Identification of A Novel Allosteric Oral CBL-B Inhibitor that Augmented T Cell Response and Enhanced NK Cell Killing *in vitro* and *in vivo*

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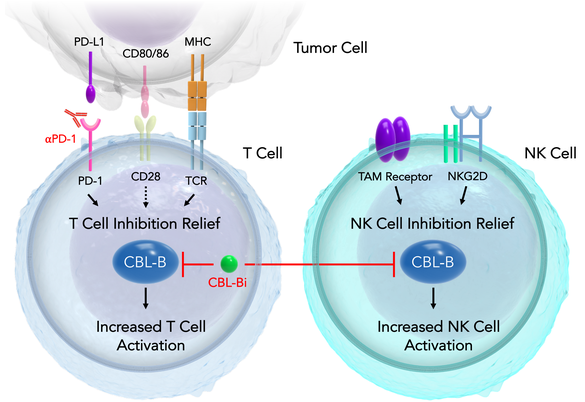
Abstract

Immunotherapies aiming to boost anti-tumor cell responses in cancer patients has been proven successful by checkpoint inhibitors targeting PD1 or CTLA-4, but the majority of cancer patients do not garner durable benefit. The E3 ubiquitin ligase CBL-B (casitas b-lineage lymphoma proto-oncogene b) has been established as a master negative regulator of T-cells and NK cells and plays an important role in immune suppression. Genetic ablation of CBL-B or functional inactivation of its E3 ligase activity in mice resulted in CD8 T-cell-mediated rejection of primary tumors in several mouse models. Based on the overwhelming evidence supporting the role of CBL-B in immune suppression, targeting CBL-B with small molecule inhibitors is attractive for cancer immunotherapy.

Here we report a member of our lead series of inhibitors, a low nanomolar potent inhibitor with picomolar binding affinity identified via application of our Smart AllosteryTM platform. This inhibitor binds to the inactive form of CBL-B, its binding mode in the identified hotspot confirmed by co-crystal structures. It inhibits the phosphorylation of CBL-B by kinases, inhibits the E3 ligase activity of CBL-B, promotes cytokine release and enhances T cell proliferation as well as NK cell activation and killing. *In vivo*, our CBL-B inhibitors efficaciously augmented the T cell response in anti-CD3 treated mice. We herein demonstrated the validation of our proprietary Smart Allostery™ platform via the prediction and drugging of a regulatory hotspot on an important immuno-oncology target that has to date been very difficult to drug.

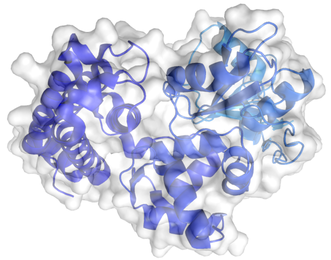
Introduction

CBL-B is a master negative regulator of T cells and NK cells. Inhibitors of CBL-B will release the inhibition and enhance the anti-tumor response of cytotoxic T cells and NK cells.



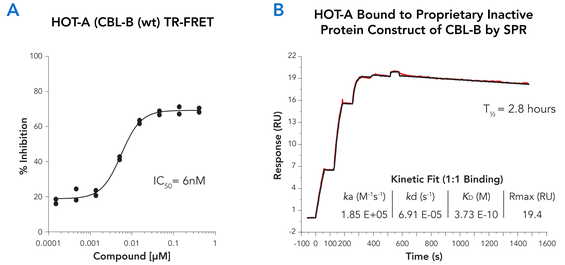
Results

Figure 1. HotSpot's Smart Allostery™ Platform



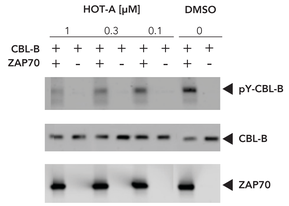
HotSpot's novel platform identified a druggable hotspot on CBL-B. Crystallization of CBL-B enabled structure-based design of the inhibitors.

Figure 2. HOT-A was Identified as a Potent CBL-B Inhibitor that Binds to the Inactive Form of CBL-B



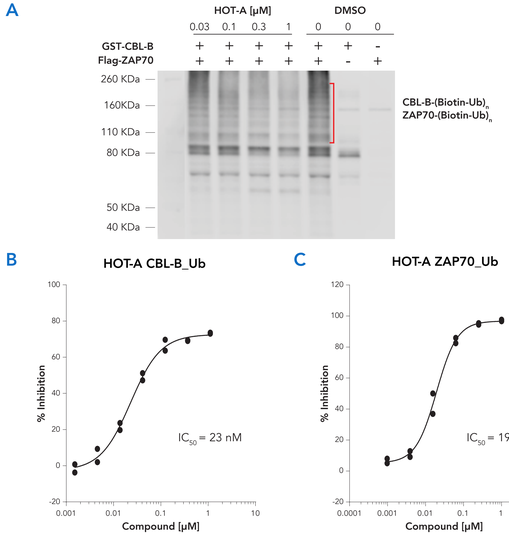
A. A TR-FRET assay which measured the interaction between the Biotin-E2 and inactive form CBL-B was developed and used for screening the compounds. HOT-A showed 6 nM potency in this assay. B. HOT-A bound to a proprietary inactive protein construct of CBL-B with a very slow off rate but not to an in-house designed active protein construct of CBL-B (data not shown) by surface plasmon resonance. Biotin-CBL-B proprietary inactive protein construct was immobilized on the surface.

