A CBLB Screening Cascade to Facilitate Novel Drug Discovery

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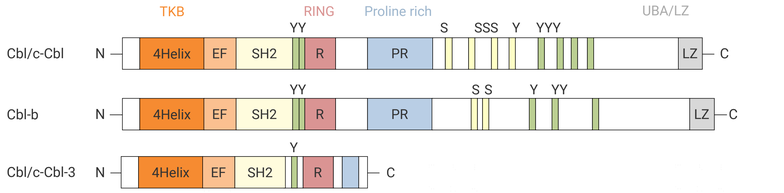
Introduction

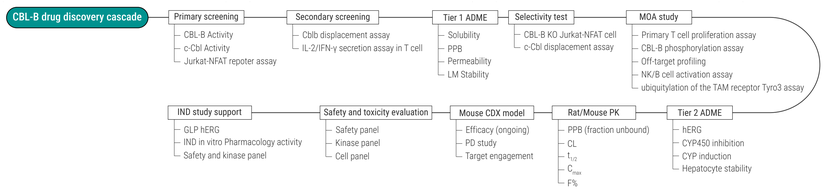
Maintaining the balance between immunity and tolerance of immune cells is strongly controlled by several sophisticated regulatory mechanisms of the immune system, among which the E3 ligase ubiquitin Casitas B cell lymphoma-b (Cbl-b) appears to be a central player.

Cbl-b is newly identified component in the ubiquitin-dependent protein degradation system, which is thought to be an important negative regulator of immune cells. Ubiquitin ligase targeting could be a novel approach to human disease therapy, such as in autoimmunity and cancer.

Here, we constructed an experimental cascade from in vitro to in vivo, which is composed of protein production, biochemical assays, cell line construction, cellular assays, and animal modeling. Our Cbl-b screening cascade can satisfy the mechanism study of Cbl-b as well as efficient and comprehensive screen of Cbl-b inhibitor, thus accelerate the novel drug discovery.

CBLB Screening Cascade

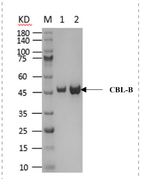




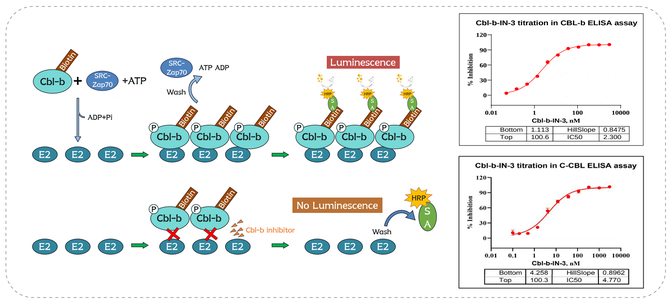
Result 1: Biochemical Assays

We have successfully purified Cbl-b proteins. The Cbl-b displacement and activity were analyzed by TR-FRET, ELISA and HTRF assay. These assays can be used for rapid and efficient screening of Cbl-b inhibitor. Representative data are shown below.

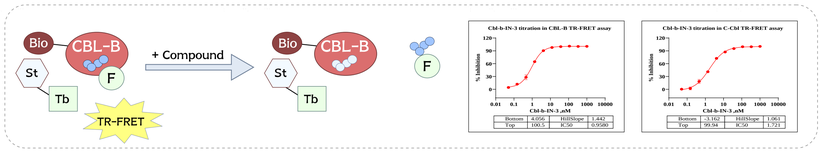
• SDS-PAGE



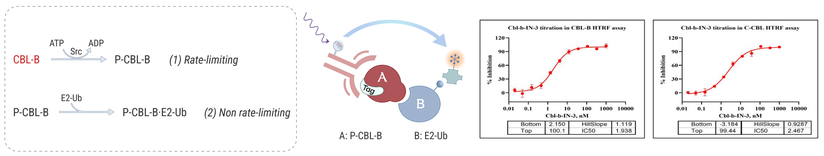
• Cbl-b ELISA assay: Featuring our patented method for high-throughput screening of Cbl-b inhibitors



• Cbl-b displacement assay

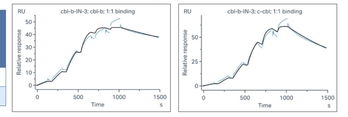


• Cbl-b HTRF assay



We have developed SPR assay to test the single cycle kinetics of Cbl-b inhibitor and Cbl-b and c-Cbl. Representative data are shown below.

