Targeting the Kynurenine Pathway for the Treatment of Cisplatin-Resistant Lung Cancer

Dan J.M. Nguyen1, George Theodoropoulos1, Ying-Ying Li1, Chunjing Wu1, Wei Sha1, Lynn G. Feun2, Theodore J. Lampidis3, Niramol Savaraj1,2, and Medhi Wangpaichitr1,4

ABSTRACT

Cisplatin resistance is a major barrier in the effective treatment of lung cancer. Cisplatin-resistant (CR) lung cancer cells do not primarily use glucose but rather consume amino acids such as glutamine and tryptophan (Trp) for survival. CR cells activate the kynurenine (KYN) pathway (KP) to cope with excessive reactive oxygen species (ROS) and maintain homeostasis for growth and proliferation. Consequently, indoleamine 2,3-dioxygenase-1 (IDO1) becomes an essential enzyme for CR cells’ survival because it initiates and regulates the first step in the KP. Increased IDO1 activities and ROS levels are found in CR cells versus cisplatin-sensitive lung cancer. Importantly, significantly greater KYN/Trp ratio (P = 0.005) is detected in serum of patients who fail cisplatin when compared with naïve treatment. Knocking down IDO1 using shRNA or IDO1 inhibitors heightens ROS levels and results in a significant growth inhibitory effect only on CR cells and not on cisplatin-sensitive cells. Exposing CR cells to antioxidant (TIRON) results in suppression of IDO1 activity and confers resistance to IDO1 inhibition, indicating an interrelationship between ROS and IDO1. Because KYN plays a critical role in reprogramming naïve T cells to the immune-suppressive regulatory T-cell (T-reg) phenotype, we observed higher expression of TGFβ, FoxP3, and CD4+CD25+ in mice bearing CR tumors compared with tumors from cisplatin-sensitive counterparts.

Implications: Findings suggest that the enzyme-inhibitory activity and antitumor efficacy of IDO1 inhibitors rely in part on ROS levels, arguing that IDO1 expression alone may be insufficient to determine the clinical benefits for this class of experimental cancer drugs. Importantly, IDO1 inhibitors may be more suitable to treat patients with lung cancer who failed cisplatin therapy than naïve treatment patients.

Introduction

Surgery is the best treatment approach for early-stage lung cancer; nonetheless, most patients already have locally advanced or metastatic disease at the time of diagnosis. Anti-PD1 (pembrolizumab) in combination with chemotherapy was recently approved as first-line treatment of patients with non–small cell lung cancer (NSCLC) with stage 3B who are not candidates for surgical resection. However, patients’ tumors must express PD-L1 and have no EGFR or anaplastic lymphoma kinase mutation in order to receive this therapy. Although these combination therapies offer a longer duration of response than other second-line chemotherapy, the overall response rate in NSCLC is low (15%–20%; ref. 1). Subsequently, cisplatin-based chemotherapy alone and in combination with radiation remains the primary modality of treatment. Despite early positive responses to cisplatin, all patients with lung cancer will develop drug resistance; thus, cisplatin resistance remains the major obstacle for the effective treatment of lung cancer.

Our research focus has been to elucidate molecular differences in metabolic heterogeneity observed among patients with sensitive and resistant tumors. We have previously reported that cisplatin-resistant (CR) lung cancer cells possessed increased numbers of mitochondria and consumed higher rates of oxygen than nonresistant cancer cells, resulting in significantly higher (2-fold to 3-fold) basal levels of reactive oxygen species (ROS; refs. 2, 3). In addition, we showed that CR tumors are no longer addicted to glucose for energy and do not utilize classic aerobic glycolysis [Warburg effect; (4, 5)]. By switching to oxidative metabolism (OXMET), CR cells increase cellular energy or metabolic demand and outstrip the glutamine supply, making glutamine the conditionally essential amino acid for cell survival. Besides glutamine, CR cells are also auxotrophic for arginine due to the lack of argininosuccinate synthase 1 (ASS1), which is a key enzyme in the synthesis of arginine from citrulline (2, 6, 7). Lack of ASS1 expression therefore makes arginine an essential amino acid for CR tumors as well. Because of these phenomena, CR cells are collectively dependent more on OXMET for survival and are susceptible to amino acid deprivation.

Here we show that these metabolic derangements have led us to the discovery that L-tryptophan (Trp) catabolism is also upregulated in CR cells. Although Trp is an essential amino acid, required for protein synthesis and as the precursor of serotonin and melatonin, the catabolism of Trp can also generate kynurenine (KYN) via the kynurenine pathway (KP). This pathway is responsible for the catabolism of approximately 95% of ingested Trp not used for protein synthesis or the serotonin pathway (8, 9) and not limited to its originally derived role in the biogenesis of nicotinamide adenine dinucleotide (NAD+; ref. 10). At the first step, Trp is oxidized through indoleamine 2,3-dioxygenase-1 and 2 (IDO1 and IDO2) or Trp 2,3-dioxygenase-2 (TDO2) to form formylkynurenine, which is later degraded to KYN (11). However, TDO2 is primarily present in the

1Department of Veterans Affairs, Miami VA Healthcare System, Research Service, Miami, Florida. 2Department of Medicine, Hematology/Oncology, Miller School of Medicine, University of Miami, Miami, Florida. 3Department of Cell Biology, Miller School of Medicine, University of Miami, Miami, Florida. 4Department of Surgery, Cardiothoracic Surgery, Miller School of Medicine, University of Miami, Miami, Florida.

