Combined Administration of the Dual $A_{2a}R/A_{2b}R$ Antagonist Etrumadenant with a Reduced Chemotherapy Regimen Leads to Enhanced Tumor Efficacy and Survival

SITC Annual Meeting Nov 1-5th, 2023 #793

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Background

- Chemotherapy remains the standard of care for numerous cancer indications; however, in addition to having poor overall tolerability, a negative effect of such regimens is the extracellular release of adenosine triphosphate, which is rapidly converted to immunosuppressive adenosine by the enzymes CD39 and CD73 (Figure 1).
- Extracellular adenosine drives immunosuppression by activating the $A_{2a}R$ and $A_{2b}R$ adenosine receptors on immune cells, thereby inhibiting their activity and enabling tumor growth and survival.
- We have previously shown that etrumadenant (etruma), a dual $A_{2a}R/A_{2b}R$ antagonist, prevents adenosine-mediated immunosuppression *in vitro* and combines with immunogenic chemotherapy to enable greater control of mouse syngeneic tumors.
- In our current work, we sought to evaluate the ability of etruma to enhance the anti-tumor immune activity of priming doses of chemotherapy, compared to an extended chemotherapy regimen alone.
- Further, we evaluated the effect of discontinuing etruma dosing post chemotherapy and found an extended treatment with etruma did not result in greater tumor control or survival.
- Etruma is currently being studied in several clinical trials in combination with chemotherapy or other immune-enhancing regimens.

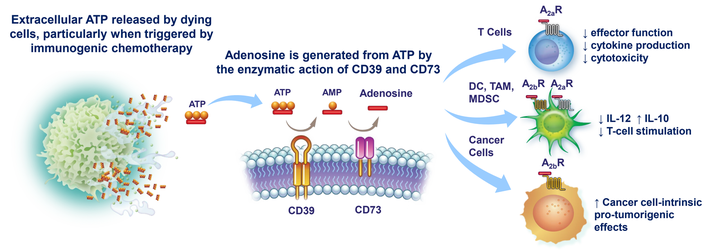

Figure 1. Diagram of adenosine production from ATP released into the TME by immunogenic chemotherapy. Hydrolysis of ATP by the ectoenzymes CD39 and CD73 produces adenosine, which exerts immunosuppressive effects by binding to adenosine receptors expressed on immune cells and may promote cell growth via-$A_{2b}R$-mediated signaling on cancer cells.

Materials and Methods

- **Isolated immune cell experiments:** Immune cell populations were sorted from healthy donors. $CD8^+$ 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.
- **Cancer cell line assay:** Different cancer cell lines were treated with 5 μM NECA with and without different adenosine receptor antagonists (2 μM). RNA was extracted and gene expression analysis via real time PCR was performed both at steady state and after treatment
- **Mouse *in vivo* studies:** Mice were inoculated with syngeneic cancer cells: 4T1, AT3-OVA or B16F10. Once tumors were established (50 or 100 mm^3), mice were treated with either oxaliplatin (5 mg/kg Q4D, 10 mg/kg Q4D) or doxorubicin (5 mg/kg, Q7D), alone or in combination with etruma (100 mg/kg BID). Gross anatomic, histological and flow cytometric analyses of CD45+ or CD8+ T cells were performed on tumor or lung tissue.

Table 1: Potency and Selectivity of Adenosine Receptor Antagonists *

Potency (nM)	Etrumadenant (etruma)	$A_{2a}R$ antagonist[a]	$A_{2b}R$ 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 patent application WO2018178338
* Potency data generated by measuring cAMP levels in CHO cells stably expressing $A_{2a}R$ or $A_{2b}R$ following stimulation with NECA

Results

Adenosine Signaling Drives Suppression Through $A_{2a}R$ and $A_{2b}R$ in Immune Cells, an Effect that is Reversed by Etrumadenant