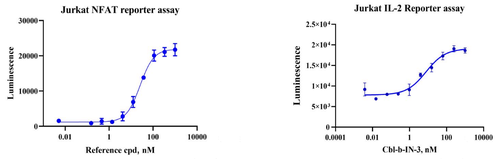
Immobilized ligand	Injection variables Single cycle kinetics 1 Solution	General Kinetics model	1:1 binding ka (1/Ms)	Kd (1/s)	KD (M)	Rmax (RU)	Quality Kinetics Chi² (RU²)	U-value
cbl-b	cbl-b-IN-3	1:1 binding	1.42e+05	3.97e-04	2.80e-09	45.7	4.40e+00	5
c-cbl	cbl-b-IN-3	1:1 binding	7.95e+04	9.67e-04	1.22e-08	61.8	5.60e+00	2



Result 2: Cellular Assays

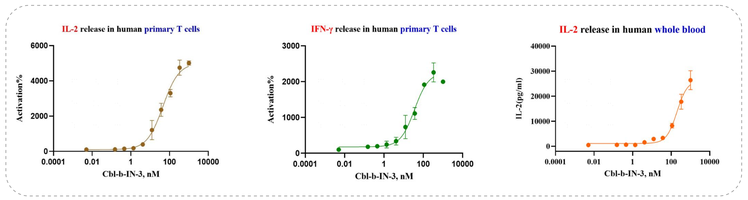
We have successfully constructed Cbl-b reporter assays. Jurkat NFAT Cbl-b KO reporter cell line and Juekat-IL2-Luc reporter cell line were ready to use. We have developed T cell activation and NK cell activation assays. Also, we have established Mixed Lymphocyte Reaction (MLR) assay and Adenosine suppression assay.

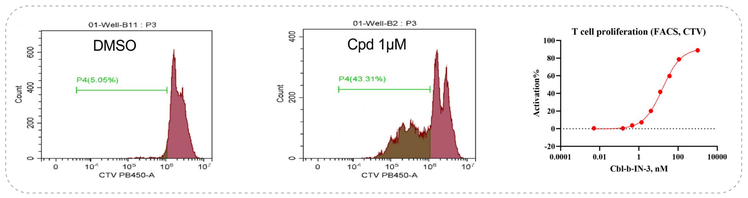
T cell reporter assay: The Jurkat reporter cells were treated with different concentrations of compound, the NFAT pathway and IL-2 expression were promoted in a dose-response effect.

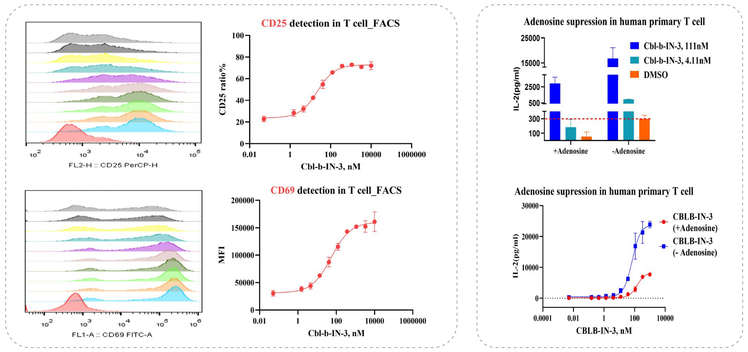


T cell activation assay: T cell or the whole blood were treated with compound in the present of anti-CD3/28 antibodies, the cytokine levels were measured in the supernatant, CTV labeled T cells proliferation and cell surface marker CD69 and CD25 were analyzed by FACS.

Adenosine suppression assay: T cells were treated with different concentrations of compound in the present of anti-CD3/28 antibodies and adenosine, the cytokine production were detected.



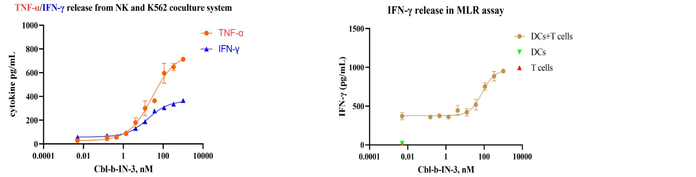




The Cbl-b inhibition promote the IL-2 and IFN-γ release in the primary T cells or human whole blood, which also promote the T cell proliferation and T cell activation marker expression including CD25 and CD69. Inhibition of Cbl-b also reversed the adenosine suppressed T cell that enhanced the IL-2 release.

