A Novel Allosteric CBL-B Inhibitor with Differentiated Immune Enhancing Activity in Preclinical Models

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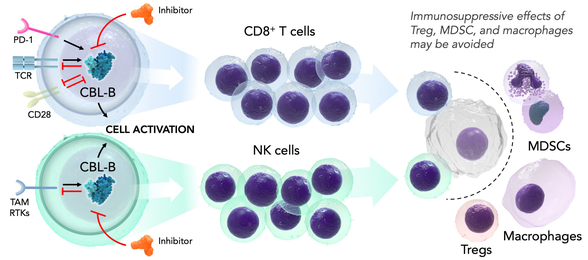
Introduction

- E3 ligase Casitas B-Lineage Lymphoma Proto-Oncogene B (CBL-B) is a key negative modulator of T-cell receptor and co-stimulatory regulation.[1]
- CBL-B inhibition lowers the threshold of antigen-specific T cell activation, even in the absence of co-stimulatory signaling or the presence of an immune suppressive environment.[2-5]
- Genetic ablation of CBL-B or functional inactivation of its E3 ligase activity in mice or primary human T cells enhances immune-mediated tumor growth control.[6-8]
- CBL-B inhibition may address the suboptimal response to current immunotherapies due to low inflammation, no/low co-stimulation signal or a high immune suppressive environment.

CBL-B Inhibition Enhances Anti-tumor Immunity Through Several Key Biological Mechanisms



1 Lowers threshold for effector cell activation

2 Drives effector cell proliferation and inflammation

3 Reduces susceptibility to suppression

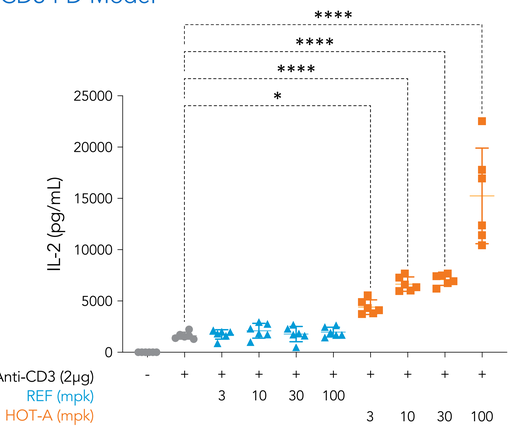
Results

Table 1. HotSpot CBL-B Inhibitor, HOT-A, Shows Potent Immunostimulatory Activity *in vitro*

Compound	Biochemical Assay	Human T Cell Assay			
	TR-FRET CBL-B IC_{50} (nM)	CD4 proliferation EC_{50} (nM)	CD8 proliferation EC_{50} (nM)	IL-2 EC_{50} (nM)	IFNγ EC_{50} (nM)
HOT-A	6	0.8	0.6	18	30
REF	15	5	6	230	510

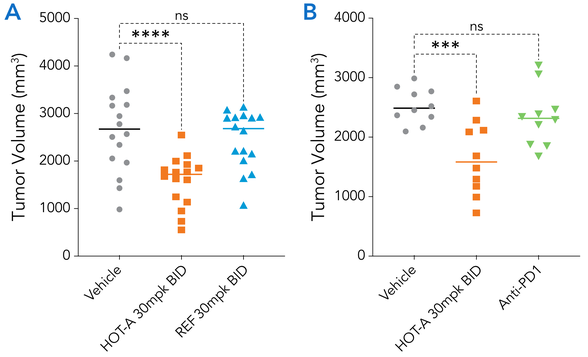
TR-FRET assay measures the interaction between the Biotin-E2 and CBL-B; Human T cell assay characterizes the effect of compounds on anti-CD3-stimulated human CD4+ and CD8+ T cells without anti-CD28 costimulation. IL-2 (24hr) and IFNγ (72hr) secretion were measured from supernatant by ELISA. The frequency of T cell proliferation at 72 hours was assessed through T cell labeled cell trace violet dye dilution by flow cytometry. REF: synthesized small molecule based on example 519 from patent WO 2019/148005 A1.[9]

Figure 1. HOT-A Enhanced IL-2 Release in the *in vivo* Anti-CD3 PD Model



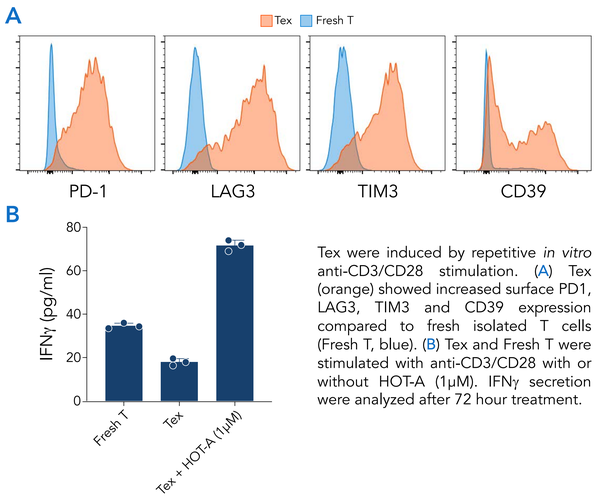
Compound HOT-A or REF was orally (PO) administered to female BALB/c mice at indicated doses one hour before the intraperitoneal (IP) injection of anti-CD3 antibody. Serum IL-2 levels were measured 4 hours after the anti-CD3 injection by MSD.

