# Adenosine and AMP Gene Expression Profiles Predict Response to Adenosine Pathway Therapies and Indicate a Need for Dual Blockade of CD73 and A2AR with CD73 Inhibitors

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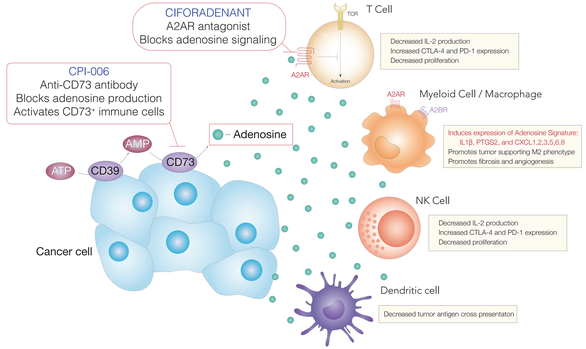
## ADENOSINE INHIBITS ANTI-TUMOR IMMUNITY




- Extracellular adenosine has a short half life and it is not feasible to routinely measure in human tumors
- Determine genes/proteins modulated by adenosine as a surrogate to identify patients with adenosine rich tumors

## ADENOSINE INDUCES A SPECIFIC GENE SIGNATURE

### HUMAN PBMCs STIMULATED WITH NECA, A STABLE ADENOSINE ANALOG

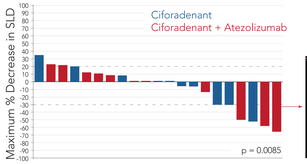

### ADENOSINE SIGNATURE - NANOSTRING

| | Adjusted p Value | Function |
|---|---|---|
| IL23A | 1.44E-04 | Increases angiogenesis and reduces CD8+ T-cell infiltration |
| SLC11A1 | 1.27E-03 | Natural resistance-associated macrophage protein 1 |
| CXCL2 | 1.27E-03 | MIP2a: macrophage inflammatory protein 2, alpha |
| CXCL7 | 1.27E-03 | PPBP. Pro-Platelet Basic Protein |
| CXCL6 | 1.40E-03 | GCP2: Granulocyte chemotactic protein 2 |
| CXCL3 | 1.40E-03 | Controls migration and adhesion of monocytes |
| IL-6 | 1.48E-03 | Pro- and anti-inflammatory cytokine |
| IL-1α | 1.73E-03 | Inflammation |
| CXCL8 | 1.98E-03 | IL-8. Nutrophil chemotactic factor |
| CXCL5 | 1.98E-03 | Attracts and activates neutrophils |
| THBS1 | 2.28E-03 | Multiple functions. Inhibits angiogenesis & immune regulation |
| IL-1β | 2.38E-03 | Inflammation |
| PTGS2 | 2.65E-03 | COX-2. Elevated during inflammation and cancer |
| IL-24 | 2.70E-03 | Cell survival and proliferation. Activates STAT1/3 |
| CXCL1 | 3.92E-03 | Neutrophil chemotractant |
| CD86 | 5.31E-03 | B7-2: Costimulatory signal for T cell activation and survival |
| CLEC5A | 5.82E-03 | Interacts with DAP-12 and may play a role in cell activation |
| CD14 | 6.24E-03 | Expressed by myeloid cells |

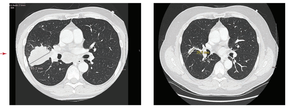
Human PBMCS were isolated from buffy coat samples by density centrifugation with Histopaque 1077 (400*g*, 30 min). Cells were washed and resuspended at a density 2*10^6 cells/ml in RPMI + 10% human serum. PBMCs were stimulated with DMSO or 5'-N-Ethylcarboxamidoadenosine (NECA) at 0.3, 1, or 10 μM for one hour. T cells were then activated with anti-CD3 + anti-CD28 antibodies and incubated for 48 hrs. Purified RNA was collected using a Qiagen RNAEasy Kit and gene expression analysis was performed using the NanoString PanCancer Immune Panel with PLUS codeset. Normalized counts were obtained using NanoString nSolver Software. Log2 transformed expression data were fit to a linear model comprised of donor and treatment effects. Genes which showed a statistically significant treatment effect were identified in 3 initial donors. Adjusted p-values were used to correct for multiple hypothesis testing using the Benjamini-Hochberg procedure.

