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Inhibition of equilibrative nucleoside transporter 1 relieves intracellular adenosine-mediated immune suppression

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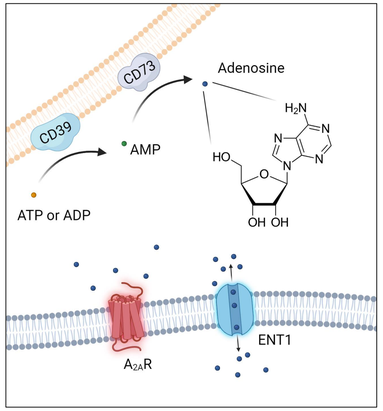
Introduction

The profound benefit of immune checkpoint blockade (ICB) for cancer therapy is restricted to limited subsets of patients with specific cancers. Novel targets and combination therapies are needed to improve treatment outcomes.

Factors contributing to ICB resistance include local accumulation of immunosuppressive metabolites such as the nucleoside adenosine. Adenosine promotes immune suppression mainly through the A_{2A} receptor ($A_{2A}R$), expressed by tumor-infiltrating immune cells.

Pharmacological inhibition of adenosine generation (e.g. the enzymes CD39 and CD73) and signaling (e.g. $A_{2A}R$) are active areas of clinical investigation, however so far only limited clinical benefit has been reported.

Adenosine can enter cells through equilibrative nucleoside transporters such as ENT1. Whether and how intracellular adenosine influences anti-cancer immune responses is unknown.



Here, we show that high concentrations of extracellular adenosine found within the TME can be taken up by T cells and impair their expansion and effector function. Furthermore, we have discovered and characterized EOS301984, a highly potent ENT1 antagonist which is currently under investigation in a Phase 1 clinical study in solid cancers.

Acknowledgements

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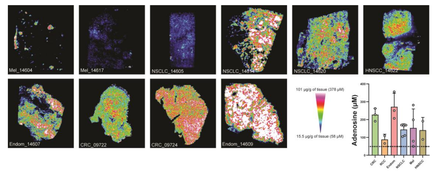
- Nucleoside transporter inhibition assays were performed at SOLVO Biotechnology (https://www.solvobiotech.com/)
- Human cell line xenograft experiments were performed at TransCure bioServices (https://transcurebioservices.com/)
- Introduction and conclusion schematic created in BioRender

1. Very high adenosine concentrations are present within human tumors

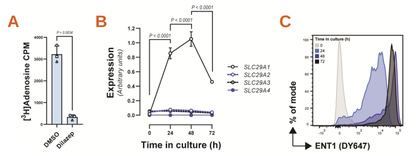
High tumor adenosine concentrations have important implications for cancer immunotherapy



Adenosine concentrations were assessed in tissue sections from 19 resected and frozen human tumors by quantitative mass spectrometry imaging (QMSI). Concentrations of adenosine exceeded 100 µM in many samples.

2. Adenosine enters activated human T cells via ENT1

High adenosine concentrations could exert intracellular effects on T cell function



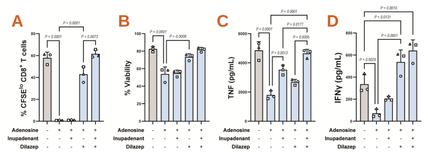
A. CD3/CD28-activated human T cells took up [^{3}H]adenosine which was blocked by the ENT1 inhibitor dilazep.

B. Human T cells upregulated *SLC29A1* (ENT1) transcription upon CD3/CD28 activation. Transcription of other ENT and CNT genes was not significantly affected by activation.

C. Human T cells upregulated ENT1 protein upon CD3/CD28 activation.

3. Adenosine uptake suppresses human T cell function and proliferation

ENT1 inhibition is an innovative approach to shield T cells from high tumor adenosine concentrations

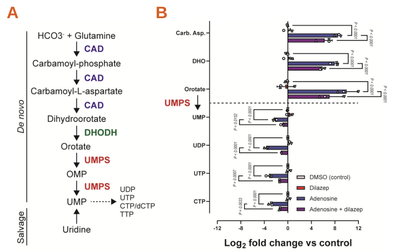


A. Adenosine (100 µM) suppressed the proliferation (CFSE dilution) of T cells following CD3/CD28 stimulation, which was restored by the ENT1 inhibitor dilazep and further rescued through combination with the $A_{2A}R$ antagonist inupadenant.

B-D. Summary data for T cell viability, TNF and IFNγ production, respectively, from experiments performed as in **A**. Symbols represent 3 donors.

4. Adenosine uptake suppresses *de novo* pyrimidine synthesis in human T cells

A novel suppressive mechanism of action of adenosine that is reversible via ENT1 inhibition

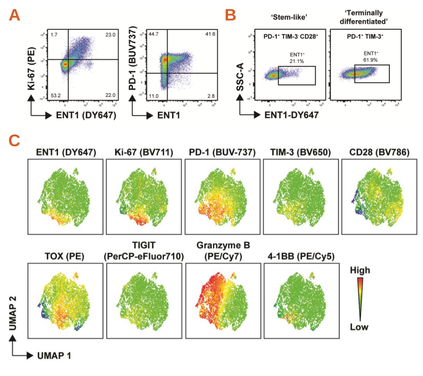


A. Pyrimidine *de novo* synthesis and salvage pathways.

B. T cells were CD3/CD28-activated for 24h in the presence of adenosine and/or the ENT1 inhibitor dilazep and pyrimidine metabolites were quantified by LC/MS and normalized to control stimulated cells. Metabolites upstream of UMPS were increased by adenosine and this effect was reduced by dilazep, whilst metabolites downstream of UMPS were reduced by adenosine with restoration by dilazep.

