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Authors
Fong, Lawrence
Hotson, Andrew
Powderly, John D
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Adenosine A2A Receptor Blockade as an Immunotherapy for Treatment-Refractory Renal Cell Cancer

Lawrence Fong[1], **Andrew Hotson**[2], **John D. Powderly**[3], **Mario Sznol**[4], **Rebecca S. Heist**[5], **Toni K. Choueiri**[6], **Saby George**[7], **Brett G.M. Hughes**[8], **Matthew D. Hellmann**[9], **Dale R. Shepard**[10], **Brian I. Rini**[10], **Shivaani Kummar**[11], **Amy M. Weise**[12], **Matthew J. Riese**[13], **Ben Markman**[14], **Leisha A. Emens**[15], **Daruka Mahadevan**[16], **Jason J. Luke**[17], **Ginna Laport**[2], **Joshua D. Brody**[18], **Leonel Hernandez-Aya**[19], **Philip Bonomi**[20], **Jonathan W. Goldman**[21], **Lyudmyla Berim**[22], **Daniel J. Renouf**[23], **Rachel A. Goodwin**[24], **Brian Munneke**[2], **Po Y. Ho**[2], **Jessica Hsieh**[2], **Ian McCaffery**[2], **Long Kwei**[2], **Stephen B. Willingham**[2], **Richard A. Miller**[2]

[1]UCSF Helen Diller Family Comprehensive Cancer Center, San Francisco, CA 94158

[2]Corvus Pharmaceuticals, Burlingame, CA 94010

[3]Carolina BioOncology Institute, Huntersville, NC 28078

[4]Yale University Cancer Center, Yale University, New Haven, CT 06510

[5]Massachusetts General Hospital, Harvard University, Boston, MA 02114

[6]Dana Farber Cancer Institute, Boston, MA 02215

[7]Roswell Park Cancer Institute, Buffalo, NY 14203

[8]Royal Brisbane Hospital, Herston and University of Queensland, Brisbane, Australia, Herston QLD 4029

[9]Memorial Sloan Kettering Cancer Center, New York, NY 10065

[10]Cleveland Clinic Foundation, Cleveland, OH 44109

[11]Stanford University School of Medicine, Stanford, CA 94305

[12]Karmanos Cancer Institute, Wayne State University, Detroit, MI 48201

[13]Medical College of Wisconsin, Wauwatosa, WI 53226

[14]Monash Health and Monash University, Melbourne, Clayton VIC 3800, Australia

[15]UPMC Hillman Cancer Center, Pittsburgh, PA 15232

[16]University of Arizona Cancer Center, Tucson, AZ 85719

[17]University of Chicago Medical Center for Care and Discovery, Chicago, IL IL 60637

[18]Icahn School of Medicine at Mount Sinai, New York, NY 10029

[19]Washington University Siteman Cancer Center, St Louis, MO 63110

[20]Rush University Medical Center, Chicago, IL 60612

[21]Ronald Reagan UCLA Medical Center, Los Angeles, CA 90095

[22]University of Nebraska Medical Center, Omaha, NE 68198

[23]BC Cancer - Vancouver, Vancouver, V5Z 4E6, BC

[24]The Ottawa Hospital Cancer Centre, Ottawa, ON K1H 8L6, Canada

Abstract

Adenosine mediates immunosuppression within the tumor microenvironment through triggering adenosine 2A receptors (A2AR) on immune cells. To determine whether this pathway could be targeted as an immunotherapy, we performed a phase 1 clinical trial with a small molecule A2AR antagonist. We find that this molecule can safely block adenosine signaling in vivo. In a cohort of 68 renal cell cancer (RCC) patients, we also observe clinical responses alone and in combination with an anti-PD-L1 antibody, including subjects who had progressed on PD-(L)1 inhibitors. Durable clinical benefit is associated with increased recruitment of CD8^{+} T cells into the tumor. Treament can also broaden the circulating T cell repertoire. Clinical responses are associated with an adenosine-regulated gene expression signature in pre-treatment tumor biopsies. A2AR signaling, therefore, represents a targetable immune checkpoint distinct from PD-(L)1 that restricts anti-tumor immunity.

INTRODUCTION

Overcoming immunosuppressive barriers within the tumor microenvironment has become an important strategy in treating cancer in the era of immunotherapy.[1] Accumulation of the nucleoside adenosine in the tumor microenvironment has been shown to inhibit the anti-tumor function of various immune cells, including cytotoxic T cells and natural killer cells, by binding to cell surface adenosine 2A receptor (A2AR).[2–9] Adenosine further restricts anti-tumor immunity by augmenting the immunosuppressive activity of myeloid and regulatory T (Treg) cells.[10–13] Adenosine is generated in tumors through the coordinated activity of the ectonucleotidases CD39 (also known as ENTPD1) and CD73 (also known as 5'-NT and NT5E) that together convert extracellular adenosine triphosphate (ATP), an inflammation-inducing factor, to adenosine. In turn, adenosine inhibits the pro-inflammatory effects of ATP released by injured or dying cells, and its generation can be co-opted by tumors as a mechanism to suppress anti-tumor immunity.[4, 14]

Renal cell carcinoma (RCC) may be particularly influenced by the effects of adenosine in the tumor microenvironment. The adenosine pathway genes *ADORA2A* (A2AR) and *NT5E* (CD73) are both highly expressed in RCC compared to other solid tumor histologies (Figure S1). Intra-tumoral hypoxia may contribute to the the production of extracellular adenosine in RCC tumors by upregulating CD39 and CD73 expression and stimulating the release of intracellular ATP.[2, 15–18] Adenosine pathway genes may also be induced as a

consequence of somatic mutations in the von Hippel–Lindau (VHL) gene, which are common in RCC, that increase levels of hypoxia inducible factor-1 (HIF-1) and HIF-2 activity to mimic conditions of intra-tumoral hypoxia.[2, 16, 19]

The treatment landscape of RCC has evolved dramatically in recent years, with promising results and/or approvals for therapies targeting the PD-(L)1 pathway alone or in combination with anti-CTLA-4, VEGF inhibitors, and tyrosine kinase inhibitors (TKIs).[20–22] However, complete remissions remain uncommon and metastatic RCC is still by in large incurable, with responses short lived in later lines of therapy. Studies in animal models have shown that prior treatment with anti-PD-1 antibodies results in increased expression of A2AR and CD73, suggesting that the adenosine pathway may contribute to therapeutic resistance to immunotherapy.[23, 24] There is a need for new combination therapies that prevent or overcome resistance to PD-(L)1 blockade, and for biomarkers to identify and predict resistance mechanisms with the goal of selecting the most appropriate therapy.

