LiLis: harnessing new E3 ligases for targeted protein degradation platform

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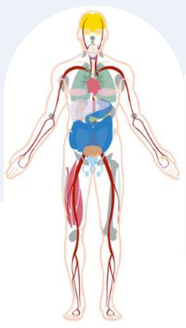
Introduction

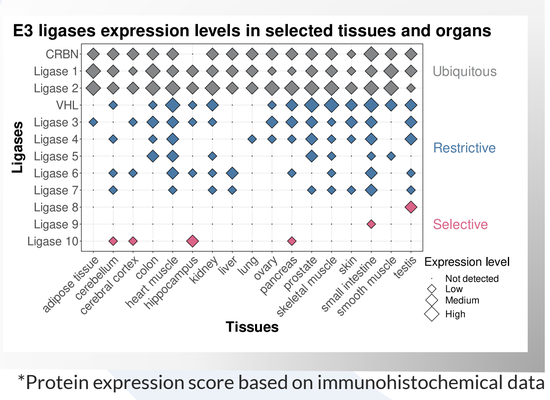
PROTAC design and development center on the use of primarily VHL and Cereblon ligands. However, more than 600 E3 ligases are known to function in human cells, suggesting a largely untapped pool of E3 ligases that are potentially hijackable for targeted protein degradation. To expand the scope of the PROTAC modality, we embarked on developing of the novel E3 ligase ligands (LiLi) platform. Here, we give an overview of the platform's components and outline the processes that led to identifying of novel PROTAC handles for unprecedented E3 ligases.

Target selection: E3 ligase databsae

To aid identification of E3 ligases with desirable biological properties, we assembled an SQL database that combines:

- the expression profiles of ubiquitination-related proteins across tissues and cell types
- the results generated in internal global proteome profiling experiments (399 ligases determined among 10325 proteins identified in-house)





*Protein expression score based on immunohistochemical data

Main advantages of the E3 database:

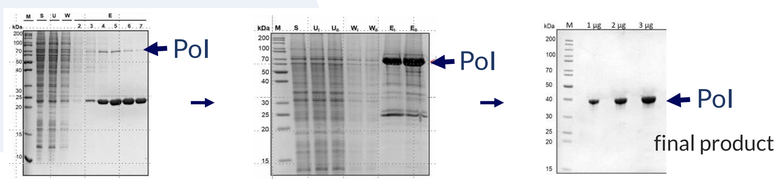
- prioritization of ligases based on their expression profiles:
  - ligases expressed selectively in the tissue of interest
  - to select optimal ligases for particular PoI
- open-end structure of the database easily updated with freshly generated data

Production of recombinant E3 proteins

We have strong protein production and engineering capability and can generate structural-biology grade reagents in-house. Production of recombinant proteins is carried out in bacteria or insect cells. So far, we managed to obtain high-homogeneity and high-purity samples of ~30 E3 ligases.

Recombinant production of E3 sequences may be problematic, as they typically engage in transient interactions with other proteins. Iterative construct optimization is often necessary to improve the stability or expression yields. In our hands, the introduction of peptide linkers and fusion proteins has been particularly useful.

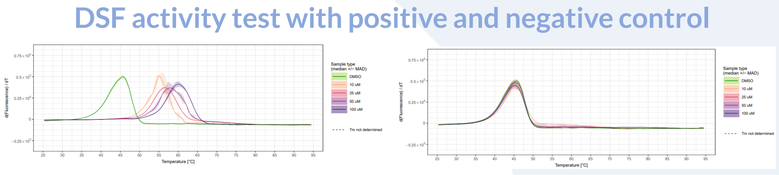
Iterative optimization of protein production protocol



QC and activity data of every protein sample are stored in the CDD Vault repository.

CDD VAULT® Complexity Simplified

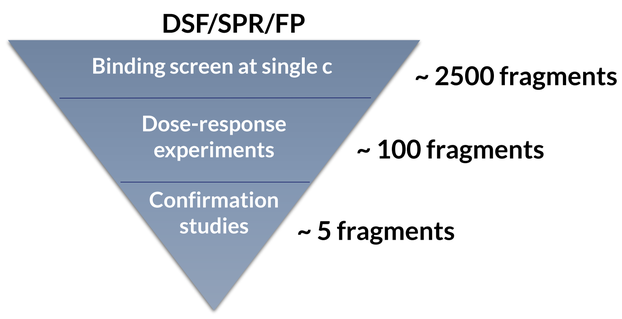
DSF activity test with positive and negative control

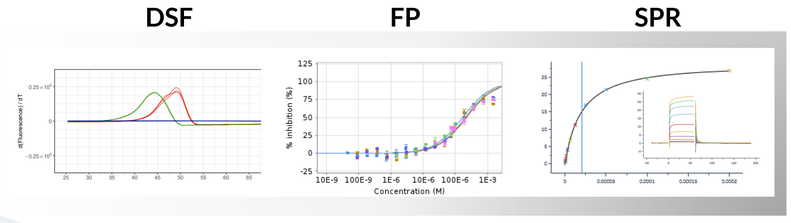


Compound	Positive control				Negative control			
Concentration [µM]	10	25	50	100	10	25	50	100
ΔTm [°C]	9.1	11.1	12.9	14.4	-0.1	-0.2	-0.2	-0.4

Ligandability assesment

Early assessment of the chemical tractability of the E3 protein is achieved by means of fragment screening. We utilize a collection of 2500 Ro3-compliant fragments. Binding is assessed using biophysical methods like DSF, SPR, or FP competition assays.



