APPROACH TO RATIONAL IDENTIFICATION OF LEAD MOLECULAR GLUE DEGRADERS FOR CASEIN KINASE 1α

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ABSTRACT

There has been a tremendous explosion of interest in the targeted protein degradation (TPD) in drug discovery in recent years. One approach that has been widely exploited is the use of proteolysis targeting chimeras (PROTACs). Here, a heterobifunctional compound with 2 ligands linked by a flexible/rigid spacer has one ligand that binds to an E3 ligase while the other binds to a protein of interest (POI). This action brings the POI in close proximity to the E3 ligase for polyubiquitination and subsequent degradation by cellular proteasome machinery. The limitations of this approach are that it has usually led to the identification of compounds that are outside of the rule of five, have molecular weights >500 and in most cases, are not orally bioavailable. An alternative TPD approach involves the use of molecular glue degraders (MGD). To date there are few examples of cases where a rational approach has been used to identify potent MGDs and most MGDs have been discovered by serendipity or by happenstance. Unlike PROTACs, MGDs generally have molecular weights of <500, satisfy the rule of five and are orally bioavailable. MGDs could be exploited to target a plethora of proteins that do not have a well-defined binding pocket (currently considered undruggable) where traditional small molecule approaches fail.

CRBN and CK1α have a relatively weak native protein-protein interaction with Kd of about 2 μM. These types of interactions, between the domain of one protein with the sequence motif of the other, are generally weak as they possess only a small buried surface area (BSA). The goal of a MGD is to increase these interactions (if present) and stabilize the resultant ternary complex. Indeed, when Lenalidomide binds to CRBN, it increases its affinity for CK1α and the resultant ternary complex has a Kd of about 75 nM. This could also be viewed as Lenalidomide binding to the binary complex of CRBN-CK1α and strengthening the interaction within the ternary complex by increasing the BSA interactions between CK1α and CRBN. In a recent report[1], a series of MGDs have been designed based on the stabilization of the ternary complex, in silico.

In this poster we report our findings on the binary and ternary complex formation, cellular target engagement, target ubiquitination and CK1α degradation. After screening compounds in these assays, two potent CK1α degraders, CC-885 and **cmpd-006** have been identified.

RESULTS AND DISCUSSION

Cmpd-001 - **004** were chosen based on the substitution pattern and the commercial availability of the analogs. Docking of the **cmpd-001** in the CRBN-Lenalidomide-CK1α crystal structure[2] (PDB: 5FQD; 2.45 Å resolution) revealed that absence of glutarimide hydrogen bonding and the steric clash of N-Me with W386 indole weakened its interaction in the Lenalidomide pocket. This apparently resulted in loss of CRBN binding in the binary assay and hence no ternary complex could form. Virtual screening was carried out using Enamine Lenalidomide library after prepping the crystal structure[2] (PDB: 5FQD) as described in the materials and methods section. **Cmpd-005** was chosen from the top ranked sulfonamide cluster and **cmpd-006** from the overall top ranked compounds and that are commercially available.

The reference compound Lenalidomide along with Thalidomide, CC-885, CC-90009, **cmpd-001** – **006** were evaluated for affinity in E3 ligase, Cereblon (CRBN) assay (**Fig. 1**) as described briefly in materials and methods section. Lenalidomide had an affinity of 73 nM. **Cmpd-005** had the highest affinity of the evaluated compounds at 10 nM followed by CC-885 at 12 nM and CC-90009 at 33 nM. These 3 compounds had higher affinity for CRBN than Lenalidomide. **Cmpd-001** had no detectable affinity for the ligase. The rest of the compounds shown in **Table 1** had relatively low affinity in the sub micromolar range except **cmpd-006** that had an IC_{50} of 93 nM.

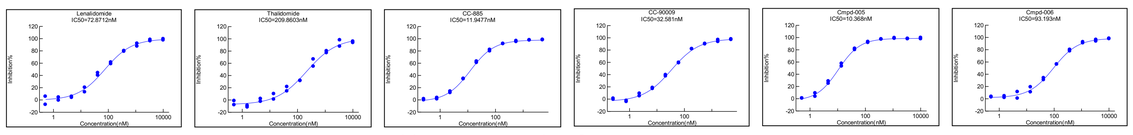


Fig.1 CRBN-cmpd binary complex formation assay_HTRF assay

Then the compounds were evaluated in ternary complex CRBN-MG-CK1α formation assay. The dose response curves are shown in the **Fig. 2**. Relative and absolute EC_{50} values in nM are shown in **Table 1**. CC-885 and **cmpd-006** were the most potent in the assay with EC_{50} of 27 nM and 38 nM, and top activation of 99% and 182%, respectively. Both the compounds were more potent in the ternary complex formation than Lenalidomide. Though the **cmpd-005** had a relative EC_{50} of 33 nM the top activation was only 54%. Thalidomide and **cmpd-001** – **004** did not form ternary complex.