Figure 3. HOT-A Inhibited CBL-B Phosphorylation by ZAP70



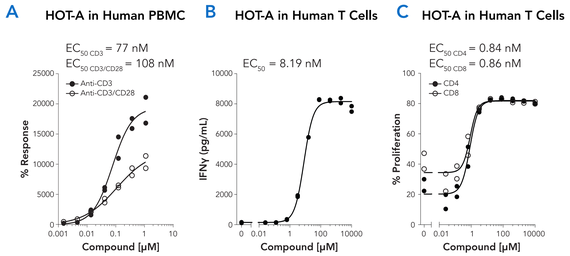
CBL-B phosphorylation was carried out in a kinase reaction with recombinant proteins of CBL-B and ZAP70. After reaction, phosphorylated CBL-B, total CBL-B and ZAP70 were detected with anti-phosphotyrosine antibody (4G10), anti-CBL-B antibody and anti-ZAP70 antibody on Western blots. HOT-A inhibited CBL-B phosphorylation by ZAP70 in a dose dependent manner.

Figure 4. HOT-A Inhibited CBL-B E3 Ligase Activity



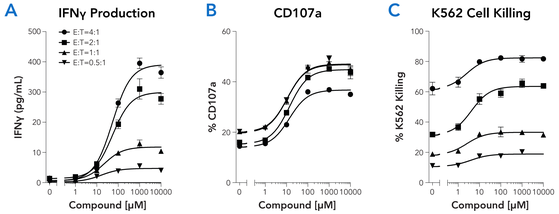
The Ubiquitination reaction was constituted by recombinant proteins of CBL-B and ZAP70 in the presence of Biotin-Ub, E1, E2 and ATP. In the reaction, ZAP70 activated CBL-B, which in turn mediated the Ubiquitination on ZAP70 and auto-Ubiquitination on CBL-B. The Biotin-Ub on ZAP70 and CBL-B were detected by WB (A), ELISA of CBL-B-(Biotin-Ub)$_n$ (B) or MSD ZAP70-(Biotin-Ub)$_n$ (C). HOT-A inhibited the production of both CBL-B-(Biotin-Ub)$_n$ and ZAP70-(Biotin-Ub)$_n$.

Figure 5. HOT-A Increased T Cell Response in Both Human PBMC and Isolated T Cells



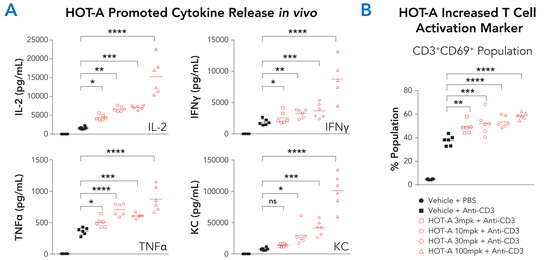
A. HOT-A increased anti-CD3 and anti-CD3/CD28 stimulated IL-2 production in human PBMC. The amount of IL-2 production was normalized to anti-CD3 or anti-CD3/CD28 stimulation. B. HOT-A increased anti-CD3 stimulated IFNγ production in human T cells. C. HOT-A increased anti-CD3 stimulated CD4 and CD8 T cell proliferation by flow cytometry.

Figure 6. HOT-A Increased NK Cell Activation and Enhanced Cell Killing



Human NK cells were tested for their ability to kill tumor targets by adding K562 cells labeled with Celltrace Violet dye at different ratios. After incubation, IFNγ were measured by ELISA (A); Degranulation marker of CD107a was detected by flow cytometry (B); K562 killing was measured by 7AAD (C).

Figure 7. HOT-A Promoted the T Cell Response in Anti-CD3 Treated Mice



Compound HOT-A was orally (PO, methylcellulose/water) administrated to BALB/c mice at indicated doses one hour before the parenteral (IP) injection of anti-CD3 antibody. Serum cytokines were measured 4 hours after the anti-CD3 injection by MSD (A) and cell surface activation markers were measured by FACS analysis (B).

Conclusions

- HotSpot's proprietary Smart AllosteryTM platform successfully predicted a druggable regulatory hotspot on the highly sought-after protein CBL-B.
- Utilizing state of the art structure-based design, we identified and optimized a series of potent allosteric inhibitors of CBL-B, including HOT-A. This inhibitor bound to the inactive form of CBL-B, inhibited the E2-E3 interaction and E3 ligase activity of CBL-B.
- HOT-A potently augmented the T cell response and NK cell activation and cell killing.
- HOT-A efficaciously promoted the T cell response in anti-CD3 treated mice after oral dosing.
- HOT-A series of CBL-B inhibitors are en route to clinical development for immunotherapy.