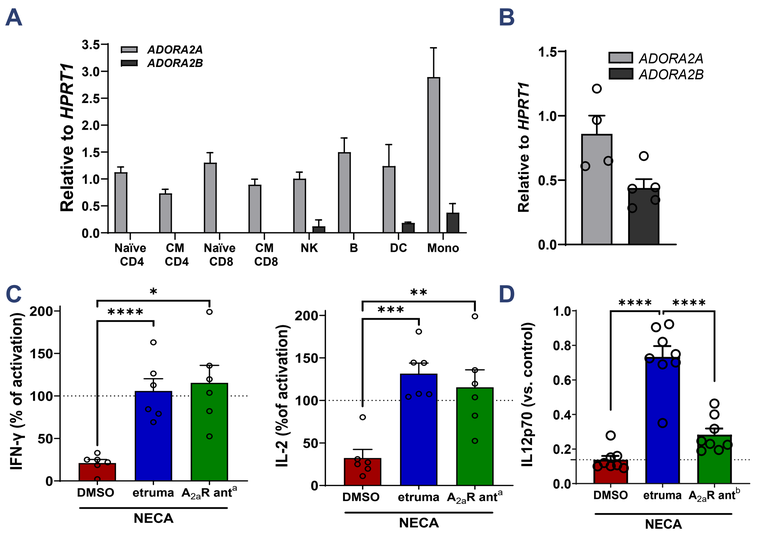

Figure 2. (A) Adenosine receptor gene expression of sorted human immune cells from healthy donors. **(B)** Real-time PCR for adenosine receptor *ADORA2A* and *ADORA2B* expression in DCs enriched from healthy human blood shows similar expression of each receptor. **(C)** IFN-γ (left) and IL-2 (right) production from primary human $CD8^+$ T cells activated for 72 h 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)** IL-12p70 suppression by NECA (5 μM) in DCs was rescued by etruma (1 μM) after 24 hours in culture whereas blockade of $A_{2a}R$ alone was less effective. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. See Table 1 for details on $A_{2a}R$ antagonists a and b.

Results

$A_{2b}R$ is Highly Expressed on Cancer Cell Lines and Drives Gene Expression Changes that are Blocked by Etrumadenant

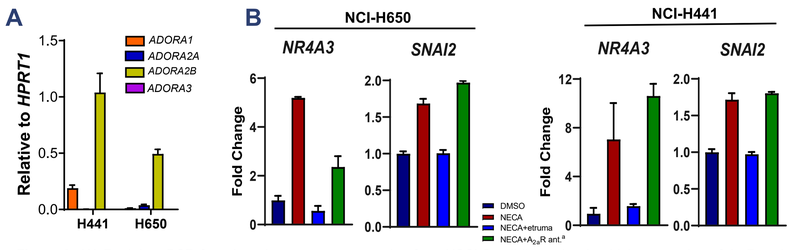

Figure 3. (A) Real-time PCR for adenosine receptor expression from NSCLC cell lines showing high expression of $A_{2b}R$ and substantially lower levels of expression for all other adenosine receptors. **(B)** Real-time PCR from NSCLC cell lines that were stimulated with NECA (5 μM) in the presence of dual vs $A_{2a}R$-selective antagonists (2 μM, see table 1 for details) showing that etruma is capable of blocking NECA-stimulated gene expression changes driven by $A_{2b}R$.

