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Methods for Assessment of the "Adenosine Fingerprint" in Clinical Trials of AB928

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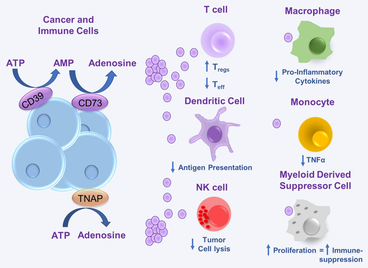
Introduction

The tumor microenvironment (TME) contains high levels of immunosuppressive adenosine (ADO), which activates the $A_{2a}R$ and $A_{2b}R$ receptors on immune cells, leading to an ineffective anti-tumor response. Ecto-5'-nucleotidase (CD73) and tissue non-specific alkaline phosphatase (TNAP) are primarily responsible for the conversion of extracellular adenosine mono-phosphate (AMP) to ADO and exhibit both membrane-bound and secreted forms. We have previously shown that AB928, a dual $A_{2a}R/A_{2b}R$ antagonist, rescues the immuno-suppressive effects of ADO in experimental 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 used to define an "adenosine fingerprint" to identify tumor types and patients most sensitive to adenosine inhibition by AB928.



Materials and Methods

pCREB Assay: Whole blood from healthy volunteers or cancer subjects was stimulated *ex vivo* with the adenosine agonist NECA. Levels of CREB phosphorylation (pCREB) were assessed by flow cytometry and AB928 plasma concentrations were determined using LC-MS/MS.

CD73 ELISA: Circulating levels of CD73 were quantified in matched serum and plasma samples from healthy donors and cancer patient samples with an in-house ELISA. Validation studies were performed to correlate CD73 levels between serum and plasma and to ensure acceptable assay parallelism and inter-assay variation.

AMPase Activity: AMP hydrolytic activity in serum from healthy volunteers or patients with solid tissue tumors was assessed using the AMP Glo™ (Promega) assay. CD73 and TNAP inhibitor cocktails were used to assess the relative contribution of each enzyme to AMPase activity.

Gene Expression: Expression of CD73 (*NT5E*) and TNAP (*ALPL*) on select tumor types were derived from RNASeq in The Cancer Genome Atlas (TCGA) database. Data is displayed as log2 transformed expression of counts per million.

Histology: Immunohistochemistry (IHC) was performed using CD73 (Cell Signaling, D7F9A) and TNAP (Sino Biological, R034) on formalin fixed paraffin embedded (FFPE) human tissue. QuPath software was used for quantification and H-score = 3x(high intensity staining area) + 2x(medium intensity staining area) + (low intensity staining area). .

Results

AB928 Inhibits $A_{2a}R$ Activation Following Dosing with ≥75 mg in Healthy Volunteers and Cancer Subjects

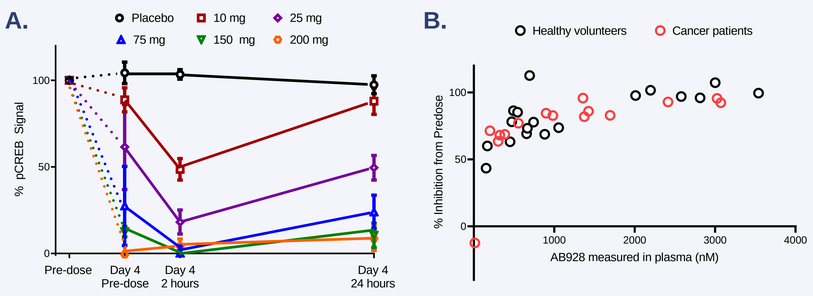


Figure 1. (A.) AB928 suppresses NECA-induced pCREB activation in CD8+ T cells of healthy volunteers. (B.) Healthy volunteers and cancer patients exhibit a similar pattern of pCREB inhibition and an overlapping PK/PD relationship.

Development of a Validated ELISA Assay to Quantify Soluble CD73 in Peripheral Blood

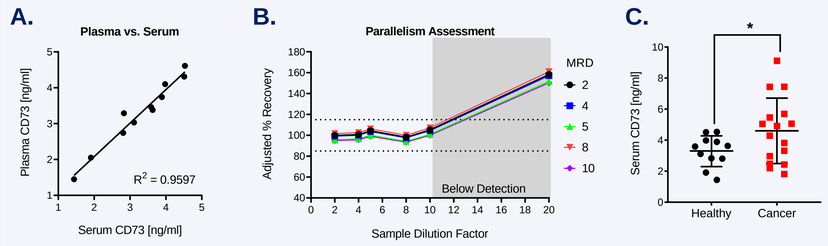


Figure 2. (A.) Serum and sodium heparin plasma from healthy donors were evaluated in an ELISA to measure soluble CD73 and were highly correlated. (B.) Parallelism assessment was performed to identify the quantitative range of the assay. (C.) Soluble CD73 levels in serum are elevated in cancer patients compared to healthy controls. *p<0.05

Determination of AMP Hydrolysis by CD73 and TNAP in Serum Using an AMP Glo™ Assay

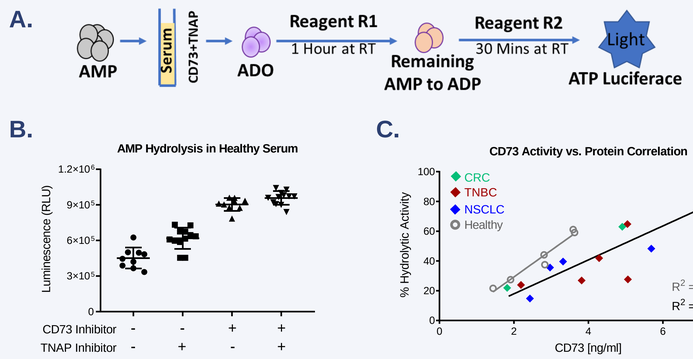


Figure 3. (A.) Healthy volunteer serum was tested in the presence of CD73 and/or TNAP inhibitors. (B.) Average CVs across multiple conditions show the assay is robust for the determination of AMP hydrolysis in human serum. (C.) Healthy volunteer and cancer patient serum display a strong correlation between CD73 protein concentration and AMP hydrolysis.