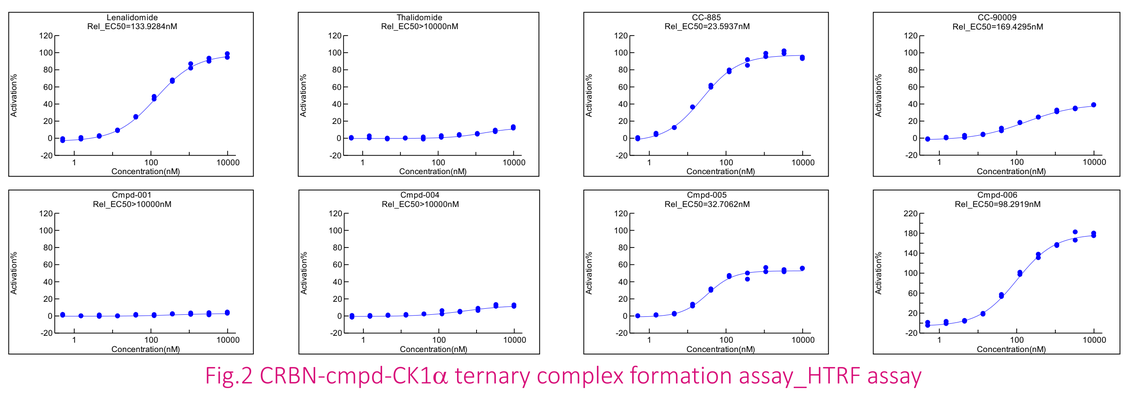
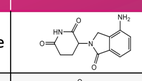
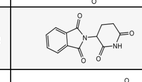
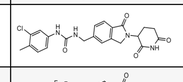
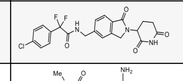
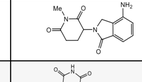
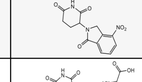
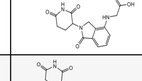
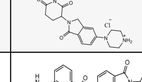
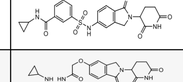
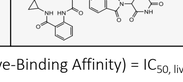


Fig.2 CRBN-cmpd-CK1α ternary complex formation assay_HTRF assay

Compound	Structure	Binary Complex (E3)_HTRF IC_{50} (nM)	Ternary Complex (CRBN-MG-CK1α) EC_{50} (nM)		Top Activation	Cellular Target Engagement (NanoBRET) IC_{50} (μM)			
			Relative	Absolute		Live Cell	Permeabilized Cell	RBA[a]	AI[b]
Lenalidomide		72.87	133.93	149.69	100.00%	0.147	0.172	0.855	1
Thalidomide		209.86	>10000	>10000	13.30%	0.660	2.645	0.249	0.292
CC-885		11.95	23.59	26.85	99.09%	0.013	0.025	0.515	0.602
CC-90009		32.58	169.43	>10000	41.32%	0.059	0.234	0.254	0.297
Cmpd-001		>10000	>10000	>10000	3.35%	NA	NA	NA	NA
Cmpd-002		340.28	>10000	>10000	24.87%	0.619	2.867	0.216	0.252
Cmpd-003		320.85	>10000	>10000	20.51%	1.899	0.767	2.477	2.896
Cmpd-004		126.66	>10000	>10000	13.10%	0.427	0.693	0.616	0.721
Cmpd-005		10.37	32.71	288.42	53.91%	0.204	0.262	0.774	0.905
Cmpd-006		93.19	98.29	37.91	181.83%	1.435	0.017	86.655	101.303

a) RBA (Relative-Binding Affinity) = $IC_{50,\ live\text{-}cell\ mode}$ / $IC_{50,\ permeabilized\text{-}cell\ mode}$ b) AI (Availability Index) = RBA_{ligand} / $RBA_{permeable\ control}$

Table 1 Structures and Assay Results

Ternary complex formation, in silico, was visualized in MOE. The final frames of a 15ns molecular dynamics simulation of ternary complexes of CC-885, **cmpd-005** & **006** were shown in **Fig. 3**. Glutarimide and isoindolone moieties of the ligands interact with CRBN H378 and N351, respectively. The sidechains of CC-885 and **cmpd-005** engaged in hydrogen bonding acceptor interaction with the K18 sidechain amine of CK1α and the sidechain of the **cmpd-006** had hydrogen bonding donor interaction with the E377 sidechain carbonyl of CRBN.