Etrumadenant Increases B16F10 Tumor Control in Combination with anti-PD-1

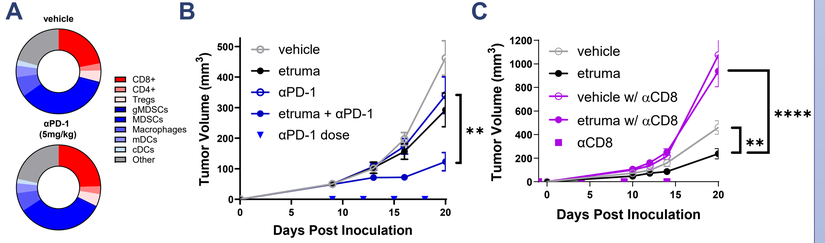

Figure 4. (A) Profiling of tumor-infiltrating immune cells in the B16F10 tumor in vehicle vs αPD-1 treated tumors. **(B)** Growth of B16F10 tumors in mice administered αPD-1 (5 mg/kg, Q3D) with and without etruma (100 mg/kg, PO, BID) starting at a tumor volume of 50 mm^3. **(C)** Animals were dosed prophylactically with αCD8 (25 mg/kg, IP, Q5D) and one day later inoculated with B16F10 tumor cells and concurrently dosed with etruma (100 mg/kg, PO, BID). Loss of $CD8+$ T cells results in rapid tumor growth which etruma is unable to alleviate. Etruma treatment enhances the immune response by inhibiting immunosuppression in B16F10 tumor model. **p<0.01, ****p<0.0001.

Etrumadenant Combined with Oxaliplatin Provides AT3-OVA Tumor Control

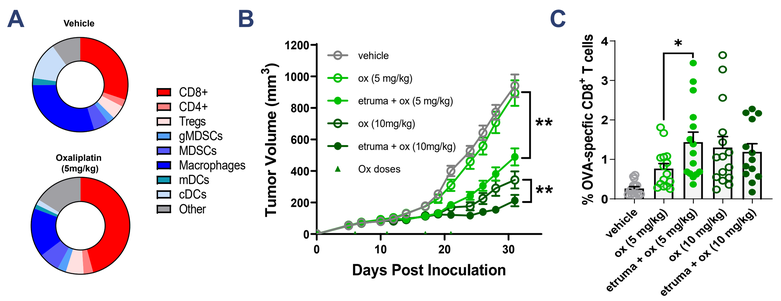

Figure 5. (A) Profiling of tumor-infiltrating immune cells in the AT3-OVA tumor in vehicle vs oxaliplatin-treated tumors. **(B)** Tumor volume from AT3-OVA model. Dosing started at 50 mm^3, etruma (100 mg/kg, PO, BID) and oxaliplatin (ox, 5 mg/kg or 10 mg/kg, Q4Dx4), as indicated. Etruma combined with either ox dose significantly reduced tumor burden (p<0.01). **(C)** Flow cytometric analysis of OVA-specific tumor infiltrating $CD8^+$ T cells displayed as % of total CD45+ cells. Etruma combined with 5 mg/kg ox significantly increased antigen-specific $CD8^+$ T cells into tumor microenvironment (p<0.05). *p<0.05, **p<0.01.

A Reduced Doxorubicin Regimen with Etrumadenant Results in Comparable AT3-OVA Tumor Growth Inhibition