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Corresponding Authors: Medhi Wangpaichitr, Miami VA Healthcare System, 1201 NW 16th Street, Research 151, Miami, FL 33125. Phone: 305-575-7000 ext.4496; Fax: 305-575-3575; E-mail: mwangpaichitr@med.miami.edu and Niramol Savaraj, nsavaraj@med.miami.edu

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liver and is not expressed in lung tissue or lung tumor (12). IDO2 is expressed in human tumors (13), and recent genetic data have implicated it functionally in drug-resistant pancreatic ductal adenocarcinoma resistant to DNA-damaging radiotherapy (14). But the significance of the relationship between cisplatin resistance and IDO2 activity in lung cancer remains elusive and needs to be explored in future.

IDO1 is the unique enzyme similar to superoxide dismutase-1 (SOD1) that exploits superoxide as a substrate (15, 16). We have previously reported that CR cells possessing elevated basal levels of ROS also expressed higher superoxide dismutase-1 (SOD1) when compared with parental cell counterparts (3). Increasing SOD1 levels and IDO1 activities are crucial mechanisms for the capture of ROS/superoxide and maintenance of homeostasis in CR cells. Importantly, increased IDO1 activity results in greater KYN production, which may otherwise be lethal to normal cells (3). These findings are supported by recent reports that IDO levels impact outcomes in patients with lung cancer and gliomas receiving radiotherapy that damages DNA as well as immunoradiotherapy (19, 20). Hence, links have been established between metabolic alterations in oxidative processes and amino acid catabolism and tumor responsiveness to treatment that may be utilized to affect outcomes.

Here, we show that IDO1 activity is dependent on ROS levels in CR cells. Increased basal level of ROS heights IDO1 activity and in turn hyperactivates the KP (T-cell) phenotype (17, 18). Thus, it seems that adaptations in CR cells confer a survival advantage in an environment with elevated basal levels of ROS, which may otherwise be lethal to normal cells (3). These findings are supported by recent reports that IDO levels impact outcomes in patients with lung cancer and gliomas receiving radiotherapy that damages DNA as well as immunoradiotherapy (19, 20). Hence, links have been established between metabolic alterations in oxidative processes and amino acid catabolism and tumor responsiveness to treatment that may be utilized to affect outcomes.

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**Materials and Methods**

**Cell lines and reagents**

Two pairs of parental versus cisplatin-resistant human NSCLC cells (A vs. ALC and FA vs. FC) and a pair of mouse Lewis lung cells (LLC vs. LLC-CR) were used. Cell line “A” was established with pleural fluid from a patient with adenocarcinoma. FA was established from a poorly differentiated squamous cell carcinoma. Cellular characteristics have been previously characterized (21, 22). The culture medium and the tryptophan cells were collected and lysed, and subjected to radioactivity analysis.

**Measurement of patients’ plasma**

**Animal studies**

Procedures and mice protocol were approved by the Institutional Animal Care and Use Committee of the Miami VA Healthcare System (Animal Welfare Assurance Number: A3739-01). Male or female C57BL6/N mice (age 5 weeks, Jackson laboratory) were used to establish in vivo allograft using LLC versus LLC-CR. Mice were inoculated subcutaneously with 2.5 × 10⁷ cells on the dorsal lumbar region. Tumor growth was evaluated twice a week by measuring tumor volume according to the following formula: tumor volume = width² × length × 0.5. Experiment was ended when either W or L reached the final set value of 10 mm.

**Results and discussion**

**Growth inhibition and cytotoxicity assay**

Cells were seeded in 24-well dishes and treated with various concentrations of IDO1 inhibitors (i.e., Epacadostat). The procedure was described previously (21, 25). Briefly, the culture media and the tryptophan cells were collected and this mixture was centrifuged at 400 × g for 5 minutes. The supernatant was discarded, resuspended in 1 mL of Hank’s buffer, and assayed for live cells and death cells using trypan blue exclusion method.

**Western blot analysis**

Cells were seeded at 1 × 10⁵/mL onto 60-mm dishes, treated, collected, lysed, and immunoblotted with indicated antibody. Detailed procedure was described in our previous publications (21, 25). Briefly, cell lysis was completed by sonication and the total protein was separated on an SDS-PAGE, transferred onto a polyvinylidene difluoride membrane (Millipore), and immunoblotted with indicated primary antibody. Antibody to IDO1 (cat#NB1-8702) was purchased from Nuvos. Antibodies to: hypoxia-inducible factor-1α (HIF1α; cat#12905) were purchased from BD Biosciences; Phospho-Aryl Hydrocarbon Receptor (AHRR) (AHRR; cat#GTX113124) and FoxP3 (GTX107737) were purchased from GeneTex; AHR (cat# A1451) was purchased from ABEconal; Antibody to LAT1 (cat#5347) was purchased from Cell Signaling. All antibody dilutions were at 1:1,000, except for Actin (Sigma; cat#A5441) which was diluted at 1:10,000. Bands were measured using a molecular imager ChemiDoc system with Quality One software (Bio-Rad).
H2O2 was measured by incubating with 10 μM NBT (saturated with O2) in growth medium for 24 hours in plate reader. Basal oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) was measured with an absolute measure of oxygen use and the level of H2O2 was analyzed by AMROX H2O2 Redox Assay Kit (Biotium). The samples were analyzed either by luminometry (excitation at 485 nm and emission at 520 nm) or by fluorometry using a Multimode plate reader (BioTek). LAT1 localization was analyzed with Cytosensor (Beckman). For cell surface LAT1 staining, a1 Antibody (clone 4B7; Millipore cat# MABF850) which has been conjugated to peroxidase (HRP) solution was added for 30 minutes. Then the cells were washed once with PBS and centrifuged to remove unpermeable reagents. Cells were resuspended in 500 μL of PBS and analyzed either by fluorometer [FLUOstar OPTIMA, BMG Labtech (excitation at 485 nm and emission at 520 nm)] or by flow cytometer (CytoFLEX, Beckman).

