Introduction

The high levels of adenosine (ADO) found in the tumor microenvironment have been shown to inhibit immune responses through activation of the $A_2A$ and $A_3$ receptors on immune cells. The extracellular enzymes ecto-5'-nucleotidase (CD73) and tissue non-specific alkaline phosphatase (TNPAP) catalyze the extracellular conversion of adenosine monophosphate (AMP) into ADO. We have previously shown that AB928, a dual $A_2A$/$A_2B$ antagonist, blocks the immunosuppressive effects of ADO in human cell culture systems and in mouse syngeneic tumor models. Herein, we describe the development of assays to measure the expression and activity of adenosine-generating enzymes in human tumor samples and peripheral blood. These assays are being implemented in ongoing clinical trials with AB928, to identify tumor types and patients most sensitive to adenosine receptor antagonism.

Methods

Gene expression data were extracted from The Cancer Genome Atlas (TCGA). CD73 and TNPAP immunohistochemistry (IHC) was performed on sections of formalin fixed paraffin embedded (FFPE) tumor tissue. Circulating levels of CD73 were quantified with an in-house developed CD73 ELISA and total AMP-ase enzymatic activity in plasma was determined using an AMP-Glo assay.

TCGA Analysis of Human Tumors for CD73 and TNAP

![TCGA Analysis of Human Tumors for CD73 and TNAP](image)

**Figure 1.** Ratio of CD73 versus TNAP expression from RNAseq in The Cancer Genome Atlas (TCGA) samples. Numbers indicate a ratio of log2 counts per million per sample. Tumors with a high CD73 expression are shown on the left whereas a higher TNAP expression are shown on the right. Prostate and non-TNBC breast tumors were low for both CD73 and TNAP.

CD73 is Detectable and Quantifiable via IHC in Human Tumors

**Figure 2.** (A.) Representative images of immunostaining for CD73 (brown) and CD8 (purple) on human FFPE tumor samples. Strong staining can be seen in both tumor (left, middle) and stromal cells (right). (B.) Quantification of IHC staining area from cores of tumor microarrays stained with CD73 (left panel). Strong correlation was seen with gene expression patterns from TCGA database (right) where each dot represents one tumor type. T: tumor, S: stroma.

IHC Robustly Identifies TNAP Expression Across Several Human Tumor Types

**Figure 3.** (A.) Representative images of immunostaining for TNAP (brown) on human FFPE tumor samples. Strong staining can be seen in ovarian and NSCLC tumors whereas liver and CRC exhibit much less staining. (B.) Quantification of staining area from cores of tumor microarrays stained with TNAP.

A Robust Assay to Quantify Soluble CD73 in Peripheral Blood

**Figure 4.** (A.) Serum and sodium heparin plasma from healthy donors were evaluated in an ELISA to measure soluble CD73 and were highly correlated. (B.) Parallelism assessment was performed to identify the Minimum Required Dilution (MRD = 4) for sufficient recovery (+ 3σ% ) and to identify the quantitative range of the assay. Donor 1 is shown, gray background = dilutions below the lower limit of quantification (LLOQ).

Reliable Determination of AMP Hydrolysis in Serum Using AMP Glo™

**Figure 5.** AMP hydrolysis in the periphery is determined using a biochemical assay (A.) Healthy volunteer serum (n=6) was tested in the presence of CD73 and/or TNAP inhibitors. (B.) Assay validation parameters were tested (n=2) and average CVs across multiple conditions shows the assay is robust for the determination of AMP hydrolysis in human serum. (C.) Cancer patient serum (n=12) displays a strong correlation between CD73 protein concentration and AMP hydrolysis.

Conclusions

- We have developed robust assays to measure tumoral and peripheral levels of adenosine generating enzymes.
- These assays are being implemented to assess the adenosine generating machinery of subjects in clinical studies with AB928.