Ciforadenant (previously known as CPI-444) is a small molecule that potently and selectively binds A2AR, and competitively inhibits the binding and signaling of adenosine. [25] Ciforadenant has been shown to be active in multiple preclinical tumor models both as a monotherapy and in combination with anti-PD-(L)-1.[25, 26] We conducted a first-in-human Phase 1 dose-escalation study with ciforadenant monotherapy and combination with atezolizumab in pateints with advanced refractory cancers (Figure S2). The primary objectives were to 1) evaluate the safety and tolerability of multiple doses of ciforadenant administered on a daily schedule to subjects with selected incurable cancers as single agent and in combination with atezolizumab, 2) identify a recommended dose and schedule for further study of ciforadenant on the basis of safety, pharmacokinetic (PK), and pharmacodynamic (PD) data, and 3) evaluate the anti-tumor activity of ciforadenant as single agent and in combination with atezolizumab. Secondary objectives included a characterization of ciforadenant pharmacokinetics, biomarkers associated with the efficacy or safety of ciforadenant, and PD effects of ciforadenant on lymphocyte substes, cytokine production, immune function, tumor immunohistochemistrym or gene expression patterns. Based on the observation of early evidence of anti-tumor activity in patients with RCC, we expanded the study (Phase 1b) to gain more experience with monotherapy and combination therapy in this disease. Here we report the safety and efficacy of adenosine blockade in patients with advanced refractory RCC. We have also identified a gene expression signature that associates with treatment related disease control, which may be useful as a predictive biomarker.

RESULTS

PATIENTS CHARACTERISTICS

A total of 68 patients with RCC were enrolled over a 24 month period ending in April 2018. Thirty-three patients received ciforadenant monotherapy and 35 patients received the combination of ciforadenant and atezolizumab. Median on-treatment time was 5.0 (1.0, 21.7) months. Baseline demographics and disease characteristics are shown in Table 1. All patients had documented disease progression at the time of study entry and had failed multiple previous therapies (median=3) including TKIs and anti-PD-1 antibodies (Table 1).

More than 72 percent of patients were resistant or refractory to previous anti-PD-(L)1 antibody treatment; median time since last dose of anti-PD-(L)1 was 3.1 months (range 1.2 – 70.4 months) and 1.7 months (range 0.9–23.6 months) for monotherapy and combination therapy cohorts, respectively (Table 1). Nine percent of evaluable patients had 5% PD-L1 expression on tumor or immune cells in pretreatment tumor biopsy specimens (Table 1).

TREATMENT RELATED ADVERSE EVENTS

Immune-related adverse events were observed in patients receiving combination therapy and resolved upon discontinuation of treatment (Table 2). Grade 3 or 4 adverse events were infrequent with ciforadenant monotherapy. There were no treatment or disease related deaths while on therapy. At the time of data cut-off, 3% of monotherapy and 17% of combination-treated patients remained on therapy. 65% of patients discontinued therapy due to disease progression.

MODULATION OF A2AR SIGNALLING

Pharmacokinetic and pharmacodynamic studies were conducted in a Phase 1 portion of the study which enrolled patients with multiple different histologies. Signaling through A2AR induces phosphorylation of CREB downstream of protein kinase A activation.[27] *In vivo* blocking of the adenosine pathway by ciforadenant was examined by determining if A2AR on patient's peripheral blood lymphocytes could be stimulated *ex vivo* with the adenosine receptor agonist NECA, as determined by measurement of phosphorylation of CREB by flow cytometry. There was an exposure-response relationship between plasma drug concentrations and inhibition of CREB phosphorylation (pCREB), with nearly complete inhibition at drug levels exceeding 2000 ng/mL (Figure 1A). Pharmacokinetic measurements revealed that plasma C_{min} and C_{max} concentrations exceeding 2000 ng/mL were consistently achieved at the 100 mg BID dose of ciforadenant, and this dose was selected for efficacy evaluation during the expansion stage of this study. There were no significant differences in pharmacokinetics between ciforadenant monotherapy and combination treatment (Table S1).

EFFICACY

RECIST-defined partial responses were seen in 1 of 33 (3%) RCC patients treated with ciforadenant monotherapy (Figure S3A) and 4 of 35 (11%) RCC patients receiving the combination (Figure S3B). An additional 24% (15 of 63 evaluable) of patients experienced tumor regression that did not meet the RECIST criteria for a partial response (Figure 1B).

17% of patients receiving ciforadenant monotherapy and 39% of patients in the combination groups had confirmed disease control for at least 6 months (Table 3); The median progression-free survival was 4.1 months and 5.8 months for ciforadenant monotherapy and combination treatment, respectively (Figure 1C). The estimated overall survival (OS) exceeded 90% at 25 months for the combination group and is over 69% at 16 months for the ciforadenant monotherapy group (Figure 1D).

Significant tumor regression was observed in heavily pre-treated patients receiving either ciforadenant monotherapy or combination treatment, including patients who failed prior therapy with anti-PD-(L)1 therapy. The median time to best tumor response was 3.4 and 5.5

months for monotherapy and combination, respectively. The kinetics of tumor response were prolonged in some patients as seen in the spider plots (Figure S4). Of note, this included one patient receiving ciforadenant monotherapy who demonstrated initial tumor progression followed by durable tumor regression lasting almost one year (Figure S4) while on continuous therapy. This patient was scored as having progressive disease.

CIFORADENANT EFFICACY IS ASSOCIATED WITH CD8+ T CELL INFILTRATION

The extent of CD8+ T cell infiltration present in pre-treatment and on-treatment tumor biopsies was evaluated using immunohistochemistry. Increases in tumor infiltrating CD8+ T cells were significantly higher in patients with at least 6-month disease control compared to patients with shorter periods of disease control (Figure 2A, left panel). Representative images of CD8+ T cell infiltration into the tumor microenvironment following ciforadenant monotherapy are shown in Figure 2A, right panel. We did not observe an association between tumor response and baseline CD8+ T cell infiltration or CD73 expression, as assayed by both NanoString (Figures S5A–B, S5E) and immunohistochemistry (Figure S5C–S5E)

CIFORADENANT EFFICACY IS ASSOCIATED WITH DIVERSIFICATION OF TCR REPERITOIRE

We have previously shown that CTLA-4 blockade can modulate the TCR reperitoire.[28] The effect of adenosine blockade on the TCR repertoire was investigated by sequencing the TCR Vβ genes in patients receiving ciforadenant alone and in combination with atezolizumab. The Morisita Index, which is a measure of change in TCR repertoire in the peripheral blood on drug treatment, was greater (median=0.15, SD=0.23) in subjects with a more diverse baseline TCR repertoire than in subjects with a higher clonality (median=0.03, SD=0.01) (Figure 2B). These results would suggest that ciforadenant exerts immunomodulatory effects on the adaptive compartment in patients with broader TCR repertoires that may include pre-existing tumor-reactive T cells. 8 of the 13 patients with diverse baseline TCR repertoires, but none of patients with more narrow repertoires, exhibited a Morisita Index above 0.1 compared to post-treatment samples (Figure 2B), a threshold previously shown to be associated with anti-CTLA-4 mediated changes in TCR repertoire.[28] Similar findings were observed in both the ciforadenant monotherapy and the ciforadenant plus atezolizumab combination group.