Real-time assay of oxygen consumption

Simultaneous multiparameter metabolic analysis of cell populations in culture was performed in the Seahorse XF24 extracellular flux analyzer (Seahorse Bioscience) as described by Wu and colleagues (26). All cell lines were cultured in growth medium for 24 hours in plate reader before real-time metabolic analysis. At the start of the assay, growth medium was removed and replaced with assay medium. Basal oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) of the cells were measured. Oligomycin, Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP), and rotenone were used to inhibit ATP synthase, uncouple OXPHOS, and inhibit mitochondrial complex 1, respectively. After XF assay, cells were harvested by trypsin-ethylenediaminetetraacetic acid treatment and counted. The number of cells per well was used to normalize OCR and ECAR.

Assay of intracellular ROS/H2O2

As previously described (3), cells were collected and intracellular H2O2 was measured by incubating with 10 μmol/L of CM-H2DCFDA (Life Technologies; cat#C2938) at 37°C for 30 minutes in the dark. Then the cells were washed once with PBS and centrifuged to remove impermeable reagents. Cells were resuspended in 500 μL of PBS and analyzed either by fluorometer [FLUOstar OPTIMA, BMG Labtech (excitation at 485 nm and emission at 520 nm)] or by flow cytometer (CytoFLEX, Beckman).

LAT1 localization

For cell surface LAT1 staining, fluorochrome-conjugated antibodies were used to detect LAT1/CDC98 (BioLegend; cat#315603). Cells were washed in PBS and resuspended in 250 μL of FACS buffer (PBS, 1% fetal calf serum, 0.1% Na3HPO4) with LAT antibody. Cells were incubated for 30 minutes on ice, followed by three washes, and resuspended in 500 μL of PBS. Live cells were gated according to their forward scatter and side scatter. Data were acquired using CytoFLEX (Beckman) flow cytometer.

Trp uptake

Seeded cells were cultured in complete RPMI media for 24 hours, at which point media was replaced with fresh Trp and serum-free RPMI media with “hot” (5 μCi L-[5-3H(N)] Trp; ref. 2). Next, the hot medium was removed, and cells were washed three times with fresh, “cold” RPMI serum-free medium with Trp. Then cells were lysed with 500 μL of 1 N NaOH, collected in 1.5-ml Eppendorf tubes and gently vortexed (2). Protein analysis was conducted on 250-μL samples using the Micro BCA Protein Assay Kit (Thermo Fisher Scientific; cat#23235). Radioactivity in the remaining sample was measured as counts per minute, and normalized to protein, using a PerkinElmer Tri-Carb 2810TR liquid scintillation spectrometer with QuantaSmart (2).

IDO1 activity assay

An IDO1 assay kit (BioVision; cat#K972-100) was used to measure IDO1 activity. Briefly, 5 × 106 cells were seeded onto 100-mm dishes, treated, collected, and homogenized with IDO1 assay buffer. IDO1 activities were calculated by the amount of N-formylkynurenine produced/(Reaction time × Amount of protein in well). Samples were measured at Ex/Em = 402/488 nm.

NAD+ concentration assay

A NAD/NADH assay kit (Cayman Chemical; cat#600480) was used to measure total cellular NAD+ concentration. Cells were seeded in 96-well at 1 × 104 and grow overnight. Total NAD+ was detected at 450 nm according to manufacturer’s instruction.

Amino acid analyzer

Physiologic fluids or culture medium were collected and the Biochrom 30 amino acid analyzer (using ion exchange chromatography) was used to analyze free amino acids (30–40 in the complete panel) in samples (2). Values were reported compared with normal ranges in controls. Samples used in ion exchange chromatography are separated in an analytical fashion with an ion exchange column and buffers of increasing pH and ionic strength. After postcolumn derivatization with ninhydrin, and colorimetric intensity recordings at 570 nm and 440 nm, the amino acids separated by chromatography were detected (2). Amino acid concentration was calculated by comparing the peak area of a particular amino acid to the peak area of an internal standard of known concentration and then multiplying by its specific response factor from calibration.

IHC staining

IHC staining was performed according to our routine methodology with some modification (2). Target retrieval solution (citric buffer; pH 6.0) was used to enhance the staining. Samples were incubated overnight with CD4 (GeneTex; cat#GTX59084), CD25 (Biorybt; cat#orb389314), and TGFβ (Cell Signaling; cat#3709) primary antibodies at 1:100 in antibody dilution solution (Dako S3022). Anti-IDO1 Antibody (clone 4B7; Millipore cat# MABF850) which has been validated in IDO+/− mouse was diluted at 1:50. Secondary antibody solution (LSAB2 Kits Dako, biotinylated antibody solution; cat#K0675) was added for 30 minutes, washed, and streptavidin conjugated to peroxidase (HRP) solution was added for 30 minutes. DAB Chromogen (Dako, cat#K3466) was used and then counterstained with hematoxylin.

