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Abstract 256

Dual $A_{2a}R/A_{2b}R$ Antagonism with Etrumadenant Eliminates the Suppressive Effects of Adenosine on Immune and Cancer Cells in the Tumor Microenvironment

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Introduction

- High levels of extracellular adenosine generated in the tumor microenvironment (TME) engage A_{2a} and A_{2b} adenosine receptors on immune cells, resulting in immunosuppression (Figure 1).
- Expression of $A_{2a}R$ and $A_{2b}R$ can vary by cell type with T cells predominantly expressing $A_{2a}R$, while myeloid cells express both $A_{2a}R$ and $A_{2b}R$, and some cancer cells primarily expressing $A_{2b}R$.
- Etrumadenant (AB928) is a selective, small-molecule, dual $A_{2a}R/A_{2b}R$ antagonist with minimal penetration across the blood brain barrier. It was specifically designed to potently block the immunosuppressive effects of adenosine in the TME. We have previously shown that etrumadenant blocks the immunosuppressive effects of adenosine in immune cells and enhances anti-tumor immune responses in mouse syngeneic tumors.
- Here we describe the contribution of these receptors to adenosine-mediated phenotypes in immune and cancer cells and provide a mechanistic rationale for stimulation of anti-tumor immune responses with the dual adenosine receptor antagonist etrumadenant.

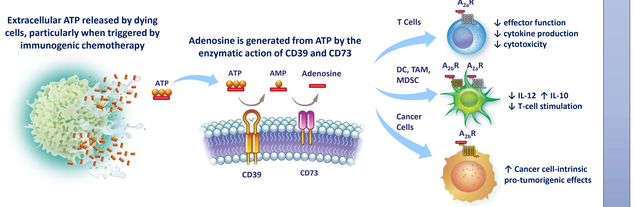

Figure 1. Diagram of adenosine production from ATP released into the TME. Hydrolysis of ATP by the ecto-enzymes CD39 and CD73 produces adenosine, which exerts immunosuppressive effects by binding to adenosine receptors expressed on immune cells and promotes a suppressive tumor microenvironment through $A_{2b}R$-mediated signaling on cancer cells.

Methods

- **Isolated immune cell experiments:** $CD8^+$ T cells and dendritic cells (DC) were isolated from healthy human blood by negative selection. DC were matured with LPS/IFN-γ for 24 hours in the presence of NECA (synthetic adenosine receptor agonist) +/- antagonists, then analyzed by RNA sequencing and cytokine bead array.
- **PBMC CXCL5 production:** PBMC were freshly isolated from healthy human blood and incubated with anti-CD2/CD3/CD28 beads in the presence of adenosine receptor agonists and antagonists for 24 hours. Supernatants were collected and analyzed for CXCL5 production by ELISA.
- **TAM and MDSC experiments**: TAM and MDSC were obtained from B16F10 or LLC tumors by sequential magnetic bead isolation TAM (F4/80+), gMDSC (Ly6G+), mMDSC (GR-1+). Cells were stimulated with NECA +/- antagonists for 24 hours and then RNA was extracted for NanoString gene expression analysis.
- **RNAseq data analysis:** TruSeq Stranded Total RNA libraries from DC were sequenced at 50mil 150bp PE reads. Genes were quantified using STAR alignment and Salmon quantification against Gencode 38. Differential expression analysis was performed adjusting for donor effects. Pathway analysis was performed using wilcoxGST and the sparrow package "camera", which accounts for inter-gene correlations. Hallmark gene sets were downloaded from MSigDB, GNE Myeloid signature was obtained from McDermott et al. (2018), and adenosine response was derived experimentally using monocyte-derived dendritic cells analyzed by Nanostring.

Table 1: Potency and Selectivity of Adenosine Receptor Antagonists *

Potency (nM)	Etrumadenant (etruma)	$A_{2a}R$ antagonist A	$A_{2a}R$ antagonist B
$A_{2a}R$	1.4	1.5	0.2
$A_{2b}R$	2.0	123	141

[A] Proprietary $A_{2a}R$-selective adenosine receptor antagonist
[B] Compound 35 from WO2018178338 (iTeos Therapeutics)
* Potency data generated at Arcus Biosciences

Results

Adenosine Signaling Drives Immunosuppression in T Cells Through $A_{2a}R$ Which is Reversed by Etrumadenant