Figure 2. HOT-A Showed Enhanced Tumor Growth Inhibition Versus Key Comparators in the CT26 Tumor Model



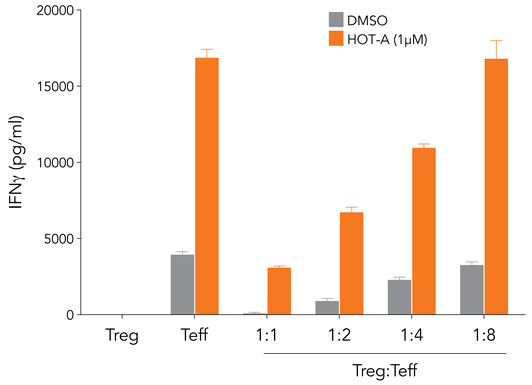
Female BALB/c mice were inoculated with CT26 colorectal carcinoma cells, randomized, and treated with vehicle, HOT-A (30mpk, BID, PO), REF (30mpk, BID, PO), anti-PD1 (10mpk, BIW, IP) or isotype control. (A) Individual tumor volume on day 17 in vehicle, HOT-A or REF treated tumor-bearing mice. (B) Individual tumor volume on day 13 in vehicle + isotype control (Vehicle), HOT-A + isotype control (HOT-A 30mpk BID) or vehicle + anti-PD1 (Anti-PD1) treated tumor-bearing mice.

Figure 3. CBL-B Inhibition Restored IFNγ Secretion in the Exhausted T Cells (Tex)



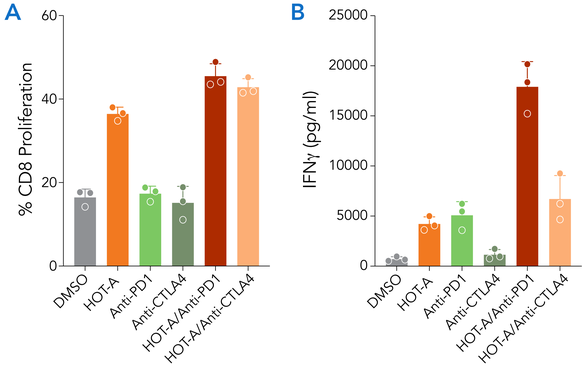
Tex were induced by repetitive *in vitro* anti-CD3/CD28 stimulation. (A) Tex (orange) showed increased surface PD1, LAG3, TIM3 and CD39 expression compared to fresh isolated T cells (Fresh T, blue). (B) Tex and Fresh T were stimulated with anti-CD3/CD28 with or without HOT-A (1μM). IFNγ secretion were analyzed after 72 hour treatment.

Figure 4. CBL-B Inhibition Restored the Teff Response to Treg Suppression



Human CD4+CD127lowCD25+ regulatory T cells were isolated from human PBMC. After *in vitro* expansion for 14 days, coculture of Treg and Teff at various ratios were set up in the presence of anti-CD3/CD28 stimulation and HOT-A (1μM). IFNγ secretion was measured at 72 hours after the coculture.

Figure 5. CBL-B Inhibition Enhances MLR Responses as Monotherapy with Further Enhancement in Combination with Anti-PD1



T cells from donor one PBMC were isolated and labeled with CellTraceViolet (CTV). Labeled T cells were then mixed and cultured with dendrite cells from a different donor (allo DC). After 4 day coculture in the presence or absence of HOT-A (1μM), anti-PD1 (10μg/ml) or anti-CTLA4 (10μg/ml), (A) proliferation was measured by the frequency of T cells with decreased CTV fluorescence and (B) IFNγ secretion was assessed by ELISA.

Conclusions

- We have identified HOT-A, a novel allosteric small molecule that potently inhibits CBL-B E3 ubiquitin ligase activity.
- HOT-A demonstrated superior activity *in vitro* and *in vivo* compared to key comparators.
- HOT-A showed an ability to reinvigorate exhaustive T cells. Additionally, treatment with HOT-A restored the Teff response in the presence of Treg-mediated suppression.
- HOT-A synergizes with anti-PD1 to enhance T cell proliferation and IFNγ in MLR assay.
- Our allosteric CBL-B inhibitors enhance key parameters associated with T cell activation and reduce susceptibility to immune suppression, supporting their further progression toward clinical development.

References

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