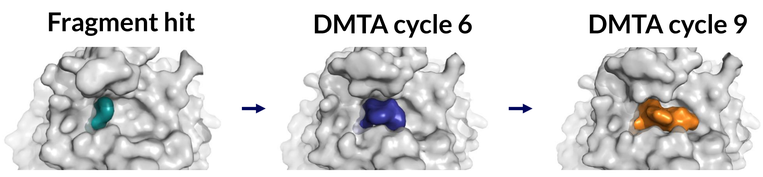


Ligases that yield robust fragment hits are progressed further into crystallization trials.

Structural biology

We managed to develop X-ray crystallography systems for 8 ligases. Knowledge of fragments binding poses facilitates structure-based drug discovery and enables progress of the hit-to-lead phase for some of the E3 targets, e.g., for ligase A we went from 50 uM initial hit to a 20 nM lead compound in 580 compounds, for ligase B we reached sub-µM affinity in 230 compounds, and for ligase C in 10 compounds.

Stucture guided hit-to-lead process for ligase B



DSF ΔTm= 3.8 deg
FP pKi= 4.2
SPR pKD= 4.4

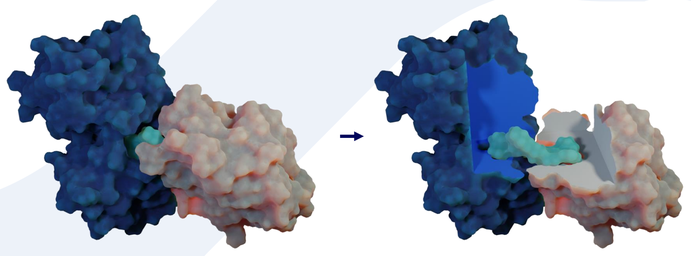
DSF ΔTm= 10.4 deg
FP pKi= 6.4
SPR pKD= 6.8

DSF ΔTm= 13.0 deg
FP pKi= 7.6
SPR pKD= 7.9

We have generated >100 X-ray structures of E3 ligase components with small molecule ligands. As soon as compounds with sufficient affinity are identified, we determine the positions of exit vectors.

Discovery of bifunctional degraders

Once E3 ligands with appropriate exit vectors are identified, we synthesize first bifunctional degraders against model proteins. Design of model bifunctional molecules is based on molecular modeling (e.g., degraders modeling protocol of MOE).



We employ two orthogonal approaches:

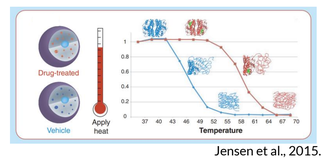
- Kinase degrader achieved by coupling E3 ligand with promiscuous kinase inhibitor
- Halo-tag directed molecule obtained by attaching a chloroalkane moiety to E3 warhead.

In vitro binding assays are used to confirm the formation of ternary complexes such as HTRF, AlphaLisa, and SPR.

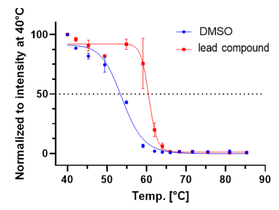
Cellular assays

Examples of cellular assays employed by us:

- confirmation of E3 ligase engagement by small molecules with intracellular thermal stabilization by ligands, or bioluminescence resonance energy transfer



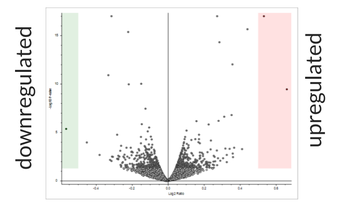
Jensen et al., 2015.

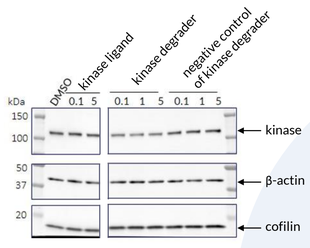


Intracellular stabilization assay: ΔTm=6.74 deg; DSF ΔTm=7.72 deg

- assessment of E3 ligase inhibition effects: global proteomics of cells treated with E3 ligand

SNU-182 liver cancer cell line





- degradation of model substrates: Western-blot based assays, or global proteome measurements

Conclusions

- We have established a drug discovery pipeline to generate novel E3 ligase binders and transform these into bifunctional degraders
- We found small molecule binders for 3 E3 ligase targets
- E3s explored by us are highly attractive in „protacability" assesments that appeared in literature recently
- An overview of chemical properties of E3s ligands is shown below:

	Ligase A ligand	Ligase B ligand	Ligase C ligand
MW	440	430	201
LogP	4.1	1.6	1.0
TPSA	72.6	101.2	68
HAC	32	30	15
ΔTm	7.7	13.8	14.5
pKd	**7.6**	**7.9**	**6.3**
Solubility (uM)	94	>100	-
Chemical stability $t_{1/2}$ (min) (pH 1.6 ; 7.4)	> 120 > 120	-	-
Plasma stability $t_{1/2}$ (min)	> 120	-	-
PAMPA (10^{-6} cm/s)	39.5	5.59	-
CACO-2 (10^{-6} cm/s)	7.3	4.2	-

- Currently we are exploring degradation of model proteins by first generation bifunctional degraders

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