NK cell activation assay: NK cells were pre-treated IL-2 and then co-cultured with K562 cells, the TNF-α and IFN-γ production were detected in the present of compound.

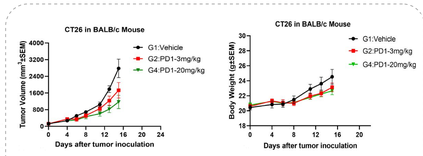
Mixed Lymphocyte Reaction (MLR) assay: T cells and mature dendritic cells from different donors were co-cultured with compound for 48 hours and IFN-γ levels in the cellular supernatant were detected.



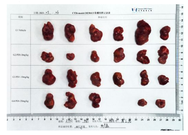
Inhibition of Cbl-b enhances the NK cell response to target cells, which promotes the TNF-α and IFN-γ release from NK cell in the present of K562 cells. Inhibition of Cbl-b enhances the T cell response to DCs, which promotes IFN-γ release in MLR assay.

Result 3: Animal Modeling

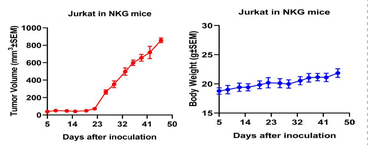
CDX modeling utilizing different cell lines has been established for the efficacy study as shown below to expand the drug discovery cascade to the in vivo experiments.



	TGI(%)
PD1-3mg/kg	39.55
PD1-10mg/kg	33.46
PD1-20mg/kg	60.51

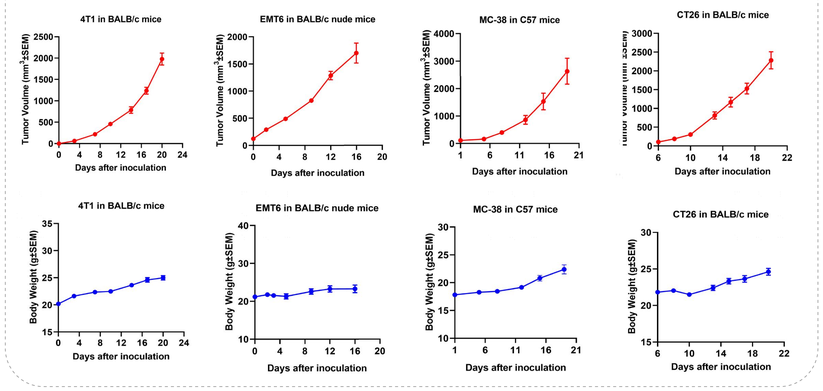


Animal	NKG mice ♀ 6-8w
Cell line	Jurkat
Cancer type	Human T-lymphocytic Leukemia
Injected Cell Mumbers	5*10^6/Mouse



Animal	BALB/c mice ♀ 6-8w	Animal	BALB/c nude mice ♀ 6-8w
Cell line	4T1	Cell line	EMT6
Cancer type	Mice Breast Cancer	Cancer type	Mice Breast Cancer
Injected Cell Mumbers	1*10^6/Mouse	Injected Cell Mumbers	1*10^5/Mouse

Animal	C57 mice ♀ 7w	Animal	BALB/c mice ♀ 6-8w
Cell line	MC38	Cell line	CT26
Cancer type	Mice Colon Cancer	Cancer type	Mice Colon Cancer
Injected Cell Mumbers	5*10^5/Mouse	Injected Cell Mumbers	5*10^5/Mouse



Besides in vivo CDX modeling, we can also provide PK/PD study to in-depth evaluate the efficacy of the potential drug compounds. Furthermore, our safety and kinase panel screening capabilities can provide the pre-clinical Adverse Drug Reactions (ADRs) evaluation and prediction.

Conclusions

Degradation inhibition of Cbl-b targets could be regarded as a rational approach in autoimmune diseases. Moreover, anti-cancer immune responses might be increased by the inhibition of the Cbl-b activity. Therefore, targeting the immunological gate keeper Cbl-b opens new avenues to treat cancer and autoimmunity diseases. Our Cbl-b screening cascade can provide comprehensive compound evaluation across in vitro and in vivo platforms, thus serve as an efficient screening platform for new drug discovery.

References

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[2] Lutz-Nicoladoni, C., Wolf, D., & Sopper, S. (2015). Modulation of immune cell functions by the E3 ligase Cbl-b. Frontiers in oncology, 5, 58.

[3] Mueller, D. L. (2004). E3 ubiquitin ligases as T cell anergy factors. Nature immunology, 5(9), 883-890.