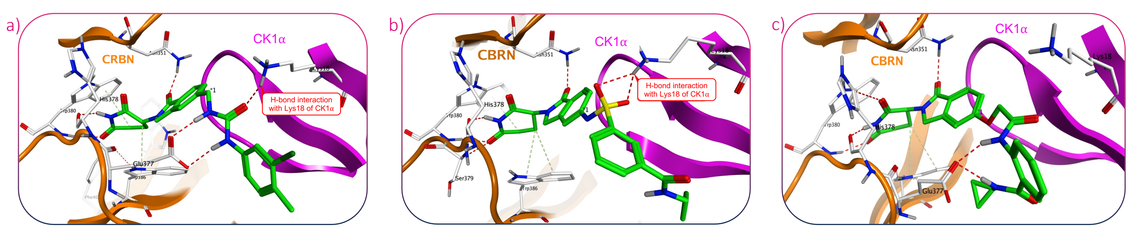


Fig.3 Ternary complexes final frame at 15 ns, CRBN in brown, CK1α in pink, ligand in green a) CC-885, b) **Cmpd-005** and c) **Cmpd-006**

In the cellular target engagement studies (**Fig. 4**), all compounds except **cmpd-001**, **003** & **006** had better membrane permeability than Lenalidomide. The inactivity of **cmpd-001** was due to its inability to form ternary complex as mentioned above. **Cmpd-003** had a poor cellular permeability as shown by their availability index (AI) value of 2.89. Notably, though **cmpd-006** showed the least cellular permeability (AI = 101.3), the IC_{50} in permeabilized cell is 17 nM (**Table 1**). This indicates that **cmpd-006** has a strong affinity for CRBN in permeabilized cell mode, which is consistent with the results of the ternary complex formation assay.

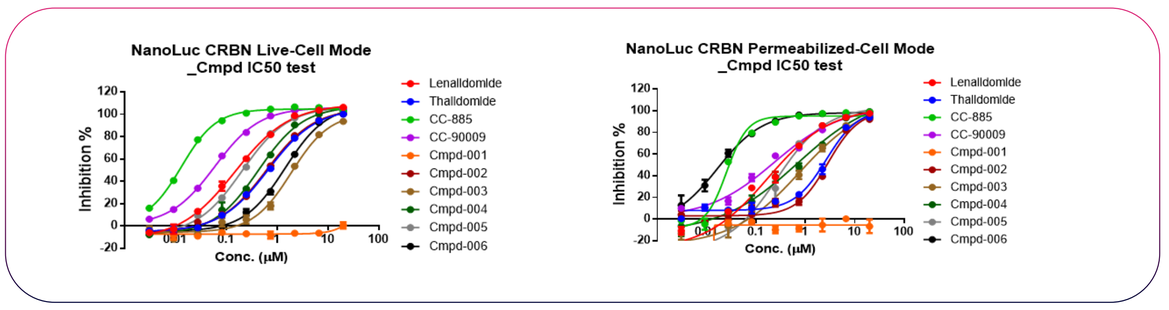


Fig.4 Cellular target engagement assay_NanoBRET assay

Western blot was done to detect the effects of the compounds on *in vitro* ubiquitination of CK1α. Lenalidomide and CC-885 promoted ubiquitination of CK1α after 24 hours of reaction, more obvious after 48 hours (**Fig. 5**). Ubiquitination was also observed with **cmpd-005** & **006**. Thalidomide, CC-90009 and **cmpd-001** – **004** showed minimal ubiquitination.

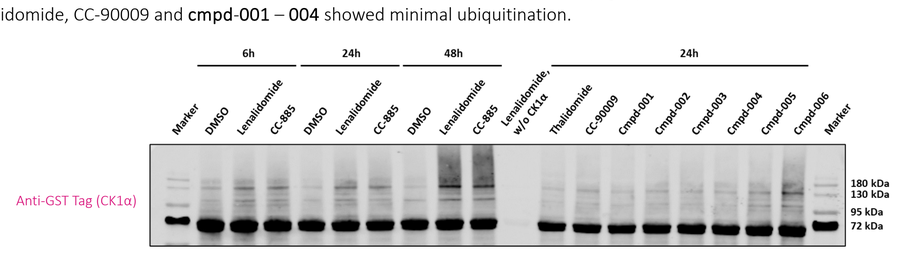


Fig.5 CK1α ubiquitination assay _WB assay

In the cellular CK1α degradation assay, CC-885 and **cmpd-006** were effective (**Fig. 6**). CC-885 also showed dose dependent degradation of CK1α starting at 0.01 μM to 10 μM. Like Lenalidomide, **cmpd-006** also showed dose dependent degradation at 0.01 μM and 0.1 μM. Thalidomide, CC-90009, and **cmpd-001** – **005** did not show any detectable CK1α degradation.