Immunofluorescent staining

Cells were treated with and without AHR antagonist, washed with PBS, and fixed with 4% paraformaldehyde for 30 minutes at room temperature. Cells were incubated with PBS containing 0.2% Triton X-100 (antibody diluent) for 15 minutes at room temperature and subsequently blocked with PBS containing 5% BSA for 1 hour. Cells were incubated with anti-AHR antibody (cat#GTX113124 at 1:1,000 in

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antibody diluent) overnight at 4°C. Secondary antibody (1:250; Alexa Fluor 555) was added for 1 hour. DAPI (Vector Laboratories; cat#H1500) was used for nuclei staining. Cells were examined under a fluorescence microscope (Keyence BZ-X710) to determine localization of AHR. Our Keyence microscope also equipped with hybrid cell count (an algorithm software that allows accurate quantification for phase contrast).

Statistical analysis
All statistical analyses were performed from three separate biological replicates that were isolated and analyzed in technical triplicates. Separate measurements using the two-tailed t test and the results were expressed as mean ± SD. A P value of less than 0.01 or 0.05 was considered as statistically very significant and significant, respectively. The medium or plasma concentrations of KYN or Trp, as well as KYN/Trp ratios approximated a parametric distribution; therefore, data were presented as means ± SD. Because of the small sample size, the associations between serum biomarker levels and time point variables were evaluated with a Mann–Whitney U test.

Results
The KP contributes to higher KYN/Trp ratio in patients who fail cisplatin
We have reported that CR lung cancer cells were less dependent on the glycolytic pathway but instead utilized mitochondria for biogenesis to catalyze glutamine as a prerequisite for KYN production (2, 6). Here, we first compared oxygen consumption among resistant and sensitive cells using the Seahorse flux analyzer. In response to adding oligomycin, FCCP, and rotenone (Supplementary Fig. S1A), CR cells consumed significantly higher rates of oxygen (Fig. 1A) and thus had higher levels of ATP production than their parental counterparts (Supplementary Fig. S1B). Adding to these metabolic alterations and the reliance on amino acids, we further discovered that CR cells also uptake significantly higher amounts of Trp (Fig. 1B). It is noteworthy that even though Trp can be used for de novo biosynthesis of NAD⁺ (essential coenzyme of all organisms’ redox system), Trp is less efficient and a poor NAD⁺ precursor in vivo. Trp will be diverted only to NAD⁺ synthesis when its supply exceeds enzymatic capacity (27). Thus, the main source of NAD⁺ is from salvage pathways, which require the uptake of other NAD⁺ precursors such as nicotinic acid. Consistent with these findings, we have found that although uptake of Trp is significantly increased in CR cells as shown in Figure 1B, the NAD⁺ levels and quinoline acid phosphoribosyltransferase (QRT), a key enzyme in NAD⁺ biosynthesis, expressions were significantly decreased when compared with parental cell counterparts (Fig. 1C and D). Hence, these findings demonstrate that CR cells actively utilized KP but did not engage in de novo synthesis of NAD⁺.

Next, we evaluated Trp levels in cell culture and found that Trp concentrations were significantly reduced in CR cells’ media (Fig. 1E). We then assayed for extracellular KYN levels (Fig. 1F), and used the KYN/Trp ratio as an indicator of IDO1 activity. A significantly higher mean KYN/Trp ratio in CR cells’ media (as compared with parental counterparts) implied that IDO1 activity was elevated in CR cells (Fig. 1G). To gain insight into the physiologic role of KP, we assayed the KYN/Trp ratio in serum of patients with cancer prior to and after cisplatin/radiotherapy. All patients with cancer from our study possessed higher basal levels of KYN/Trp ratio when compared with healthy controls. Patients (patients 1–3) who failed cisplatin possessed significantly higher KYN/Trp ratios when compared with the pre-treatment baseline (Fig. 1H). Consistently, we did not find an increase in KYN/Trp ratio in patients (patients 4–6) who continued to respond to cisplatin treatment.

Together, our data illustrate that CR cells consumed Trp at a higher rate specifically for KYN production but not for production of NAD⁺. Importantly patients who failed cisplatin therapy, in our small sampling, possessed significantly higher ratio of KYN/Trp, the indicator of IDO1 activity. These results suggest that increased IDO activity is involved in disease progression in lung cancer, possibly through immunosuppressive effects.

The KYN/AHR/ARNT axis is a crucial modulator of metabolism in CR cells
Recent discoveries revealed that KYN can serve as endogenous ligand for the Aryl Hydrocarbon Receptor (AHR) in cancer cells (28). KYN formed a complex with AHR and translocated into the nucleus to modulate their target genes as a ligand-activated transcription factor (29). To further elucidate the unique role of AHR, immunofluorescence staining showed that AHR localized with high intensity to the nucleus of CR cells, whereas less accumulation was observed in parental counterparts, implicating that KP is specifically active in CR cells (Fig. 2A). To determine that AHR can be activated by KYN, we added exogenous KYN (100 μmol/L) and assessed AHR localization. As depicted, treatment with KYN increased the nuclear distribution of AHR in both parental and CR cells. We then determined that KYN was specific to AHR activation in CR cells by inhibiting AHR translocation with either 10 μmol/L of DMF (an AHR antagonist) or 1 μmol/L of CH-223191 (AHR inhibitor). AHR was significantly less accumulated in the nucleus after treatment. Importantly, exposure to AHR inhibitors also resulted in suppression of IDO1 activities, whereas addition of KYN increased IDO1 activity only in CR cells (Fig. 2B).