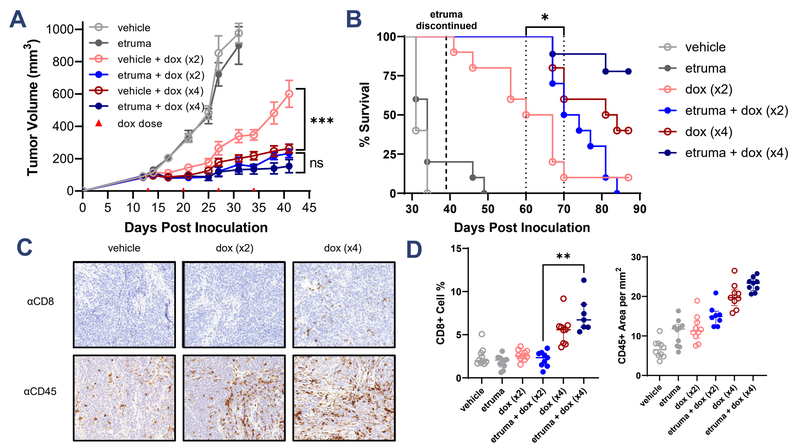

Figure 6. (A) AT3-OVA tumors were administered doxorubicin (dox, 5 mg/kg, IV, Q7D) as a 2 or 4 dose regimen in combination with etruma (100 mg/kg, PO, BID) starting at 100 mm^3, as indicated. A significant reduction in tumor volume was observed in the etruma + dox group vs vehicle + dox in the abbreviated chemotherapy group (p<0.001). Additionally, no significant increase in tumor reduction between etruma + dox (x2) vs etruma + dox (x4) groups was observed. **(B)** Kaplan-Meier survival analysis with either 2 or 4 dose dox combined with etruma. Combination treatment increases survival between shortened chemo group and matched etruma combo (p<0.05). **(C)** Representative images of immunohistochemical stains for αCD8 (top images) or αCD45 (bottom images) after 2 or 4 doses of dox. **(D)** Quantification of immunohistochemical analysis of tumor infiltrating $CD8^+$ T cells displayed as cells per mm^2 (left) or total CD45+ cells per mm^2 (right). These data suggest that the greatest therapeutic benefit of dox, when combined with etruma, may result from the initial, priming, doses of chemotherapy. While increased doses of dox increase immune infiltrate into tumor microenvironment this does not translate to an additional decrease in tumor burden. *p<0.05, **p<0.01, ***p<0.001.

Results

Etrumadenant Enhances AT3-OVA Tumor Control When Administered Concurrently with Doxorubicin

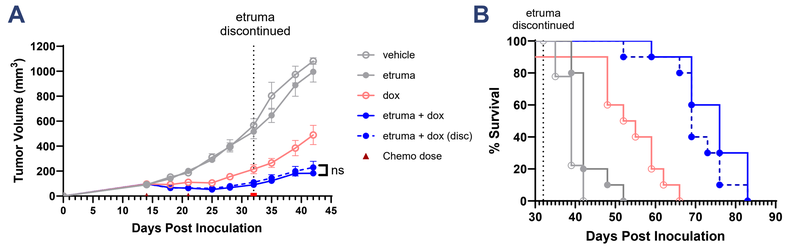

Figure 7. (A) Tumor volume from AT3-OVA model. Dosing started at 100 mm^3, etruma (100 mg/kg, PO, BID) and dox (5 mg/kg, IV, Q7Dx2). Etruma dosing was discontinued 11 days after second dox dose. No significant difference in tumor volume between continued and discontinued etruma treatment groups **(B)** Kaplan-Meier survival analysis shows comparable survival between continued and discontinued etruma dosing groups. Maximal effect of etruma treatment combined with dox occurs proximal to dox dosing when cell death and thus immunosuppression is greatest, allowing etruma to effectively block immunosuppression.

A Reduced Doxorubicin Regimen with Etrumadenant Leads to Comparable Immune Activity and Suppresses Metastasis in 4T1 Tumor Model