## SIGNATURE CORRELATES WITH TUMOR RESPONSE

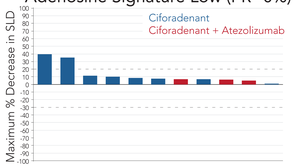
### Adenosine Signature High (PR=17%)




### Adenosine Signature High Patient

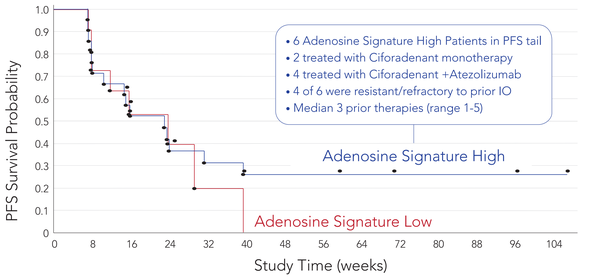



### Adenosine Signature Low (PR=0%)

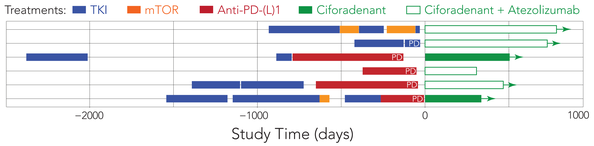



### Clinical Trial Design:

- Metastatic renal cell cancer
  - Must have progressed on prior therapy
- Ciforadenant monotherapy
  - 100 mg BID 28d cycle
- Ciforadenant + atezolizumab
  - 100 mg BID 28d cycle + 840 mg, IV, 2QW
- Median 3 prior treatments (range 0-5)
- 85% of patients were resistant/refractory to prior IO



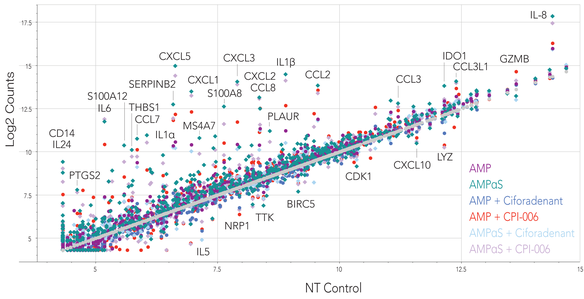

### PRIOR TREATMENTS IN ADENOSINE SIGNATURE HIGH PATIENTS WITH PROLONGED PFS




Adenosine Signature levels were determined in pre-treatment biopsy tissue. In brief, RNA was extracted from tumor tissue macrodissected from patient biopsy specimens and analyzed using the NanoString PanCancer Immune Panel. Cutoffs for determining Adenosine Signature high and low tumors were based on the mean of the Log2 NanoString counts of select Adenosine Signature genes for all subjects evaluated. The t-test for comparing the maximum percentage decrease in SLD between the adenosine positive and negative signatures is 2-sided p-value = 0.0085, using a 5% level two-sided test.

## AMP INDUCES ADENOSINE-LIKE GENE SIGNATURE

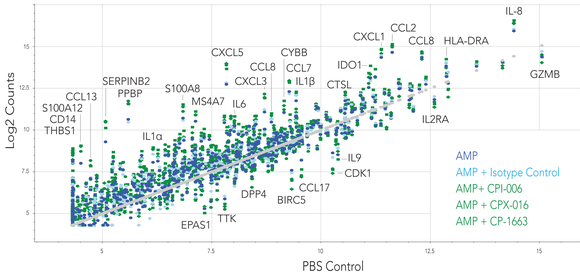
### AMP INDUCED GENE SIGNATURE IS NEARLY IDENTICAL TO ADENOSINE SIGNATURE
### AMPαS IS A NON-HYDROLIZABLE FORM OF AMP - IT DOES NOT FORM ADENOSINE




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. PBMCs were co-cultured with ciforadenant (5 μM) or CPI-006 (10 μg / mL) for 1 hour before adding AMP (150 uM) or AMPαS (150 μM). PBMCs were then stimulated after 1 hour 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 manufacturer's protocol. NanoString analysis was performed according to manufacturer's protocol on a NanoString Sprint instrument using the NanoString PanCancer Immune Panel with PLUS codeset. Normalized counts were obtained using NanoString nSolver Software. Representative data from one of >5 patients is shown above.

## CD73 ANTAGONISTS AMPLIFY AMP SIGNATURE

### CD73 ANTAGONISTS INHIBIT ADENOSINE FORMATION, BUT CONSEQUENTLY PRESERVE AMP
### PRESERVED AMP AMPLIFIES ADENOSINE-LIKE GENE SIGNATURE SUGGESTING AMP SIGNALS THROUGH A2AR




### CPI-006 AND CPX-016 ARE ANTI-CD73 ANTIBODIES. CP-1663 IS A CD73 SMALL MOLECULE INHIBITOR

Purified human PBMCs from healthy donors were co-cultured with CD73 antagonists including CPI-006 (Corvus anti-CD73 mAb, 10 μg / mL), CPX-016 (competitor anti-CD73 mAb, 10 μg / mL), an isotype control antibody, or CP-1663 (1 μM) for 1 hour before adding AMP (150 uM). PBMCs were then stimulated after 1 hour with anti-human CD3 and CD28 antibodies. RNA was purified from cells collected after incubation for 48 hours. Gene expression changes were evaluated using the NanoString PanCancer Immune Panel. Representative data from one of >3 patients is shown above.