Furthermore, we have previously demonstrated that CR cells possess very low levels of HIF1α due to metabolic reprogramming (ref. 6; Fig. 2C). ARNT or HIF1α is a known binding partner of both HIF1α and AHR (29, 30). Thus, in the absence of HIF1α, it is possible that ARNT is now available to bind and form a new partner with KYN/ AHR and initiate the transcription of genes, which favor the survival/proliferation of CR cells. Immunoblot showed that CR cells expressed higher basal level of AHR as well as its phosphorylated form (Fig. 2C). This is interesting because others have shown that AHR can be fully activated upon phosphorylation (31). To characterize the role of the KYN/AHR/ARNT axis, we assayed the expression of the AHR-target gene LAT1 [L-type amino acid transporter-1]; a known amino acid transporter for large neutral amino acids such as arginine, leucine, and Trp (32, 33)] with and without adding KYN. As shown in Figure 2C, LAT1 protein expressions were higher in CR cells; then we assayed for functional LAT1 that localized to the cell surface using the flow cytometer (Fig. 2D1). LAT1 was further augmented upon addition of KYN in CR cells (Fig. 2D2), whereas no increase in LAT1 was observed after exposing parental cells to KYN (Fig. 2D3), suggesting that HIF1α still maintained its complex with ARNT in parental cells. To determine the role of HIF1α in parental cells, we knocked down HIF1α using siRNA and exposed A−/−/HIF1α to KYN. Increased LAT1 expression was observed in A−/−/HIF1α after treatment (Fig. 2D4) and increased Trp uptake was found in CR cell after KYN treatment as anticipated. But importantly, Trp uptake was increased in A−/−/HIF1α and exposure to KYN further increased Trp uptake (Fig. 2E), suggesting that downregulation of HIF1α levels will lead to an increase in AHR/ ARNT complex formation (Fig. 2G). We used four different unique sequences to silence IDO1 expression in order to determine efficacy of silencing and consistency of effect (Fig. 2F). All knockdowns of IDO1...
(shIDO1) suppressed LAT1 (Fig. 2F) and CYP1B1 (also a known AHR target gene; Supplementary Fig. S2) expressions in CR cells. Thus, our findings strongly suggest that the KYN/AHR/ARNT axis plays the unique modulator role in CR cells’ metabolism, and increased Trp uptake can lead to further KYN secretion in CR cells.

CR tumors possess higher IDO1 activity and immunosuppressive phenotype in vivo

The tumor microenvironment with increased KYN levels can facilitate differentiation of naïve T cells into T-reg cells (17, 18), creating a highly immunosuppressive environment that favors
Figure 2.
Modulator role of KYN/AHR/ARNT axis in CR cells metabolism. A, Immunofluorescence (IF) staining of cells with 1:1,000 AHR antibody (red) and DAPI (blue nuclei). CR cells (ALC) possessed significantly higher intensity of AHR expression in the nucleus when compared with parental cells (\( P = 0.008 \)). DMF or CH223191 significantly reduced AHR accumulation in CR cells (\( P = 0.007 \), \( P < 0.001 \), respectively) whereas exposure to KYN markedly increased AHR accumulation in the nucleus of both parental and CR cells. Bar graph indicated quantification of IF intensity (RFU/cell) using hybrid cell count. B, Addition of 100 \( \mu \)mol/L of KYN increased IDO1 activity in CR cells substantially (\( P = 0.03 \)) but not significantly (NS) in parental cells. Treatment of 10 \( \mu \)mol/L of DMF or 1 \( \mu \)mol/L of CH223191 resulted in significant suppression of IDO1 activities (\( P = 0.006 \), \( P = 0.002 \), respectively). C, Immunoblot of lung cancer cell lines showed that resistant variants did not possess HIF1\( \alpha \) but expressed higher levels of AHR and LAT1. Actin was used as a loading control. D, Flow cytometry analysis of surface LAT1 in lung cancer cell lines. D1: CR cells possessed higher surface LAT1 when compared with parental counterparts. D2: Treatment of KYN at 100 \( \mu \)mol/L for 48 hours further enhanced LAT1 expression in CR cells. D3: KYN treatment did not increase LAT1 expression in parental cells. D4: Knocking down HIF1\( \alpha \) in parental cells resulted in increased LAT1 expression after KYN treatment. E, Knocking down HIF1\( \alpha \) in parental cells increased Trp uptake and further increased Trp uptake upon exposure to KYN (100 \( \mu \)mol/L). Consistently, treatment of KYN further heightened Trp uptake in CR cells (48 hours; \( P = 0.02 \), \( P = 0.006 \)). F, Knocking down IDO1 (shIDO1) in ALC suppressed LAT1 expression. sh-A to D represent four unique shRNA sequences. G, Diagram illustrating the binding partners of ARNT (HIF1\( \beta \)). When HIF1\( \alpha \) is downregulated, ARNT formed a new binding partner with AHR/KYN and initiated the transcription of genes that favored proliferation and increased Trp uptake, which can lead to further KYN secretion in CR cells.
the growth of CR cells. To determine that our in vitro cell model is indeed representative of in vivo settings, we created mouse CR cells (LLC-CR) from the Lewis lung mouse cell line (LLC). LLC-CR possessed 3- to 4-fold resistance to cisplatin (Supplementary Fig. S3) and yielded 2-fold higher basal levels of ROS (Fig. 3A). LAT1 and IDO1 activities were increased in LLC-CR, thus suggesting strong evidence of KP utilization (Fig. 3B and C).