RESPONSES TO CIFORADENANT ARE ASSOCIATED WITH EXPRESSION OF AN ADENOSINE-RELATED GENE SIGNATURE

It is not practical to routinely measure the concentration of extracellular adenosine in tumors due to its short half life (plasma $t_{1/2}$ 10 seconds).[29] Therefore, we investigated the effects of adenosine on gene expression prolife (GEP) in PBMCs to identify a potential molecular surrogate for adenosine exposure in the tumor microenvironment. Adenosine responsive genes were identified by *in vitro* stimulation of normal human peripheral blood mononuclear cells with NECA (Figure S6A). A dose-dependent increase in the expression of CXCR2 ligands (CXCL1,2,3,5,8) and mediators of neutrophil/MDSC (myeloid derived suppressor cells) biology, such as IL-23, were observed (Table S2, see Figure S6B for graphical representation of analysis). Increased expression of monocyte/macrophage

inflammatory mediators such as IL-1β, IL-6, and PTGS2 were also observed, as were increases in CD14, SLC11A1, and THBS1 (Table S2). In contrast, CXCL10 expression was decreased by NECA in a dose dependant manner (Figure S6B). These gene expression changes were also reflected in the protein levels of CXCL1, CXCL5 (both increased) and CXCL10 (decreased) in culture supernatants (Figure 3A and 3B). Addition of the A2AR antagonist ciforadenant (1 μM) to PBMC cultures fully neutralized the induction of CXCL5 by 0.1 μM and 1 μM NECA, but not at 10 μM NECA (Figure 3C). This result is expected as NECA is a much more potent agonist of A2AR (35-fold) and A2BR (72-fold) than adenosine.[30] Dose dependant increases in CCL2, CXCL1, CXCL5, and CXCL8 protein expression were also observed by intracellular flow cytometry (Figure 3D–3G). Interestingly, these protein changes occurred specifically in CD14+ monocytes and not CD8+ T cells or CD19+ B cells. (Figure 3D–3G), indicating that the source of many adenosine signature chemokines any cytokines is likely to be of monocytic lineage. Our findings suggest that adenosine signaling not only directly dampens T cell immunity, but also shifts the balance away from T effector responses and toward myeloid suppressor recruitment and functions.[25]

We next evaluated the expression of adenosine-induced genes in tumor biopsies collected from 30 patients prior to treatment initiation with ciforadenant alone or in combination with atezolizumab. Patient tumors that demonstrated high levels of adenosine gene signature expression (AdenoSig^{High}, see Methods – Adenosine Gene Signature in RCC Tumors) were almost exclusively low for an angiogenesis GEP (VEGFA, PECAM1, CD34) (Figure 4A). Gene expression of markers for baseline T cell activation neither associated with tumor response nor expression of the AdenoSig (Figure 4A). High levels (top two tertiles) of AdenoSig expression in baseline tumor biopsies was significantly associated with tumor regression (Figure 4B, p<0.008). These AdenoSig^{High} patients also demonstrated more durable PFS, the tail of the PFS curve (40+ weeks) was comprised of 5/16 subjects with high AdenoSig expression compared to 0/8 with little or no expression (Figure 2C). These results suggest ciforadenant anti-tumor activity in RCC is associated with high levels of expression of the AdenoSig in pretreatment biopsies and that the AdenoSig may be useful as a predictive biomarker to select patients more likely to respond to agents that antagonize adenosine production or signaling.

DISCUSSION

This is the first clinical report confirming the activity of adenosine pathway antagonism for cancer immunotherapy. Patients in this trial were often resistant or refractory to anti-PD-(L)1 antibodies, and had predominantly PD-L1 negative tumors, suggesting that these patients harbored tumors that were not immune suppressed through the PD-1/PD-L1 axis. The A2AR antagonist ciforadenant demonstrated monotherapy activity in immunotherapy naïve patients as well as patients who were resistant or refractory to prior anti-PD-(L)1 treatment. Although this trial was not designed to compare monotherapy to the combination, treatment with ciforadenant plus atezolizumab appeared to improve efficacy and resulted in a partial response rate of 11%, a 6-month DCR of 39%, progression free survival of 5.8 months, and 90% overall survival at 25 months. Ciforadenant treatment was well tolerated, both alone and in combination with atezolizumab. The observations of anti-tumor activity of

ciforadenant in RCC are consistent with several biological observations, including a significant association between the adenosine-related gene expression signature and tumor response. We also observed an association with T cell infiltration induced by treatment and prolonged disease control. T cell receptor diversity was also more frequently increased in patients following treatment.

Of interest in this study is the finding of encouraging disease control and survival benefit without high objective response rates. As recently reported by others in a large meta analysis of 87 clinical trials of solid tumors treated with checkpoint inhibitors, there is a lack of correlation between response rate and survival.[31] The reasons for this are uncertain, but could be due to the triggering of a persistent immune response that maintains durable tumor growth control despite the absence of an immediate elimination of tumor cells. Response rates may also be underestimated on account of the inherent problems of differentiating between tumor cell volume and residual inflammation and fibrosis following tumor elimination by computerized tomography scan.

In this study we characterized an adenosine-related gene expression signature in tumor biopsies as a surrogate biomarker to identify patients with adenosine-rich tumors (Figures 4A and 4B). In vitro stimulation of human PBMCs with A2AR agonists enabled us to identify a specific gene signature which in biopsies from RCC patients was associated with tumor responses to ciforadnant alone or in combination with atezolizumab. The efficacy data presented here suggest that resistance to anti-PD-(L)1 may be reversed by ciforadenant in the AdenoSig[High] patients. Indeed, 72% of the RCC patients enrolled in our study had received prior anti-PD-(L)1 therapy. In preclinical studies, treatment with anti-PD-1 led to increases in A2AR and CD73 expression and were associated with enhanced tumor responses to A2A receptor blockade.[24] Additionally, CD38-mediated production of adenosine has been shown to suppress anti-tumor immunity following anti-PD-1 treatment. [32] It is currently unclear if adenosine-mediated resistance to checkpoint blockade exists at the time of tumor diagnosis or evolves as a resistance mechanism during the course of anti-PD-(L)1 treatment. Interestingly, our AdenoSig substantially overlaps with an independently derived "myeloid inflammation" signature that was negatively associated with progression free survival following front-line treatment with atezolizumab in RCC.[21] We also found that high expression of the AdenoSig identified patients with low expression of an angiogenesis gene signature; low angiogenesis gene expression is associated with inferior PFS following treatment with sunitinib.[21, 33] We therefore hypothesize that the AdenoSig[High] patients will be poor responders to anti-angiogenesis agents due to the low expression of angiogenesis genes and expect ciforadenant plus anti-PD-(L)1 treatment to compare favorably to such agents.

The studies reported here with ciforadenant confirm the immune-enhancing and therapeutic potential of adenosine pathway blockade. Several other antagonists of A2AR and A2BR are currently under active clinical evaluation, both as monotherapies and in combination with PD-1 blockade, chemotherapy, or targeted agents. Early preliminary data has revealed signs of clinical activity in RCC, NSCLC, prostate cancer, endometrial cancer, anal cancer, and head & neck cancer.[34–37] In many cases, these treatments have demonstrated activity in both IO naïve and reistant/refractory patients. These promising results validate the adenosine

axis as a viable immunotherapy target, but more data will be required to determine which molecule or which combinations will be most effective, and what biomarker assays will be most informative.

