRAS mut
- Wild-type

C. PD-L1 exp.

Pearson r = 0.43
P < 0.0001

Mean ISG exp.

D. TSP-IRDS

P = 0.022

P-DL1 exp.

F. % PD-L1 (+)

H358
A549
H2030
H460
H1755
LLC

E. Early stage NSCLC

OS (%)
P = 0.048

PD-L1 low
PD-L1 high

Advanced stage NSCLC

OS (%)
P = 0.0182

IFNY
SAR (μM)

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Molecular Cancer Therapeutics

JAK2 inhibitor SAR302503 abrogates PD-L1 expression and targets therapy resistant non-small cell lung cancers

Sean Pitroda, Melinda Stack, Gene-Fu Liu, et al.

Mol Cancer Ther Published OnlineFirst February 21, 2018.