Figure 3.
Increased IDO1 activity and immune-suppressive phenotype were found in CR tumor. A, ROS analysis of mouse (LLC vs. LLC-CR) cells detected by CM-H2DCFDA probe. LLC-CR expressed 2-fold higher basal level of ROS. B, Immunoblot showed that LLC-CR also expressed higher levels of LAT1 protein. C, LLC-CR possessed higher IDO1 activity (*, P = 0.04). D, IHC staining of intratumoral CD4⁺, CD25⁺ (arrow), FoxP3⁺ (arrow), TGFβ, and IDO1. Higher T-reg densities were found in mice bearing CR tumor than control. Box graph indicated quantification of IHC (intensity/μm²) using hybrid cell count.

LLC and LLC-CR were then allografted into C57BL6/N mice. After tumors developed, we then assayed for T-reg phenotypes. CD4⁺CD25⁺ and FoxP3 (forkhead family transcriptional regulator), which are the lineage-specific marker of T-reg cells, were higher in LLC-CR when compared with parental counterpart, which further indicated the involvement of KP in CR tumors (Fig. 3D). Furthermore, we also found higher expression of TGFβ in LLC-CR tumors. TGFβ is a well-known immunosuppressive cytokine that is released by
T-reg (34) and has been shown to activate IDO1 biosynthesis (35, 36). In fact IDO1 expression was elevated in mouse allografts of LLC-CR cells compared with allografts of LLC as seen through IHC with a mouse-validated antibody (Fig. 3D; IDO1). Together, our data suggest that increased IDO1 activity found in CR cells can create a highly immunosuppressive environment that favors the growth of tumors, thus making CR tumors good candidates for IDO1 treatment therapy.

**Evaluation of IDO1 expression alone may be insufficient to determine clinical benefits**

Depletion of Trp induces signaling events in T cells leading to anergy and because Trp is a precursor for both serotonin and the KP (Fig. 4A), we assessed the expression of Trp hydroxylase (TPH), the rate-limiting step in the serotonin pathway. TPH mRNA levels were extremely low and downregulated in our NSCLC cell line models (Supplementary Fig. S4A). We then assayed for IDO1, IDO2, and TDO2 (the rate-limiting step in KP) expressions. Even in the absence of IFNγ (cytokine inducer of IDO1), IDO1 mRNA expression was significantly higher in CR cells when compared with parental counterparts (Fig. 4B). However, to our surprise, a surge in CR cells’ mRNA expression did not significantly increase IDO1 protein levels (Fig. 4C). IDO2 mRNA expressions were higher in CR cells but not as significant when compared with IDO1 (Supplementary Fig. S4B). As for TDO2, this enzyme was not expressed in NSCLC cells but expressed only in brain cancer A172 cells utilized as positive controls (Supplementary Fig. S4C), thus indicating that TDO2 was not required in the formation of KYN in lung cancer cells. We then assayed for IDO1 activity to determine whether increased Trp uptake in CR cells was being utilized by the KP. As depicted in Figure 4D, all CR cells possessed significantly higher IDO1 activity, which was further enhanced with IFNγ treatment but not in parental cells.

Our data strongly support the concept that the level of IDO1 expression alone may not be the crucial factor in determining its activity. Consequently, these results may explain why many studies have shown insignificant correlation between IDO1 expressions and clinic–pathologic parameters.

**CR cells were hypersensitive to IDO inhibitors**

To determine that IDO1 plays an important role in CR cell proliferation and survival, we assayed for growth inhibitory effects using commercially available IDO1 inhibitors (Epacadostat, PF-06840003, and NLG-919), as well as indoximod, which targets IDO2 (37). Only CR cells were hypersensitive to IDO1 inhibitors with epacadostat showing to have the best efficacy for growth inhibition at 12 μmol/L ± 0.5 (Fig. 5A). In addition, IFNγ treatment further enhanced the cytotoxicity of epacadostatin only in CR cells (Fig. 5B) providing additional validation of CR cell’s dependence on IDO. Henceforth, we selected the use of epacadostat to carry out the subsequent experiments of IDO1 inhibition. We then determined the basal level of ROS in parental versus CR cells. ALC and FC cells possessed 2.5- and 3.5-fold higher basal level of ROS (Fig. 5C, respectively), and inhibiting IDO1 led to significantly higher ROS accumulation only in CR cells (Fig. 5D). It is noteworthy that epacadostat did not affect IDO1 protein level as determined by Western blot analysis (Fig. 5E), suggesting that epacadostat inhibits IDO1 enzymatic activity and not its expression in cells.

**Sensitivity to IDO1 inhibitor is correlated with higher ROS levels**

It is also noteworthy that IFNγ raised IDO1 protein expression slightly but not significantly in both parental and CR cell counterparts. We postulate here that the expression levels of IDO1 alone may not be a sole indicator of IDO1 inhibitor sensitivity, and the efficacy of IDO1 inhibition may depend on ROS levels. In further support of this notion, we found that a stepwise increase in cisplatin resistance (Fig. 6A) is correlated with the progressive increase in ROS production (Fig. 6B).
and escalating sensitivity to epacadostat (Fig. 6C). These findings suggested that when cancer cells acquired greater resistance to cisplatin, ROS production also progressively increased. Consequently, activation of KP is amplified leading to increasing sensitivity to IDO1 inhibitor.