The patinets enrolled in this trial were heavily pretreated, with a median of 3 prior treatmens (range 1–5). It's possible that ciforadenant and other adenosine pathway antagonists will be most effective when used in earlier lines of therapy where the immune system is less compromised from prior immunosuppressive regimens. Previous studies in preclinical mouse models have suggested that the efficacy of adenosine pathway antagonists may be predicated on the presence of a sufficient number of anti-tumor T cells.[2] While it is not yet possible to prospectively screen patients to ensure a specific frequency or distribution of tumor-reactive T cells, an alternative strategy may be to administer ciforadenant in combination chimeric antigen receptor T cells (CAR-T) or ex vivo amplified tumor infiltrating lymphocytes (TILs) to exogenously supplement tumor reactive T cells. We note that ciforadenant treatment potentiated the generation of novel T cell clones appearing in the peripheral blood, however more studies will be required to determine if this effect alone is robust enough to generate an effective supply of anti-tumor T cells in otherwise deficient patient or if the the T cell-autonomous effects of A2AR blockade are more prominent in preclinical models.

The unique mechanism of action and favorable safety profile suggest that ciforadenant may be valuable, particularly in patients who have failed anti-PD-(L)1 therapy or in combination with PD-(L)1 blockade to prevent the development of resistance. While our study combined ciforadenant with a PD-L1 antagonist, there is compelling preclinical evidence for combining adenosine pathway antagonists with other immunotherapies, chemotherapy, and tumor vaccines.[8, 23, 24, 26, 38–44] Recent success combining anti-PD-1 with tyrosine kinase inhibitors (TKIs) and other angiogenesis inhibitors suggests there is rationale to explore triplet combination involving ciforadenant to further enhance responses.[21, 45, 46] Future studies are also expected to evaluate the utility of the AdenoSig as a predictive biomarker to select patients most likely to benefit from treatments based on adenosine blockade.

METHODS

PATIENTS

In the Phase 1A portion of the study, patients at least 18 years of age were eligible for enrollment if they had non-small cell lung cancer (NSCLC), clear-cell RCC, melanoma, triple negative breast (TNBC), bladder, prostate, head and neck or colorectal cancer (microsatellite instability high), and had failed approved therapies for their cancers []. Eastern Cooperative Oncology Group (ECOG) performance status of 0 or 1 and adequate hematologic, hepatic and renal functions were required. Prior treatment with an anti-PD-(L)1 or anti-CTLA-4 antibody was allowed. PD-L1 expression in the tumor was not used to select patients. This open-label, multicenter, phase 1/1b trial enrolled patients in 30 centers in the United States, Canada and Australia (see protocol design in Figure S2). Ciforadenant was evaluated at 50 mg and 100 mg twice a day for 14 days and 28 days; 200 mg once a day for 14 days, of a 28 day cycle. In patients receiving the combination, atezolizumab was

given 840 mg intravenously every 14 days. The analysis of pharmacokinetics and pharmacodynamics was performed in patients from the Phase 1A portion of the study. The selected dose of ciforadenant for the Phase 1B portion was 100 mg twice daily for 28 days as monotherapy and in combination with atezolizumab. Both RCC monotherapy and combination cohorts were expanded per protocol based on the demonstration of early signs of efficacy, defined as observing one or more responses in the first eleven patients (Figure S3). All patients with RCC that were enrolled in the Phase 1B portion of the trial are included in the safety, efficacy, and biomarker analyses reported here (see Figure S3 for overall trial design).

Patients were followed for safety during treatment and follow up and every two to three months for investigator-assessed tumor response using Response Evaluation Criteria in Solid Tumors (RECIST) version 1.1. Responses and stable disease required confirmation by subsequent CT scan. All patients continued treatment until confirmed disease progression or unacceptable toxicity. At investigators discretion, patients with disease progression could continue on therapy if they were thought to be deriving clinical benefit. Objective response, disease control rate (DCR, complete or partial response or stable disease for 3 months), and duration of response were evaluated. Progression-free survival (PFS) and overall survival were calculated using Kaplan-Meier analysis.[47]

The study was designed by the sponsor (Corvus Pharmaceuticals) and academic advisors. This trial was performed in accordance with the ethics and principles of the Declaration of Helsinki and the International Council for Harmonisation Good Clinical Practice Guidelines. All patients provided written informed consent. The protocol and informed consent forms were approved by an institutional review board or independent ethics committee at each study site. The data were collected and analyzed by the sponsor and reviewed by a data and safety monitoring committee that consisted of members from the sponsor and independent reviewers. The manuscript was written, reviewed, and approved by the authors. ClinicalTrials.gov Number .

PHARMACOKINETIC AND PHARMACODYNAMIC MEASUREMENTS

Blood was collected prior to treatment initiation and again on day 14 just prior to, and at 1.5 hour, 3 hours, 5 hours and 8 hours post administration of ciforadenant. The concentration of ciforadenant was determined by liquid chromatography with tandem mass spectrometry following protein precipitation from plasma with methanol/acetonitrile using an internal standard/peak area method. For assessment of A2AR occupancy with ciforadenant, pCREB (phosphorylated cAMP response element binding protein) measurements were conducted by flow cytometric analysis in whole blood that was stimulated with 1μM of the stable adenosine analog NECA (5'(N-ethylcarboxamido)adenosine, Sigma-Aldrich) for 15 minutes. Cells were then fixed (Lyse/Fix buffer from Becton Dickinson) and stored in methanol at −80°C for flow cytometry with antibodies including anti-CD19 (Becton Dickinson) and anti-pCREB (Cell Signaling Technologies).

ASSESSMENT OF PD-L1, CD8, AND CD73 EXPRESSION

Core needle tumor biopsies were collected prior to therapy and while on treatment (range 1–4 months on treatment, median 1.5 months), and fixed in formalin, embedded in paraffin, and processed into 5 μm sections. Immunohistochemistry (IHC) for PD-L1 protein expression was performed using the Ventana PD-L1 (SP142) assay (Ventana, Tucson AZ), and scored using a cut-off of 5% tumor cell or immune cell staining and positivity according to the diagnostic label for the assay. CD8 clone C8/144B was used to evaluate CD8 positive cell staining (HistoGeneX, Antwerpen, Belgium). To calculate the change in CD8$^+$ T cell infiltration, a noise floor of 0.5% was applied to the percentage of the tumor area positive for CD8 staining, and then Log_2 (the area of on-treatment CD8$^+$ / pre-treatment CD8$^+$) was calculated for each patient with paired biopsies. An unpooled two-tailed t-test was performed comparing the patients with DCR < 6mo and DCR   6mo to calculate the p-value. CD73 clone D7F9A was used to evaluate CD73 positive cell staining (HistoGeneX, Antwerpen, Belgium).

ANALYSIS OF CD73 AND A2AR EXPRESSION IN TUMORS

RNASeq gene expression data from The Cancer Genome Atlas (TCGA) was downloaded from the cBioPortal (http://www.cbioportal.org). cBioPortal processed and normalized the data using RSEM to translate the raw data into TPM (transcripts per million). For each indication, median tumor expression levels were calculated, as well as 2.5% and 97.5% percentiles for plotting the 95% confidence intervals. Average expression levels for *ADORA2A* and *NT5E* were determined by calculating the mean expression within each indication, and then calculating the mean of all indications.