5. ENT1 is expressed by human tumor-infiltrating PD-1$^+$ CD8$^+$ T cells

Tumor-reactive T cells are exposed to intracellular adenosine via ENT1 expression



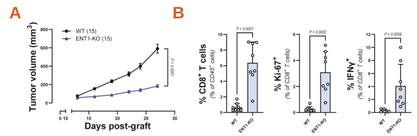
A. ENT1 expression was observed by flow cytometry on CD8$^+$ T cells from dissociated human tumors in association with Ki-67 and PD-1 expression.

B. ENT1 expression was enriched in PD-1$^+$ TIM-3$^+$ 'terminally differentiated' CD8$^+$ T cells versus 'stem-like' CD8$^+$ T cells.

C. UMAP of pooled CD8$^+$ T cell flow cytometry data from 9 tumors (4x endometrial, 4x NSCLC, 1x lung met from gastric) shows co-expression of ENT1 with various markers associated with intra-tumoral activation.

6. Deletion of ENT1 leads to potent control of tumor growth *in vivo*

ENT1 plays a key role in promoting tumor growth

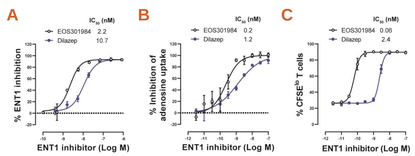


A. Growth curves of KPC s.c. tumors resistant to immuno-chemotherapy in WT and ENT1-KO mice, showing significantly reduced tumor growth in ENT1-KO mice.

B. KPC tumors from wt and ENT1-KO mice were dissociated, and TILs analyzed by flow cytometry, demonstrating increased infiltration, proliferation and IFNγ production by CD8$^+$ TILs in ENT1-KO mice.

7. EOS301984 is a potent ENT1 antagonist

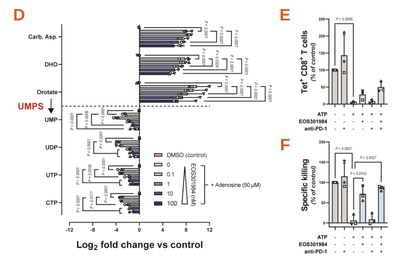
EOS301984 restores T cell activity suppressed by high adenosine concentrations with dramatically enhanced potency as compared with dilazep



A. ENT1 transport inhibition assay demonstrating that EOS301984 inhibits ENT1-mediated uridine transport with nanomolar potency.

B. EOS301984 inhibited uptake of adenosine into CD3/CD28-activated human T cells with subnanomolar potency.

C. EOS301984 restored the ability of T cells to proliferate when activated in the presence of ATP (100 µM) as a source of adenosine

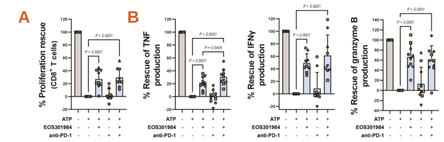


D. EOS301984 dose-dependently restored pyrimidine levels in human T cells activated in the presence of adenosine.

E-F. EOS301984 restored CMV pp65 peptide-specific T cell expansion in the presence of ATP (300 µM) as a source of adenosine, resulting in restoration of antigen-specific killing activity, respectively. No restoration was observed with anti-PD-1 monotherapy.

8. EOS301984 protects tumor-infiltrating T cells from adenosine suppression

EOS301984 may restore T cell activity in high adenosine tumors

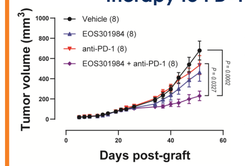


A. Human tumors were dissociated, and cells were CFSE-labelled and stimulated with CD3/CD28 microbeads and IL-2 in the presence of ATP (500 µM) as a source of adenosine. ATP restricted T cell proliferation which was partially reversed by EOS301984 alone and in combination with anti-PD-1, whilst anti-PD-1 alone had almost no effect.

B. TNF, IFNγ and granzyme B levels were assessed in culture supernatants and were suppressed by ATP but restored in the presence of EOS301984.

9. EOS301984 synergizes with anti-PD-1 in restricting tumor growth in humanized mice

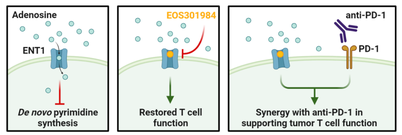
EOS301984 may extend the benefit of anti-PD-1 therapy to PD-1 resistant patients



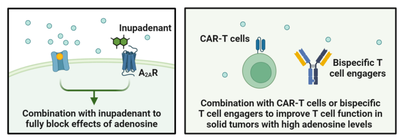
NCG mice were humanized with CD34$^+$ cells and inoculated with the anti-PD-1-resistant MDA-MB-231 cell line s.c. Tumor growth was not affected by anti-PD-1 treatment alone but was significantly reduced with a combination of EOS301984 and anti-PD-1.

Conclusions & perspectives

We have identified the uptake of adenosine through ENT1 on T cells as an important mechanism of immunosuppression within the adenosine-rich TME through inhibiting pyrimidine nucleotide synthesis. The potent ENT1 antagonist, EOS301984, relieves adenosine-mediated immunosuppression of tumor-infiltrating T cells and is currently being assessed in a Phase 1 clinical trial in advanced malignancies.



EOS301984 has potential as a combination partner beyond anti-PD-1, including with the $A_{2A}R$ antagonist inupadenant, and with CAR-T cells and bispecific T cell engagers.



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