To establish the relationship between IDO1 and ROS, in our lung cancer and CR models, we inhibited IDO1 using shRNA as previously shown (Fig. 2F). As we expected, knockdown (shIDO1) enhanced ROS production in ALC shIDOA&B due to the fact that IDO metabolizes ROS as a substrate (Fig. 6D). The addition of epacadostat to inhibit IDO only increased ROS in shCTRL and found no further effect in the absence of IDO (ALCshIDO1&nB), consistent with the specific modulation of ROS by IDO in these cells. More importantly, silencing IDO suppressed CR cell growth, which further implicated the important role of KP in proliferation of CR tumors (Fig. 6E). Moreover, the chemical inhibition of IDO with epacadostat in already knocked down cells did not result in further cell death indicating the specificity of the inhibitor for the IDO1 enzyme. Exposing CR cells to 1 mmol/L of TIRON (mitochondria-localized antioxidant; ref. 38) resulted in significant suppression of ROS production (Fig. 6F). Importantly, treatment of TIRON decreased IDO1 activity (Fig. 6G) and conferred resistance.
to epacadostat (Fig. 6H) in CR cells. Taken together, our results indicated that the IDO1 activity, as well as sensitivity to IDO1 inhibitor, was dependent on ROS levels.

**Discussion**

Over the past few years, we have discovered that metabolic reprogramming in CR cells contributes to various mechanisms of resistance to treatment (2, 6). Among these metabolic changes is the use of glutamine and fatty acids by CR cells as a carbon skeleton source in metabolism instead of glucose (2, 6). Another important finding was the detection of significantly higher basal levels of ROS accumulation in proliferating CR cells (3). Here, we have uncovered a unique metabolic difference wherein CR cells possess higher basal levels of ROS, which in turn activates the KP for Trp catabolism. Importantly, KP generates KYN, which has been shown to reprogram naive T cells to the immune-suppressive Treg phenotype. Further increase in ROS by interfering with tumor metabolism via IDO1 inhibition will selectively target these cisplatin-resistant lung cancer cells.
T cells to the immune-suppressive regulatory T-cell (T-reg) phenotype (Fig. 6).

Because ROS is a substrate for IDO1 in the oxidation reactions yielding KYN, in this study we inhibited IDO1 (i.e., IDO inhibitor (epacadostat) or shIDO) leading to higher ROS (Fig. 6). This induction in ROS accumulation pushed CR cells beyond a threshold of ROS tolerance resulting in greater growth inhibition. Moreover, epacadostat did not suppress IDO1 protein expression levels, suggesting that epacadostat inhibits IDO1 enzymatic activity and not its expression in cells. Nevertheless, these results clearly illustrate that survival of CR cells can be influenced by changes in ROS levels.

To further investigate the specific role of Trp in CR cells, we investigated the production of KYN and NAD\(^+\). As described previously, Trp is oxidized through IDO1 to generate KYN. Here, we reported that CR cells consumed Trp at a higher rate and generated more KYN but not NAD\(^+\). In fact, we have also previously reported that NMMAT2 (a cytosolic enzyme that represents the final step in the biosynthesis of NAD\(^+\) in both de novo and salvage pathways) expression was significantly lower in CR cells (2). These findings strongly confirm that Trp is being used as a precursor for KYN production in CR cells. The Trp consumption rate can also be utilized to stratify tumors on the basis of this metabolic attribute.

Increased amino acid transport [via evaluation of LAT1 (CD98)] has also been reported in many types of cancers including lung cancer, providing some insight into molecular modifications in tissue metabolism along tumor development (39, 40). In fact, all our CR cells possess higher LAT1 levels when compared with parental cells. Furthermore, we have previously reported that the IDO1 inhibitor suppressed LAT1 expression. Interestingly, we previously reported that CR cells possessed higher expression of the glutamate/cystine/xCT antiporter pump (2), and LAT1 is a major component of this plasma membrane antiporter. LAT1 facilitates the cellular uptake of extracellular cystine in exchange for intracellular glutamate and plays a key role in glutathione (GSH) synthesis. The activity of CD98/xCT-mediated cystine uptake in cancer cells is known to be highly associated with tumor growth and chemoresistance (41, 42). Thus, the important role of LAT1 in altered tumor metabolism is not limited to Trp uptake but also extends to the homeostatic regulation of antioxidant and ROS.

Interestingly, we did not find an increase in LAT1 levels in parental cells after KYN treatment, even though the IDO1 inhibitor suppressed LAT1 expression. This may be due to the fact that parental cells possessed significantly higher levels of HIF1\(\alpha\), which has a much better binding affinity to HIF1\(\beta\) than AHR (30, 43). Thus, in the presence of HIF1\(\alpha\), ARNT (HIF1\(\beta\)) is not readily available to bind and form a complex with KYN/AHR. In addition, we have previously reported that CR cells secrete less lactate (2, 6) which may very well impact the fate of T cells. Lactic acid can be taken up by neighbor cells, including immune cells, to perpetuate the glycolytic pathway and upregulate HIF1\(\alpha\) (44, 45). In fact, effector T cells (T-eff) rely on glycolysis to expand and this growth is greatly dependent on HIF1\(\alpha\) (46). A diminished amount of lactate may prevent a robust immune response to the neighboring tumor. Furthermore, mice deficient in HIF1\(\alpha\) fail to mount a strong T-cell response and have increased T-reg populations (47, 48). All of these findings support a concept wherein the metabolic modifications in CR tumors provide a growth advantage, which in turn impacts immune cells in the tumor microenvironment.