TCGA Analysis Reveals Unique Expression Patterns of CD73 and TNAP in Human Tumors

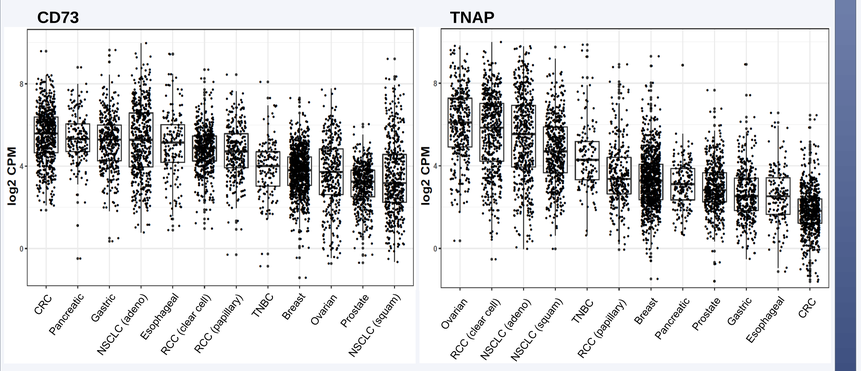


Figure 4. CD73 (left) and TNAP (right) expression from RNAseq data retrieved from The Cancer Genome Atlas (TCGA) samples. Numbers indicate a ratio of log2 counts per million per sample. Samples are ordered according to their expression with higher expressing tumors on the left and lower expressing tumors on the right.

CD73 is Detected and Quantifiable in Human Tumors via IHC

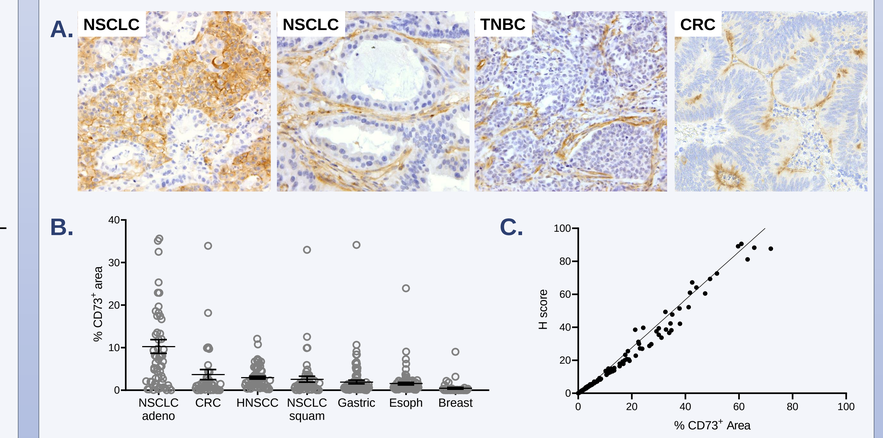


Figure 5. (A.) Representative images of immunostaining for CD73 (brown) on human FFPE tumor samples. Nuclei are stained with hematoxylin (blue) (B.) Quantification of CD73 staining area as a percentage of total tumor area. (C.) A strong correlation is seen when comparing an H-score to % staining area.

TNAP Expression is Heterogeneous Across Human Tumors from Different Indications

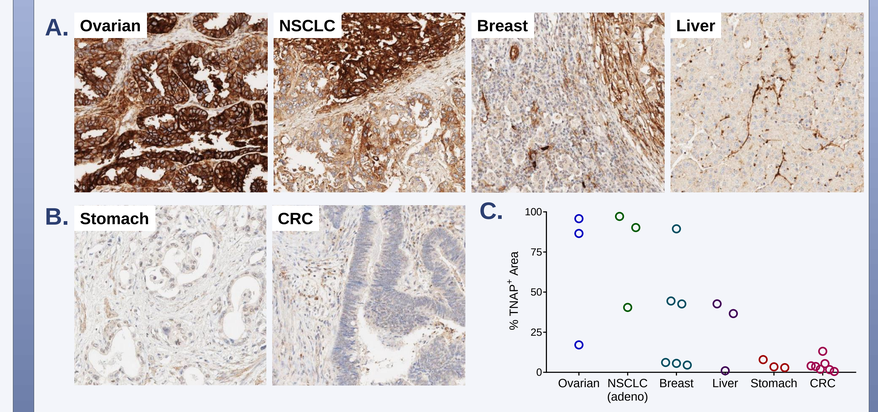


Figure 6. (A.) Representative images of immunostaining for TNAP (brown) on human FFPE tumor samples. Strong staining can be seen in ovarian and NSCLC tumors whereas liver and CRC exhibit much less staining. (C.) Quantification of TNAP staining area as a percentage of total tumor area.

Conclusions

- Robust assays have been developed to measure the peripheral levels and activity of the adenosine-generating enzyme, CD73.
- Immunohistochemical methods have been developed to measure and quantify adenosine-generating enzymes CD73 and TNAP in human tumor tissue.
- These assays are being implemented to assess the adenosine generating potential of subjects in clinical studies with AB928.
- AB928 is currently in clinical trials in combination with standard of care chemotherapeutics and an anti-PD-1 immunotherapeutic.

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