T CELL RECEPTOR (TCR) REPERITOIRE ANALYSIS

Sequencing of the CDR3 regions of human TCRβ chains was performed using the immunoSEQ® Assay (Adaptive Biotechnologies, Seattle, WA). Extracted genomic DNA was amplified in a bias-controlled multiplex PCR, followed by high-throughput sequencing and the abundance of each unique TCRβ CDR3 region was quantified.[48–50]

ADENOSINE GENE SIGNATURE

Human peripheral blood mononuclear cells (PBMCs) were isolated from buffy coat samples by density centrifugation with Histopaque 1077 (400*g, 30 min). Cells were washed and resuspended at a density $2*10^6$ cells/ml in RPMI + 10% human serum (Sigma-Aldrich, Cat. No. H4522). PBMCs (10 mL) were stimulated with DMSO or 5'-*N*-Ethylcarboxamidoadenosine (NECA, Tocris, Cat. No. 1691) at 0.1, 1, or 10 μM for one hour. T cells were then activated with anti-CD3 (clone HIT3a, 1 μg/ml) and anti-CD28 (clone CD28.2, 1 μg/ml) antibodies and incubated for 48 hours at 37 degrees. Purified RNA was collected using a Qiagen RNAEasy Kit according to manufacturers protocol. NanoString analysis was performed according to manufacturers protocol on a NanoString Sprint instrument using the NanoString PanCancer Immune Panel with PLUS codeset. Normalized counts were obtained using NanoString nSolver Software. Log_2 transformed expression data were fit to a linear model comprised of donor and treatment effects. Genes which showed a statistically significant treatment effect (i.e. gene expression level increased or decreased as

NECA level increased) were identified in 3 initial donors. Adjusted p-values were used to correct for multiple hypothesis testing using the Benjamini-Hochberg procedure. Cuture supernatants from three donors were assessed with human CXCL5 ELISA kit (R&D Systems, DX000).

INTRACELLULAR FLOW CYTOMETRY

Purified human PBMCs from three healthy donors were co-cultured with various concentrations of NECA and were stimulated with anti-human CD3 and CD28 antibody (at 1 μg/mL). Cells were kept in culture for 48 hours days. Golgi block was added 4 hours prior to intracellular flow cytometry analysis. Antibodies used in analysis include: anti-human CD8a (Clone RPA-T8, BioLegend Cat No. 301048), anti-human CD3 (Clone OKT3, BioLegend Cat No. 317322), anti-human CD4 (clone OKT4, BioLegend Cat No. 317436), anti-human CD56 (clone 5.1H11, BioLegend Cat No. 362546), anti-human CD205 (clone HD30, BioLegend Cat No. 342210) anti-human CD14 (clone 63D3, BioLegend Cat No. 367118), anti-human CD19 (clone SJ25C1, BioLegend Cat No. 363034), anti-human CXCL5 (clone J111B7, BioLegend Cat No. 524104), anti-human MCP-1 (clone 2H5, BioLegend Cat No. 505904), anti-human IL-8 (clone E8N1, BioLegend Cat No. 511408), anti-human CXCL1 (clone 20326, R&D Systems Cat No. IC275P). Data was acquired on a CytoFLEX flow cytometer (Beckman Coulter) and analyzed in FlowJo software v10.

ADENOSINE GENE SIGNATURE IN RCC TUMORS

Tumor biopsies were obtained from RCC patients prior to treatment. RNA was extracted from tumor tissue macrodissected from 5 μm sections cut from FFPE specimens. 70–100 ng of purified RNA was analyzed on the NanoString Sprint instrument using the PanCancer Immune Panel (NanoString) at HistoGeneX. Nanostring data was normalized to housekeeper genes, and bridging of normalized data between Nanostring codeset lots was performed. A noise floor of 30 counts was applied to normalized and bridged Nanostring data.

The expression of eight NECA-induced immune-related genes (IL1B, PTGS2, and CXCL1,2,3,5,6,8) were selected to comprise the 'Adenosine Signature' (AdenoSig) because they were expressed at detectable levels in the patient tumor samples from this study and were found to be significantly induced in normal PBMCs upon exposure to NECA. AdenoSig gene expression profile scores were calculated as the mean of the Log_2 value of the counts for each gene component. The distribution of the adenosine signature for all evaluated RCC patients was determined, and a cut-point at the first tertile was selected as optimal to differentiate patients with low expression from high expression. An unpooled two-tailed t-test was performed to calculate the p-value for the comparison between the AdenoSig low and AdenoSig high patient groups for the best change in the sum of the longest dimensions of the target lesions. Normalized gene expression data was z-scale transformed for heatmap visualization.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Conflict of Interest Statement:

Indicated authors are current or former employees of Corvus Pharmaceuticals. These authors may also be shareholders in Corvus Pharmaceuticals or inventors on patents held by Corvus Pharmaceuticals. LF has received research support from Abbvie, Bavarian Nordic, BMS, Corvus, Dendreon, Janssen, Merck, and Roche/Genentech. TKC and BIR have received research support and served as a consultant for Corvus and Roche/Genentech. No potential conflicts of interest directly related to this work were disclosed by the other authors.