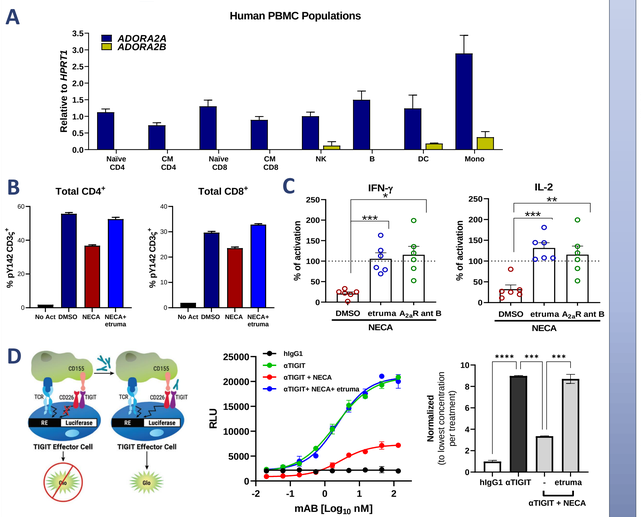

Figure 2. **(A)** Adenosine receptor gene expression of sorted human immune cells from healthy donors. **(B)** Activation of human primary CD4 and CD8 T cells with α-CD3 and subsequent phosphorylation of CD3ζ is inhibited by NECA and recovered by etrumadenant. **(C)** IFN-γ and IL-2 production from primary human $CD8^+$ T cells activated for 72h in the presence of NECA (5 μM) and adenosine receptor antagonists (1 μM). **These results demonstrate that 1 μM of the adenosine receptor antagonists used in this experiment are capable of fully suppressing adenosine-mediated suppression driven by 5 μM NECA. (D)** Jurkat-TIGIT cells were co-cultured with CD155-expressing CHO cells in the presence of α-TIGIT +/- NECA (5 μM) +/- etrumadenant (1 μM). Luciferase reporter activity was measured after 6 hours. *=p<0.05, **=p<0.01, ***=p<0.001, ****=P<0.0001

PBMC Express Low but Detectable Levels of $A_{2b}R$ Which Contributes to Adenosine Receptor-Mediated Signaling

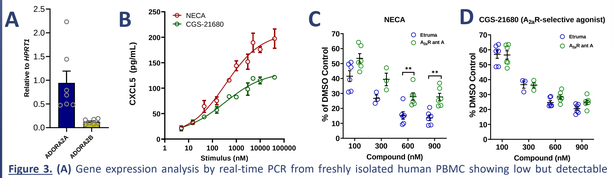

Figure 3. **(A)** Gene expression analysis by real-time PCR from freshly isolated human PBMC showing low but detectable levels of $A_{2b}R$ (ADORA2B). **(B)** Stimulation of PBMC with NECA ($A_{2a}R/A_{2b}R$ agonist) results in greater CXCL5 production when compared to stimulation with a selective $A_{2a}R$ agonist (CGS-21680). **(C)** Compared to $A_{2a}R$-selective antagonism, dual antagonism with etrumadenant results in a significantly enhanced suppression of CXCL5 production in NECA (10 μM)-treated cells **(D)** Equivalent suppression was observed with etrumadenant and an $A_{2a}R$ selective antagonist when PBMC were stimulated with CGS-21680 ($A_{2a}R$ agonist, 10 μM).

Etrumadenant Provides Greater Suppression of Adenosine-Mediated Changes than A_2R-Selective Antagonist in Isolated Human DCs

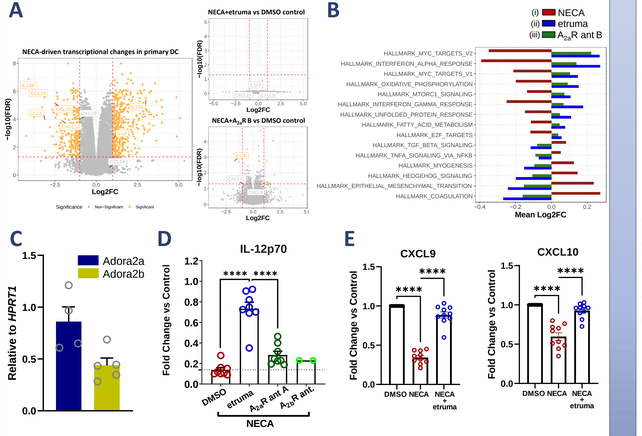

Figure 4. **(A)** Differentially expressed genes driven by NECA (5 μM) and inhibited by etrumadenant (1 μM) or $A_{2a}R$-specific antagonist (1 μM) in primary DCs (left panel, 6 donors). **Notably, there were no differentially expressed genes between the NECA + etrumadenant and DMSO control groups (top right panel) demonstrating a complete suppression of adenosine receptor signaling. In contrast, the $A_{2a}R$ antagonist was unable to completely rescue the gene expression changes stimulated by NECA (bottom right panel) (B)** Pathways altered by (i) NECA vs DMSO ($p < 0.05$), (ii) etrumadenant vs NECA, or (iii) $A_{2a}R$-selective antagonist vs NECA. Etrumadenant shows an enhanced ability to prevent NECA-driven changes relative to $A_{2a}R$ inhibition, especially for immune related gene sets. **(C)** Real-time PCR for adenosine receptors ADORA2A and ADORA2B expression in primary DCs. **(D)** IL-12p70 and **(E)** CXCL9 and CXCL10 suppression by NECA (5 μM) was rescued by etrumadenant (1 μM) after 24 hours in culture. *= p<0.05, ****=p<0.0001.