Although high IDO expression is a suggested predictor for poor prognosis and not associated with improved survival (49, 49, 51), it is often expressed in late-stage lung tumors with no study conducted in CR tumors (Supplementary Fig. S5). In addition, many studies have reported no significant correlation between IDO1 expression and clinicopathologic parameters (52, 53), although the concept of potential therapeutic efficacy for IDO1 inhibitors has been promoted (46). But the higher basal levels of ROS we have reported in CR cells will lead to a higher efficacy of the IDO1 inhibitor to decrease the level of KYN. Our studies have evaluated the metabolic background of these tumors, adding another novel level of molecular effectors that can influence IDO1 activity and any acquired resistance to the immune response. Thus, we believe that IDO1 expression level alone should not be used as a sole factor in the determination of clinical benefit/tumor response to IDO1 inhibitor.

Activation of IDO1 enzymatic activity requires ROS as a cofactor; hence, activity along with expression should be evaluated in the clinic. In previous work, we reported that parental (or naive treatment) cancer cells can upregulate both GSH and thioredoxin-1 (TRX1) antioxidant activities to keep ROS in check where CR cells cannot (3). Therefore, in the presence of active antioxidants, our findings here may explain why less IDO1 activity was found in parental tumors despite the expression of IDO1 protein. As for IDO2, a recent report showed that indoximod, which acts downstream of IDO1/2 to suppress mTORC1 activity, exerted a variety of benefits as an immunometabolic adjuvant (54). This is interesting because we have previously found that certain CR cells possessed higher mTORC1 activities, and inhibiting mTOR with CCI-779 can restore cisplatin sensitivity (22) in these CR cells. In the future, we plan to investigate the role of IDO2 in CR tumors by inhibiting both IDO2 and mTOR in our tumor models.

A recent phase III ECHO-301/KEYNOTE-252 (NCT02752074) clinical trial tested the efficacy of epacadostat in combination with pembrolizumab in patients with advanced melanoma previously untreated with PD-1 or PD-L1 checkpoint inhibitors (55). The trial evaluated the potential enhancement of anti-PD-1 therapy via IDO1 inhibition by gathering data on progression-free survival, overall survival, and adverse events but did not yield satisfactory results and was stopped early showing no indication that Epacadostat provided an increased benefit. Various limitations to the study parameters have been proposed such as the degree of adequate IDO1 inhibition within the tumor based on dosage and through sufficient drug exposure, use of combination therapies with DNA-damaging modalities, or the use of IDO and TDO inhibitor combination including dual inhibitors and AHR inhibitors (56). Perhaps future studies of such IDO and other inhibitors should include the evaluation of some metabolic factors such as elevated catabolism of Trp that affect the tumor microenvironment and the suppression of immunity (57).

Herein, we have gathered specific evidence to describe the effects of IDO, ROS, the KYN/Trp ratio in lung-mediated modifications of the tumor microenvironment, and the potential suppression of antitumor immunity. We believe that higher ROS levels together with significantly increased serum KYN/Trp ratios can be used to select patients for future treatment using IDO1 inhibitors. Such a stratification or patient grouping may increase efficacy in epacadostat trials. It is also noteworthy that even though patients with cancer from our study possessed higher basal levels of KYN/Trp ratio when compared with healthy controls, these levels were negligible when compared with patients who failed cisplatin and radiotherapy. Thus, the question remains on how high is high enough to use the KYN/Trp ratio as a biomarker for predicting the efficacy of IDO inhibitors. Our work has some limitations with only six patients, but we are currently recruiting more patients to our study who have failed cisplatin and radiotherapy.
In the context of a secondary treatment compendium based on IDO inhibition, the KYN/Trp ratio may simultaneously signal a need for a shift away from cisplatin therapy for some patients in anticipation of the development of resistance.

We firmly believe that IDO inhibitors may be more beneficial in the treatment of patients with lung cancer who failed cisplatin therapy than naïve treatment patients. The data presented herein provide a more comprehensive molecular investigation of metabolic pathway components in lung tumor cells that are resistant to cisplatin, characterizing variations that are proving to be valuable in survival mechanisms in resistant lung cancer cells.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: D.J.M. Nguyen, C. Wu, W. Sha, M. Wangpaichitr
Development of methodology: W. Sha, M. Wangpaichitr
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): D.J.M. Nguyen, C. Wu, W. Sha, M. Wangpaichitr
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): D.J.M. Nguyen, G. Theodoroopoulos, Y.-Y. Li, C. Wu, W. Sha, T.J. Lampidis, N. Savaraj, M. Wangpaichitr
Writing, review, and/or revision of the manuscript: D.J.M. Nguyen, G. Theodoroopoulos, Y.-Y. Li, L.G. Feun, T.J. Lampidis, N. Savaraj, M. Wangpaichitr
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): G. Theodoroopoulos, M. Wangpaichitr
Study supervision: M. Wangpaichitr

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Kynurenine Pathway in Cisplatin-Resistant Lung Cancer


Targeting the Kynurenine Pathway for the Treatment of Cisplatin-Resistant Lung Cancer

Dan J.M. Nguyen, George Theodoropoulos, Ying-Ying Li, et al.


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