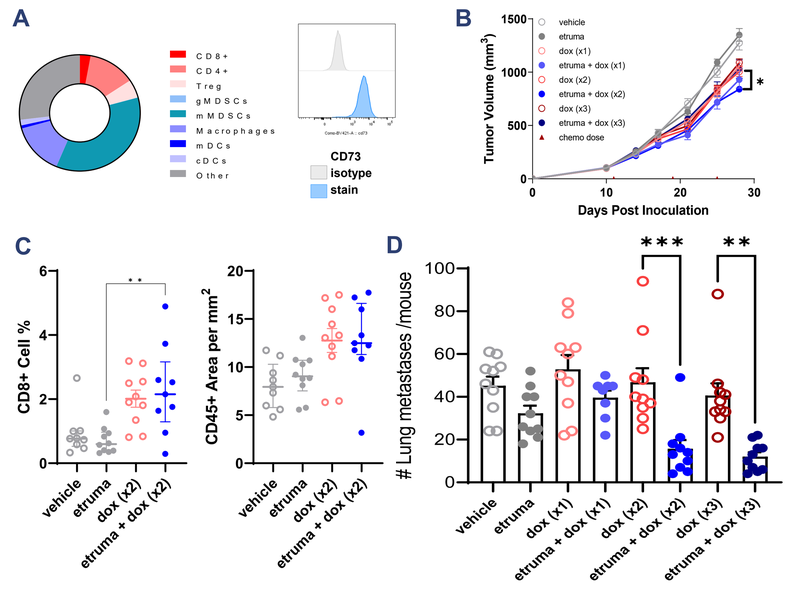

Figure 8. (A) Profiling of tumor-infiltrating immune cell shows high frequency of immunosuppressive cells in 4T1 tumors (left graph). 4T1 cells universally express CD73 in vitro as measured by flow cytometry (right). **(B)** Tumor volume from 4T1 model combined treatment of etruma with dox. Dosing started at 100 mm^3, etruma (100 mg/kg, PO, BID) and doxorubicin (5 mg/kg, IV, Q7D, 1 ,2 or 3 doses, as indicated). Chemotherapy only slightly reduces tumor burden even when combined with etruma. **(C)** Quantification of immunohistochemical analysis of $CD8^+$ T cells (cell %, left graph) and $CD45^+$ cell (area per mm^2, right graph) infiltrate in primary tumor from vehicle, etruma and 2 dose dox groups. Immune infiltrate is not increased over dox treatment alone when combined with etruma. **(D)** Number of lung metastases were quantified at the end of the study. Lung metastases were significantly decreased with two and three doses of dox combined with etruma when compared to matched dox doses alone (p<0.001 and p<0.01, respectively). From this we concluded that reduced chemotherapy doses are comparable to standard chemotherapy dosing regimen when combined with etruma. *p<0.05, **p<0.01, ***p<0.001

Summary

Results

- Our dual $A_{2a}R/A_{2b}R$ antagonist, etrumadenant, rescues NECA-induced immunosuppression on both $CD8^+$ T cells, dendritic cells, and cancer cell lines more significantly or comparably to single agent $A_{2a}R$ antagonists. These results suggests that blocking both $A_{2a}R$ and $A_{2b}R$ receptors is more effective at rescuing adenosine-mediated immunosuppression.
- In the B16F10 tumor model, a $CD8^+$ T cell enriched model, etruma combined with αPD-1 treatment significantly decreased tumor growth more effectively than either single agent. Further, depletion of $CD8^+$ T cells results in rapid tumor growth, which etruma is unable to alleviate. Based on these data, we believe etruma blocks *in vivo* immunosuppression, thus enabling an effective immune response that is enhanced when combined with αPD-1 immunotherapy.
- In the AT3-OVA tumor model, etruma combined with chemotherapy to induce immune infiltration into the tumor microenvironment and significantly reduce tumor burden. Further, we combined etruma with 2 or 4 doses of dox and found that addition of etruma significantly reduced tumor burden and increased survival in both dox dosing regimens.
- We next evaluated if extended etruma dosing further alleviates tumor burden with the 2 dox doses in the AT3-OVA tumor model and found no change to tumor burden or survival with shortened etruma treatment. These data corroborates our thinking that etruma is rescuing immunosuppression caused by ATP release due to cell death, and so etruma is most effective when elevated adenosine is present.
- Similar results were observed in 4T1 tumor-bearing mice, where etruma in combination with dox suppressed lung metastases after 2 doses of dox with no further improvement observed with a third dose of chemotherapy.
- These data suggest that etruma sustains the immune response driven by the initial doses of chemotherapy, leading to enhanced tumor control.

Conclusion

- Altogether, these data show that the combined treatment of chemotherapy with etruma leads to increased tumor control in multiple preclinical models and suggest that etruma combined with a reduced course of chemotherapy may have comparable activity as an extended chemotherapy dosing regimen.
- Further, extended treatment with etruma after chemotherapy priming does not enhance additional anti-tumor effects; thus, maximal effect of combination studies occurs around the time of chemotherapy administration.