Tumor-Resident TAM and MDSC Express Both A_2 Adenosine Receptors and Respond to Adenosine Signaling

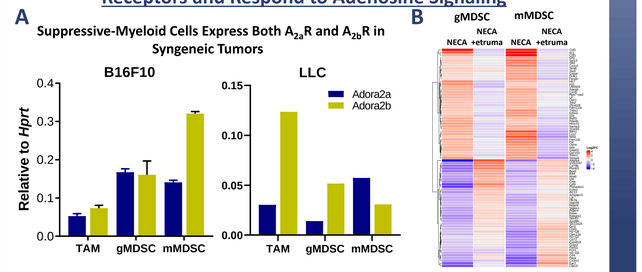

Figure 5. Mouse B16F10 or LLC tumors were dissociated and suppressive myeloid populations (TAM/MDSC) were isolated. **(A)** Real-time PCR for adenosine receptor expression in TAM and MDSCs. **(B)** Differentially expressed genes in isolated gMDSC and mMDSC from LLC tumors driven by NECA stimulation (5 μM) and inhibited by etrumadenant (1 μM) in the presence of NECA.

$A_{2b}R$ Signaling in Cancer Cell Lines Drives Gene Expression Changes Which are Blocked by Etrumadenant

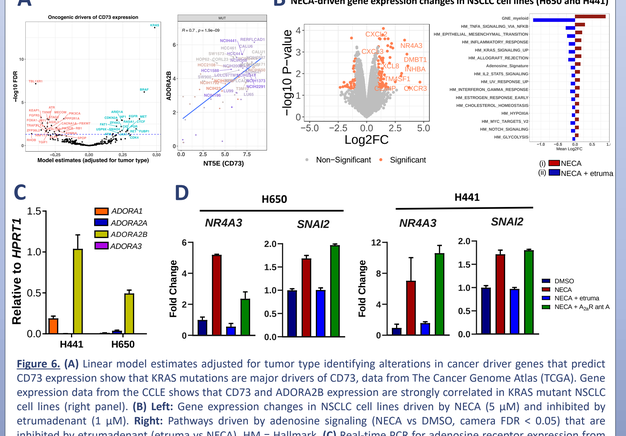

Figure 6. **(A)** Linear model estimates adjusted for tumor type identifying alterations in cancer driver genes that predict CD73 expression show that KRAS mutations are major drivers of CD73, data from The Cancer Genome Atlas (TCGA). Gene expression data from the CCLE shows that CD73 and ADORA2B expression are strongly correlated in KRAS mutant NSCLC cell lines (right panel). **(B) Left:** Gene expression changes in NSCLC cell lines driven by NECA (5 μM) and inhibited by etrumadenant (1 μM). **Right:** Pathways driven by adenosine signaling (NECA vs DMSO, camera FDR < 0.05) that are inhibited by etrumadenant (etruma vs NECA). HM = Hallmark. **(C)** Real-time PCR for adenosine receptor expression from NSCLC cell lines showing high $A_{2b}R$ expression. **(D)** Comparison of dual vs $A_{2a}R$ selective antagonists (1 μM) in blocking NECA-stimulated (5 μM) gene expression changes in NSCLC cell lines.

Etrumadenant in Combination with Doxorubicin Reduces 4T1 Syngeneic Tumor Growth and Lung Metastases

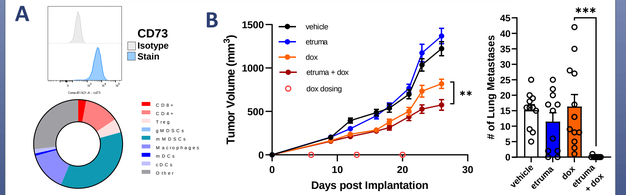

Figure 7. **(A)** 4T1 cells universally express CD73 as measured by flow cytometry (top panel). Myeloid cells comprise a large portion of the immune cell infiltrate in 4T1 tumors (bottom panel). **(B)** Tumor volume from 4T1 model. Dosing started at 50 mm^3, etrumadenant (100 mg/kg, PO, BID) and doxorubicin (6 mg/kg) dosing as indicated. Number of lung metastases were quantified at the end of the study (right panel). ** = p<0.01, ***= p<0.001.

Conclusions

- In T cells, myeloid cells, and $A_{2b}R$-expressing cancer cells, dual $A_{2a}R/A_{2b}R$ antagonism with etrumadenant prevents adenosine/NECA induced immunosuppression and gene expression changes greater than $A_{2a}R$-selective antagonism.
- These studies build upon the established rationale for targeting $A_{2a}R$ in T and NK cells, demonstrate an important role for $A_{2b}R$ in adenosine-mediated myeloid cell immuno-suppression, and provide a mechanistic rationale for stimulation of anti-tumor immune responses with the dual adenosine receptor antagonist etrumadenant.