Thus we got a new lead compound, **cmpd-006**. Lead optimization by SAR as well as focusing on ADME/PK, would potentially yield more potent analogs of **cmpd-006**. Further preclinical development of these type of compounds could pave a path potentially to clinic.

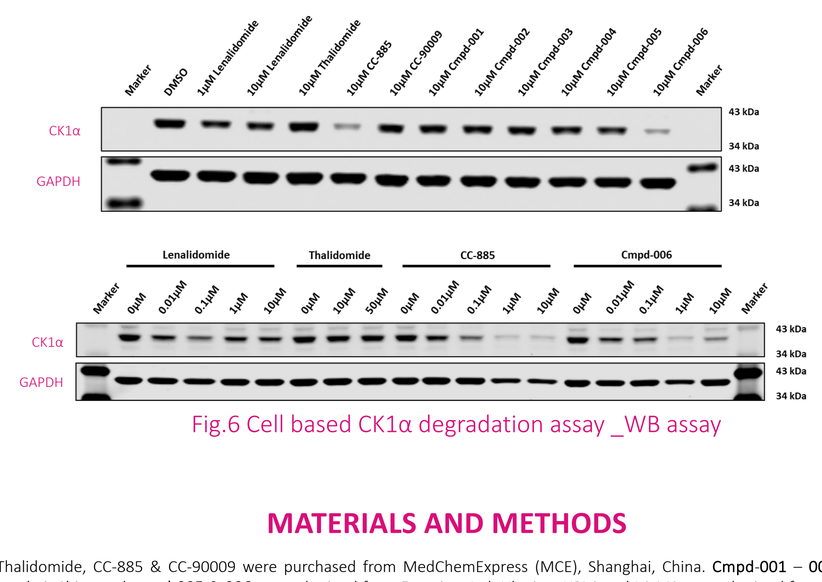


Fig.6 Cell based CK1α degradation assay _WB assay

MATERIALS AND METHODS

Lenalidomide, Thalidomide, CC-885 & CC-90009 were purchased from MedChemExpress (MCE), Shanghai, China. **Cmpd-001** – **004** were procured from BLD Pharmatech, Shanghai, China and **cmpd-005** & **006** were obtained from Enamine, Ltd, Ukraine. HPLC and LC-MS were obtained for all the compounds to confirm the purity. All the tested compounds were racemic mixtures and used as such.

Structure-Based Virtual Screening

Database preparation: The lenalidomide scaffold-based Enamine database containing 2720 compounds was used for the virtual screening experiment. The database was prepared using Wash function and the molecular flexibility of each compound in the database was then modeled by generating multiple conformers using conformations search option in MOE.
Virtual Screening: The crystal structure of ternary complex of CRBN–lenalidomide–CK1α (PDB: 5FQD)[2] was obtained from the protein data bank (PDB) and prepared for structure-based virtual screening. The complex was prepared using QuickPrep function in MOE 2022.02 (CCG, Montreal, Canada). This function was used to correct protonation and charge and to minimize the structures to a RMSD threshold of 0.1 Å using Amber10:EHT force field
The docking program in MOE was used for the docking experiment. The docking method described below was validated by redocking the lenalidomide into the ternary complex crystal structure and calculating the RMSD between the top docked pose and the bound lenalidomide conformation in the crystal structure. MOE docking (default parameters) was used to dock the database compounds into the lenalidomide binding site and the best pose for each compound was chosen based on the MOE docking score (S score). The top ranked poses were retained for cluster analysis using MACCS Structural Keys (Bit Packed) fingerprint.

CRBN-cmpd binary complex formation assay (HTRF assay)

Key Materials: His tagged CRBN E3 ligase Complex Protein, MAb Anti 6HIS-Tb cryptate HTRF reagent & Cy5 Labeled Thalidomide.
Overview: Based on the binding of Cy5-labeled thalidomide and CRBN E3 ligase, the binding of the tested compound to E3 was detected using the HTRF principle in a competitive manner through the loss of signal.
Method in brief: When the compound is ready in the 384-well plate in duplicate, CRBN E3 ligase complex protein was added and incubated with them for 10 min at 25°C. Then the detection solution containing Anti 6HIS-Tb cryptate HTRF reagent and Cy5 labeled Thalidomide was added and incubated for 2 h at 25°C. The HTRF measurement was conducted with excitation at 340 nm and emission at 615 nm and 665 nm. The HTRF signal was extracted by calculating the 665/615 nm ratio. Binding of compounds was quantified by percentage inhibition, calculated from HTRF signal relative to control signals.