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STATEMENT OF SIGNIFICANCE

This first-in-human study of an adenosine 2A receptor antagonist for cancer treatment establishes the safety and feasibility of targeting this pathway by demonstrating anti-tumor activity with single-agent and anti-PD-L1 combination therapy in patients with refractory renal cell cancer. Responding patients possess an adenosine-regulated gene expression signature in pre-treatment tumor biopsies.
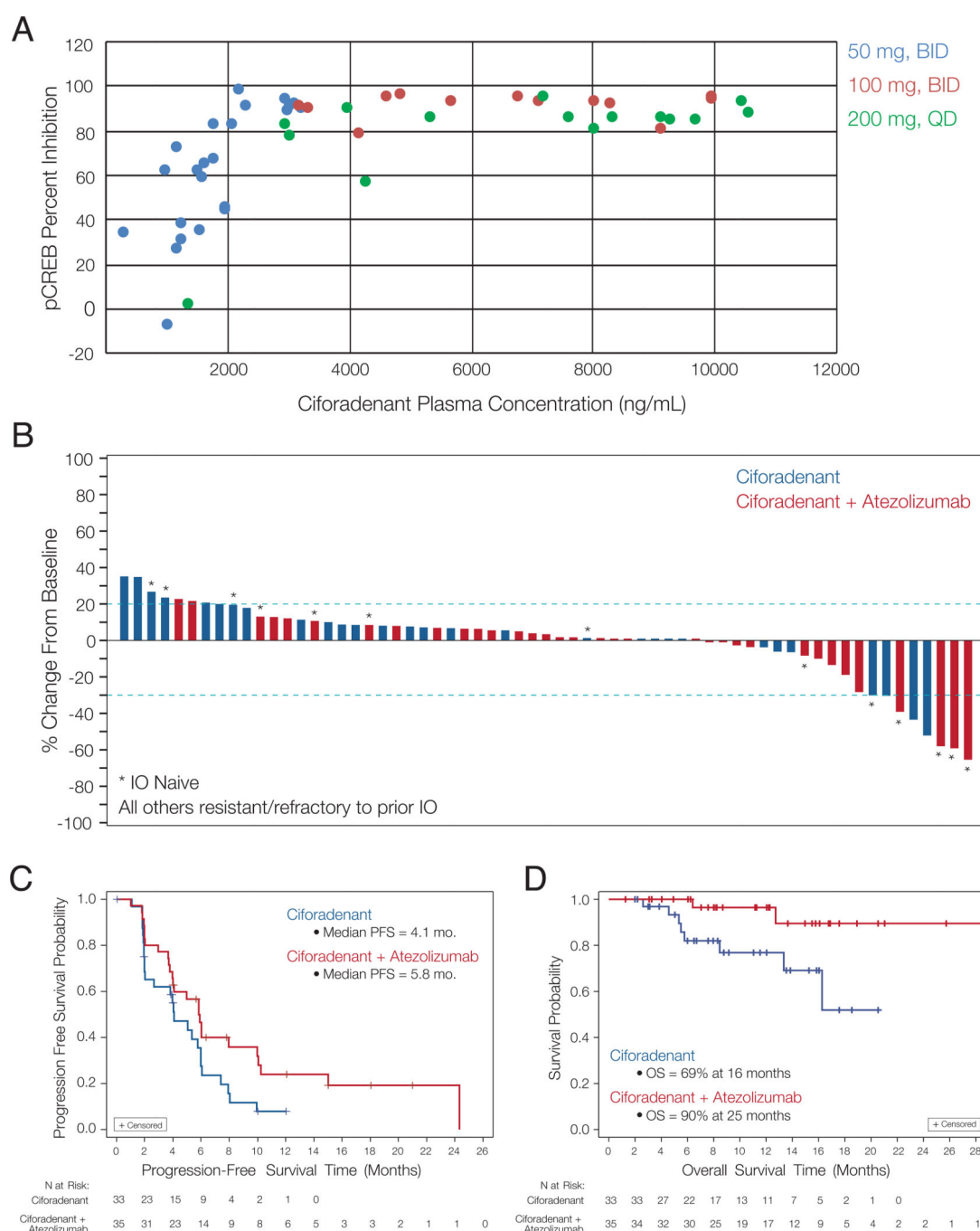
FIGURE 1: Pharmacokinetics, pharmacodynamics, and tumor response to ciforadenant alone and in combination with atezolizumab.

A) Blood was collected from subjects with different dosing regimens during an eight-hour time course on treatment day 14 and activated with exogenous 1 μM NECA. Concurrent pharmacokinetic assessment were also performed. The graph shows the relationship between plasma concentration of ciforadenant and inhibition of NECA-induced pCREB, with data from individuals dosed with 50mg BID (blue), 100mg BID (red) or 200mg QD (green). B) Waterfall plot showing best overall response in sum of longest diameter measurements of target lesions. Patients naïve to immunotherapy at time of enrollment are designated with an

asterisk. All others were resistant or refractory to prior immunotherapy treatment. C-D) Progression free survival (C) and overall survival (D) in patients treated with ciforadenant or the ciforadenant plus atezolizumab combination.
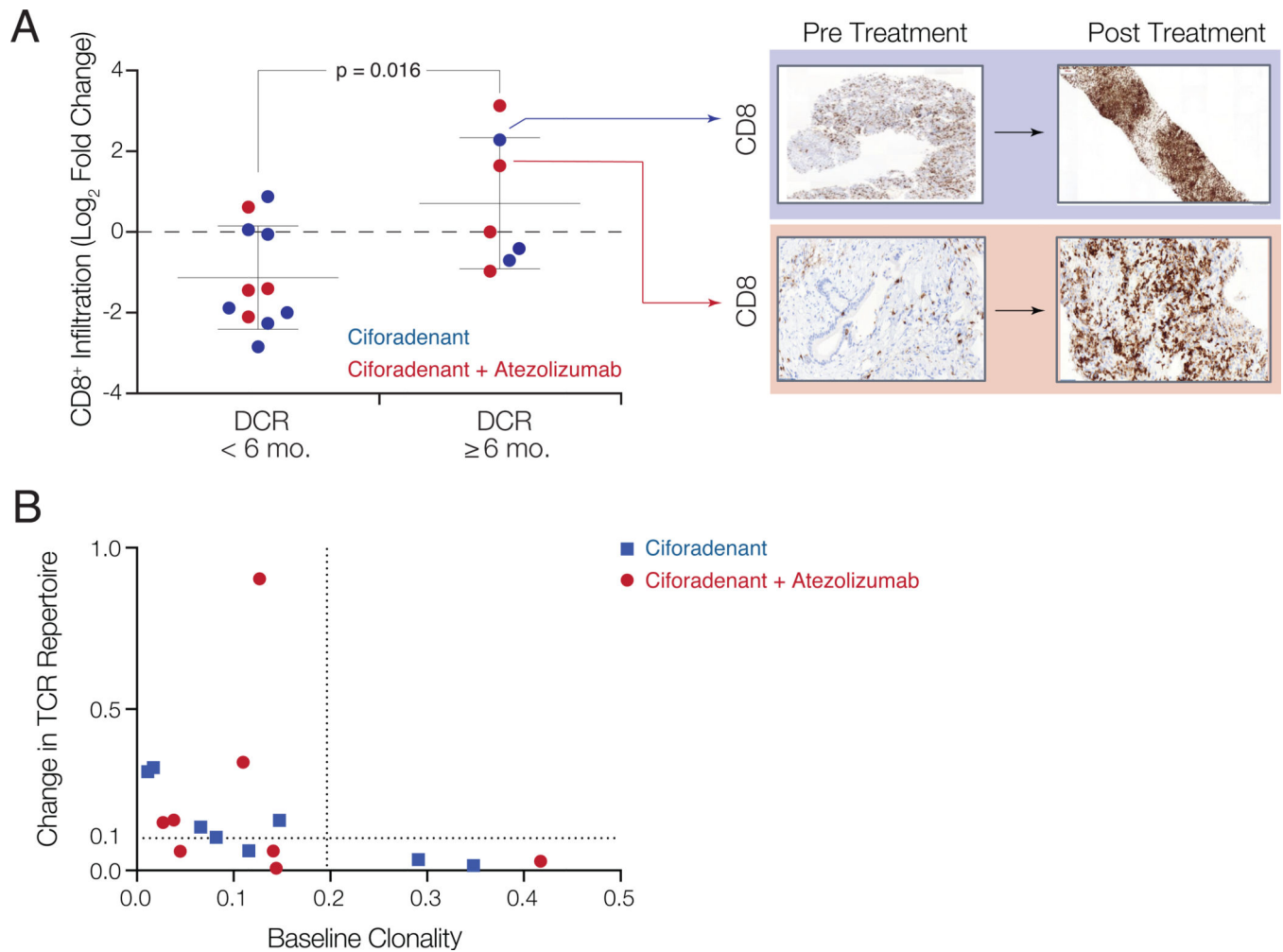
FIGURE 2: Tumor response to ciforadenant is associated with T cell infiltration and expression of an TCR diversification