## AMP IS AN ADENOSINE RECEPTOR AGONIST

### AMP AND AMPαS AGONIZE A2AR, BUT ARE WEAKER THAN ADENOSINE

| A2AR | EC50 (nM) | n |
|---|---|---|
| NECA | 0.17 | n = 2 |
| ADENOSINE | 2.7 | n = 4 |
| AMP | 235.2 | n = 4 |
| AMPαS | 228.6 | n = 4 |

| A2BR | EC50 (nM) | n |
|---|---|---|
| NECA | 1.3 | n = 2 |
| ADENOSINE | 10.8 | n = 4 |
| AMP | 227.5 | n = 4 |
| AMPαS | 427.3 | n = 4 |

| A1 | EC50 (nM) | n |
|---|---|---|
| NECA | 2.8 | n = 2 |
| ADENOSINE | 11.9 | n = 4 |
| AMP | 237.5 | n = 4 |
| AMPαS | 418.2 | n = 4 |

| A3 | EC50 (nM) | n |
|---|---|---|
| NECA | 1.7 | n = 2 |
| ADENOSINE | 12.9 | n = 4 |
| AMP | 199.0 | n = 4 |
| AMPαS | 240.1 | n = 4 |

cAMP assays were performed at Pharmaron using PerkinElmer CHO-K1 or HEK293 cells designed to stably overexpress human adenosine receptors, including hA1, hA2AR, hA2BR, and hA3. 3-fold dilutions of test compounds were evaluated, starting at a maximum concentration of 1 μM (NECA) or 10 μM (adenosine, AMP, or AMPαS). EC50 concentrations are shown along with the number of experiment replicates.

## CIFORADENANT NEUTRALIZES AMP & AMPαS

### CIFORADENANT BLOCKS AMP AND AMPαS SIGNALING AT A2AR

| A2AR | IC50 (nM) | n |
|---|---|---|
| ADENOSINE | 4.0 | n = 4 |
| AMP | 4.7 | n = 2 |
| AMPαS | 7.1 | n = 2 |

| A2BR | IC50 (nM) | n |
|---|---|---|
| ADENOSINE | 171.2 | n = 4 |
| AMP | 204.8 | n = 2 |
| AMPαS | 270.2 | n = 2 |

| A1 | IC50 (nM) | n |
|---|---|---|
| ADENOSINE | 200.9 | n = 4 |
| AMP | 111.4 | n = 2 |
| AMPαS | 62.0 | n = 2 |

| A3 | IC50 (nM) | n |
|---|---|---|
| ADENOSINE | 516.0 | n = 4 |
| AMP | 231.7 | n = 2 |
| AMPαS | 315.1 | n = 2 |

cAMP assays were performed at Pharmaron using PerkinElmer CHO-K1 or HEK293 cells designed to stably overexpress human adenosine receptors, including hA1, hA2AR, hA2BR, and hA3. Cells were stimulated with EC80 concentrations of indicated test compounds. Ciforadenant was added at a maximum concentration of 10 μM. IC50 values for ciforadenant inhibition of test compounds are shown, along with the number of experiment replicates.

## CONCLUSIONS

- A2AR agonists induce a specific gene signature in human immune cells. This "Adenosine Signature" is dominated by inflammatory myeloid cytokines and chemokines.
- **Updated clinical data confirms original reports that expression of the Adenosine Signature correlates with tumor regression in an ongoing Ph 1/1b trial in patients with advanced/refractory RCC.**
- **High Adenosine Signature expression has a statistically significant correlation with tumor response.**
- The Adenosine Signature may be used as a predictive biomarker to select patients most likely to respond to therapy with agents that antagonize adenosine production or signaling.
- AMP induces gene expression changes nearly identical to the Adenosine Signature in human immune cells, suggesting AMP can activate and signal through adenosine receptors. CD73 antagonists further amplify the AMP-associated gene expression changes as a consequence of preserving AMP.
- AMP is an agonist of adenosine receptors. AMP agonism of A2AR and A2BR can be blocked by ciforadenant.
- These data suggest treatment with CD73 antagonists will benefit from concominant blockade of A2AR to neutralize the resultant induction of AMP mediated gene expression changes.