CRBN-cmpd-CK1α ternary formation assay (HTRF assay)

Key Materials: His tagged CRBN E3 ligase Complex Protein, GST tagged CK1α protein, Anti GST-Tb cryptate HTRF reagent, Anti 6HIS-XL665 HTRF reagent.
Overview: The compounds were incubated with samples containing CRBN and target proteins, and the activation of the compound in ternary formation was detected by HTRF based on changes in the signal.
Method in brief: After compounds were transferred into the 384-well assay plate in duplicate, CRBN E3 ligase complex protein and CK1a protein were added and mixed. Then the detection solution containing Anti GST-Tb cryptate and Anti 6HIS-XL665 was added and the reaction was incubated for 2 h at 25°C. The HTRF measurement was conducted with excitation at 340 nm and emission at 615 nm and 665 nm. The HTRF signal was extracted by calculating the 665/615 nm ratio. The relative activity of compounds in inducing the formation of ternary complex was quantified by the signal over that of Lenalidomide.

Cellular target engagement assay (NanoBRET assay)

Key Materials: NanoBRET™ TE Intracellular E3 Ligase Assays kit.
Overview: Two detection modes, live-cell and permeabilized-cell, were provided to detect the compound engagement to the CRBN E3 ligase. The live-cell mode was used to measure the compound intracellular affinity and the permeable cell mode was used to measure the compound intrinsic affinity. The execution of both modes can determine the intracellular availability of the compound from the observed potency shift. In addition, normalization of this relative change in binding potency can be used to calculate a parameter called the Availability Index (AI), which quantitatively assesses the relative intracellular availability of different compounds compared to the control compound Lenalidomide.
Method in brief: For the live-cell assay mode, HEK293 cells were transfected with DDB1 Expression Vector and NanoLuc®-CRBN Fusion Vector for 24 h. Then the cells were harvested the next day, resuspended and dispensed into 384-well plates in triplicate. The prepared NanoBRET™ Tracer was then added to the cell culture medium and briefly mixed. After incubating the compound for 2 h, the complete substrate plus inhibitor solution was added and the signal was detected within 10 min. For the permeabilized-cell mode, after the same transfection and plating, the cell medium was added with tracer reagent and lightly mixed. The cells were incubated for 20 min with the addition of digitonin solution at the same time as the compound stimulation for 10 min. The complete substrate plus inhibitor solution was then added and the signal was read within 10 min.

Cell based CK1α degradation assay (WB assay)

Key Materials: Malme-3M cell line, Anti-CSNK1A1 + CSNK1A1L antibody, Anti-GAPDH antibody.
Overview: Western Blot technology was used to reflect the effect of the compound on CK1α degradation by detecting the expression of the CK1α in the cells.
Method in brief: When enough Malme-3M cells were prepared, they were stimulated with compounds for 24 h. The cells were harvested and lysed with lysis buffer. Protein concentration of cell lysate was determined by BCA kit and WB samples were prepared in such a way that each loading contained the same amount of total protein. Bands were visualized with Anti-CSNK1A1 + CSNK1A1L antibody and Anti-GAPDH antibody respectively.

CONCLUSIONS

Cereblon modulator CC-885 was a known MG degrader for GSPT1[3], BNIP3L[4], PLK1[5] and CDK4[6]. To our knowledge this is the first disclosure that CC-885 is also an efficient degrader of another oncology target CK1α. **Cmpd-006** was also a potent degrader of CK1α. Both CC-885 and **cmpd-006** were more efficient in degrading the target CK1α than the known degrader Lenalidomide. Moreover **cmpd-006** despite its poor cellular permeability showed very good potency in CK1α degradation. Lead optimization by SAR as well as focusing on ADME/PK, would potentially yield more potent analogs of **cmpd-006**. Further preclinical development of these type of compounds could pave a path potentially to clinic.

REFERENCES

1) Weiss, *et.al.* J Chem Inf Model **2023**, *63*, 2382-92; 2) Petzold, *et.al.* Nature, **2016**, *532*, 127-30; 3) Hansen *et.al.* J Med Chem **2018**, *61*, 492-503; 4) Hao, *et.al.* Acta Pharmacologica Sinica **2020**, *41*, 1246-54; 5) Li, *et.al.* Mol Ther Oncolytics **2020**, *18*, 215-25; 6) Zhao, *et.al.* Biochem Biophys Res Comm **2021**, *549*, 150-56.