A) Immunohistochemistry of CD8 was performed pre- treatment and 1 – 4 months post treatment, and the ratio of the CD8+ tumor area was determined. Representative images of CD8+ T cell infiltration following ciforadenant monotherapy treatment are shown in the right panel. B) TCR sequencing was performed on blood samples obtained pre- and post-treatment. Morisita's Index (Changes in TCR repertoire) after ciforadeant alone or in combination with atezolizumab was mapped as a function of baseline clonality. Horizontal dashed line indicates a threshold previously shown to be associated with anti-CTLA-4 mediated changes in TCR repertoire.
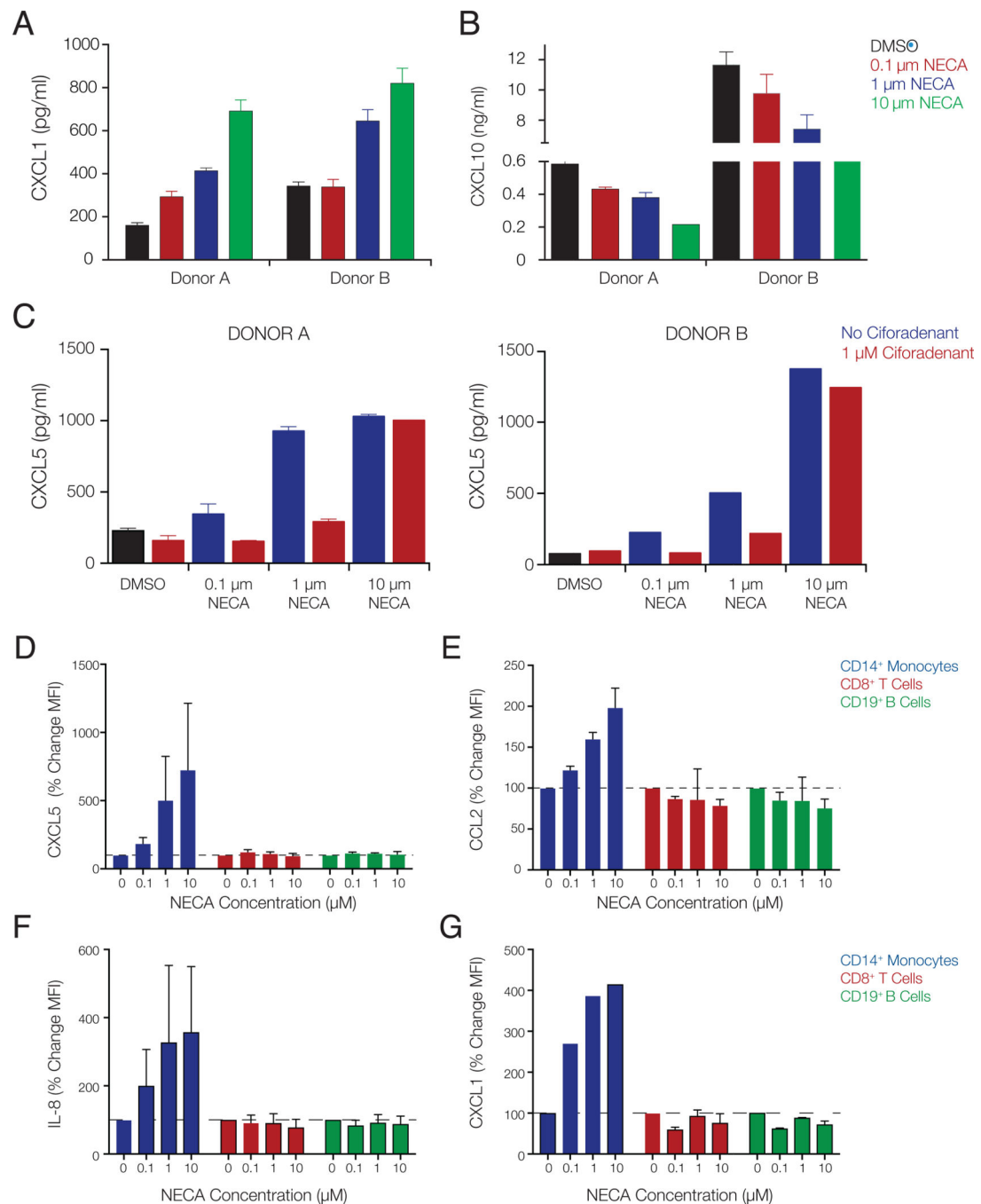
FIGURE 3: In vitro characterization of gene expression signature related to adenosine exposure
A-B) Adenosine signature related chemokine concentrations exhibited dose a dependent increase (CXCL1, panel A) or decrease (CXCL10, panel B). C) Addition of ciforadenant (1 μm) neutralizes the induction of CXCL5 by NECA as determined by ELISA. D-G) Purified human PBMCs from healthy donors were co-cultured with indicated the concentrations of NECA and were stimulated with anti-human CD3 and CD28 antibodies. Cells were kept in culture for 2 days. Golgi block was added 4 hours prior to collecting cells for intracellular flow cytometry analysis. CD14+ monocytic cells exhibited elevated expression of adenosine

signature (as determined by mean fluorescence intensity, MFI) related cytokines and chemokines including CXCL5 (D), CCl2 (E), IL-8 (F), and CXCL1 (G) as NECA concentration increased. Lymphocytes including CD8+ T cells and CD19+ B cells had minimal changes. Error bars represent SEM.
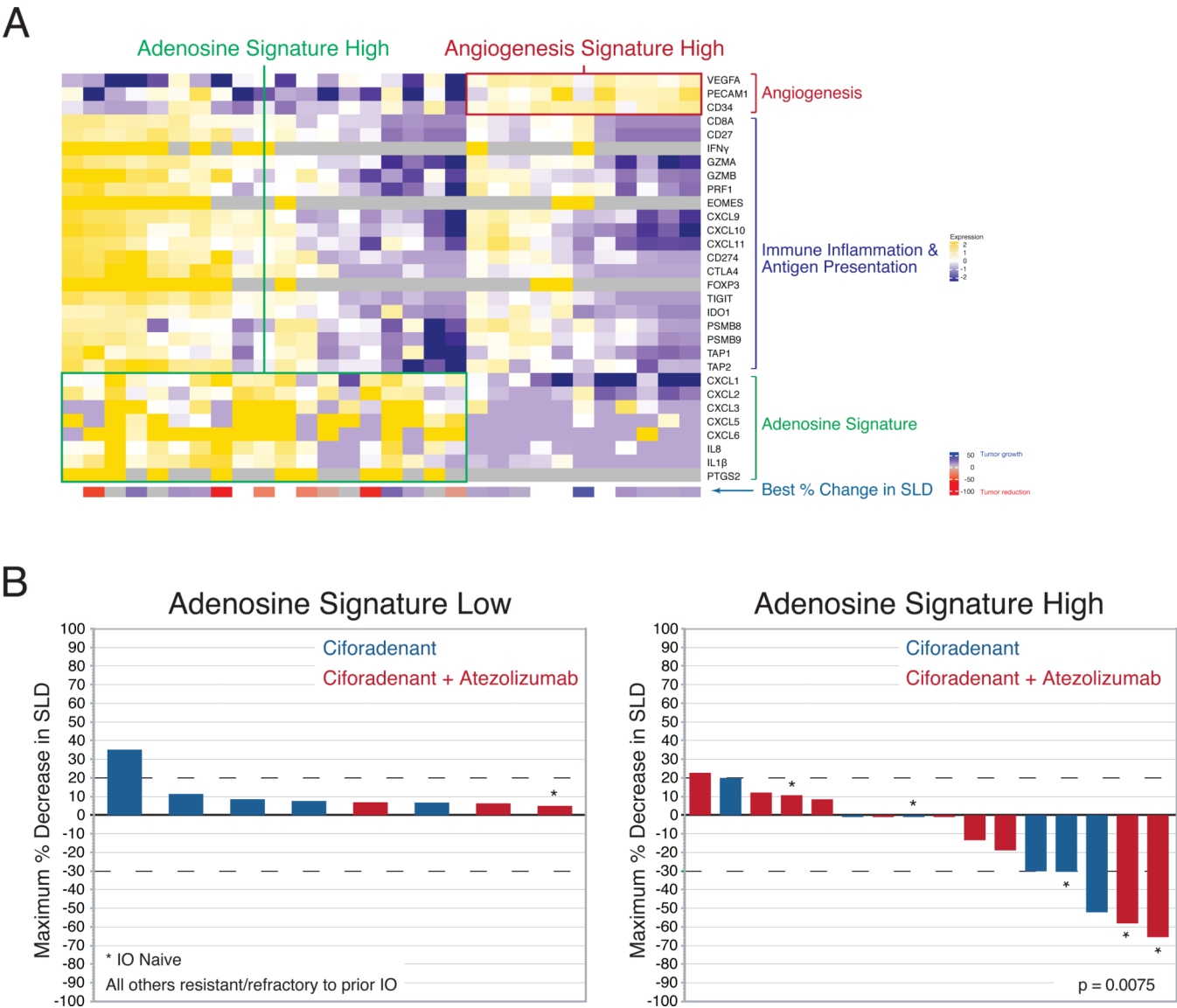
FIGURE 4: Tumor response to ciforadenant is associated with expression of an adenosine gene expression signature

A) Genes of interest (rows) were assessed from tumors collect pre-treatment from 30 patients (columns). Gene expression was z-score transformed with high (yellow) and low (purple) expression normalized for each gene. The median expression of IFNG, EOMES, FOXP3, and PTGS2 was equivalent to the noise floor so for these genes expression at the noise floor is colored gray and above the noise floor is yellow. Genes are grouped by biological functions of angiogenesis (orange), immune and antigen presentation (blue), and adenosine signature (green). B) The waterfall plot shows the best change in the sum of the longest dimensions for patients with low (left) or high (right) expression of the adenosine signature.

Table 1.

Baseline Characteristics of All Enrolled Patients

Characteristic	Ciforadenant (N=33)	Ciforadenant + Atezolizumab (N=35)
Age (years, median (range)	60 (47, 76)	65 (44, 77)
Gender, male n (%)	25 (75.8)	28 (80)
Sites of Disease, n (%)		
Lung	22 (66.7%)	27 (77.1%)
Lymph Node	19 (57.6%)	21 (60%)
Bone	16 (48.5%)	15 (42.9%)
Liver	10 (30.3)	9 (25.7%)
Number of prior tderapies		
Median, range	3 (1, 5)	3 (1, 5)
Prior IO, number of subjects n (%)	24 (72.7)	25 (71.4)
Months since prior IO		
Median, range	3.1 (1.2, 70.4)	1.7 (0.9, 23.6)
PD-L1 IHC Status		
5% PD-L1$^+$ on TC or IC, n (%)	2/27 (7.4%)	3/31 (9.7%)
Prior Anti-Cancer Therapy, n (%)		
TKI	27 (81.8)	30 (85.7)
mTor	9 (27.3)	11 (31.4)
Anti-PD-1	23 (69.7)	25 (71.4)
Anti-VEGF, bevacizumab	6 (18.2)	4 (11.4)
IL-2	7 (21.2)	9 (25.7)

Table 2.

Treatment Related Adverse Events

Event, Number of pts, (%)	Ciforadenant (N=33)		Ciforadenant + Atezolizumab (N=35)	
	Any Grade	Grade 3/4	Any Grade	Grade 3/4
Fatigue	13 (39.4)	0 (0.0)	16 (45.7)	0 (0.0)
Pruritus	7 (21.2)	0 (0.0)	9 (25.7)	0 (0.0)
Decreased appetite	4 (12.1)	1 (3.0)	6 (17.1)	0 (0.0)
Dizziness	4 (12.1)	0 (0.0)	1 (2.9)	0 (0.0)
Nausea	3 (9.1)	0 (0.0)	7 (20.0)	1 (2.9)
Pyrexia	3 (9.1)	0 (0.0)	1 (2.9)	0 (0.0)
Anemia	2 (6.1)	1 (3.0)	4 (11.4)	0 (0.0)
Arthralgia	2 (6.1)	1 (3.0)	5 (14.3)	1 (2.9)
Chills	2 (6.1)	0 (0.0)	1 (2.9)	0 (0.0)
Cough	2 (6.1)	0 (0.0)	3 (8.6)	0 (0.0)
Diarrhea	2 (6.1)	0 (0.0)	5 (14.3)	0 (0.0)
Epistaxis	2 (6.1)	0 (0.0)	0 (0.0)	0 (0.0)
Gastroesophageal reflux	2 (6.1)	0 (0.0)	0 (0.0)	0 (0.0)
Hyperhidrosis	2 (6.1)	0 (0.0)	1 (2.9)	0 (0.0)
Hypophosphatemia	2 (6.1)	0 (0.0)	3 (8.6)	1 (2.9)
Musculoskeletal chest pain	2 (6.1)	0 (0.0)	2 (5.7)	0 (0.0)
Myalgia	2 (6.1)	0 (0.0)	2 (5.7)	0 (0.0)
Edemia peripheral	2 (6.1)	1 (3.0)	1 (2.9)	0 (0.0)
Osteoarthritis	2 (6.1)	0 (0.0)	2 (5.7)	0 (0.0)
Rash	2 (6.1)	0 (0.0)	4 (11.4)	0 (0.0)
Vomiting	2 (6.1)	0 (0.0)	4 (11.4)	0 (0.0)
Abdominal pain	1 (3.0)	0 (0.0)	3 (8.6)	1 (2.9)
AST increased	1 (3.0)	0 (0.0)	2 (5.7)	1 (2.9)
Blood creatinine increased	1 (3.0)	0 (0.0)	2 (5.7)	0 (0.0)
Insomnia	1 (3.0)	0 (0.0)	2 (5.7)	0 (0.0)
Dysgeusia	0 (0.0)	0 (0.0)	2 (5.7)	0 (0.0)

Table 3.

6 Month Disease Control Rate

	Ciforadenant (N=29)	Ciforadenant + Atezolizumab (N=33)
Prior Anti-PD-(L)1	25% (5/20)	35% (8/23)
Naive	0% (0/9)	50% (5/10)
Total	17% (5/29)	39% (13/33)