Engineered Small Molecule Binding Domains for Pharmacologically Responsive Degradation and Proximity Based Therapeutics

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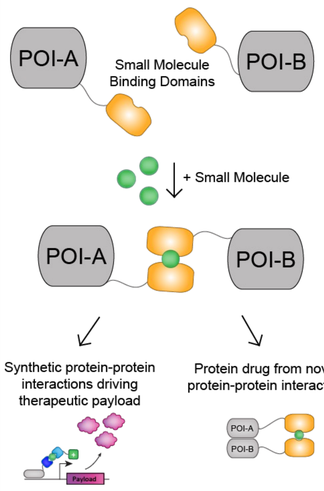
Keystone
Proximity Based Therapeutics
Abstract #1513

Expanding Therapeutic Modalities of Proximity-Inducing Agents

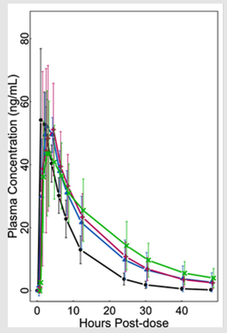
Therapeutic Applications and Design of Transcriptional Switches Regulated by FDA Approved Small Molecules



Application of SM-regulated proximity-based protein interactions. Many SM-sensor platforms have been developed, but few are suitable as a platform for proximity-based or degradation-based therapeutics. Here, we utilize IMiD Co-binders fused to Proteins of Interest (POIs) to create a SM regulated proximity-based transcriptional switch and separately, a degradation-based switch for regulation at the protein level

Available SM-based switches	FDA-approved & Convenient mode of delivery	Beneficial pharmacokinetics	Crosses Blood-Brain-Barrier (BBB)	
Grazoprevir	✓	✗	✗	*Tague, E, et al. Nat Methods 2018*
Rimiducid (rapamycin rapalogs)	✓	✓	✗	*Rivera VM, et al. Nat Med 1996*
Caffeine	▬	✗	✓	*Bojar, D, et al. Nat Commun 2018*
★ IMiD	✓	✓	✓	*Ebert, B, et al. Sci Trans Med 2021*

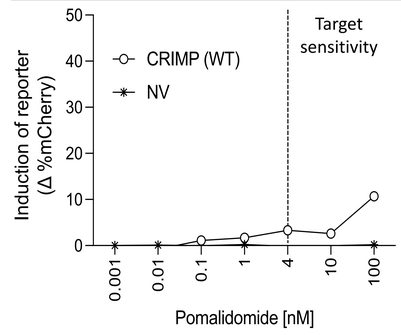
Characteristics of existing small molecule regulated switches. Few SM-sensor platforms have been developed that respond to FDA approved SMs and have beneficial pharmacokinetics for feasible therapeutic application. IMiDs were focused on for further development because of their unique ability to cross the BBB in addition to their ability to induce degradation of IKZF3/1 class of proteins.



PK profile of pomalidomide in human plasma (healthy liver (black), other colors show varying levels of hepatic impairment) (PMID: 29746728)

Based on the PK profile of pomalidomide in human plasma, concentrations peak within 6 hours of oral dosing at ~220 nM and then quickly declines to ~12 nM within 24 hours of initial does (PMID: 29746728).
In rats, it was observed that 39% of pomalidomide crosses the BBB (PMID: 23940785).
Based on these two studies, we calculated our target concentration to be 39% of 12 nM to guarantee switch function with the available amounts of pomalidomide in the brain and serum at any time as the drug is metabolized
Target concentration: 4 nM

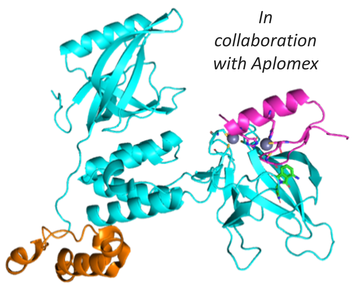
Performance of existing SM-regulated transcriptional switch



Switch responsive to 100 nM of pomalidomide, requires improvement

A transcriptional switch responsive to IMiD was built by fusing a modified version of CRBN (del.CRBN), with the DDB1 domain removed to prevent its association with E3 ubiquitin ligase complex, to a ZF DNA binding domain and co-expressing it with a second fusion of CRIMP, an IMiD co-binder derived from IKZF3 protein, to an activation domain. Switch performance was evaluated using an mCherry reporter assay (described below)

Improving Sensitivity of the Small-Molecule Binding Domains



In collaboration with Aplomex

CRBN in complex with CRIMP and pomalidomide.
DDB1 subdomain (orange) is removed to prevent CRBN (teal) from complexing with E3 Ub ligase complex. CRIMP domain (pink) complexes with CRBN only in the presence of pomalidomide (green small molecule).

Engineering an improved…

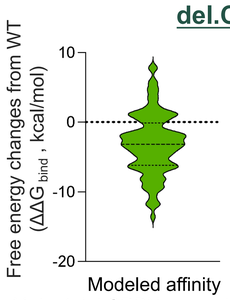
CRIMP domain

Flow-seq performed to identify candidate CRIMP mutations with improved sensitivity to pomalidomide

Library of single CRIMP mutations
↓
Sequential sorting for library members responsive to decreasing concentrations of pomalidomide
↓
Identification of CRIMP mutants enriched in sorted pools

Top enriched CRIMP mutants were selected for further validation in vitro

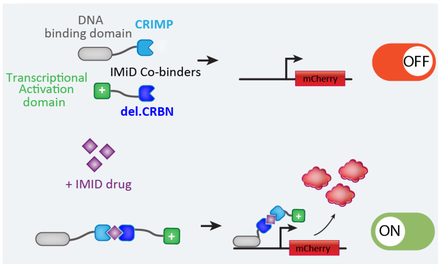
del.CRBN domain



Modeled binding affinity of del.CRBN variants, with single residue mutations, to pomalidomide.
Computational modeling of del.CRBN variants in complex with pomalidomide was performed in collaboration with Aplomex.

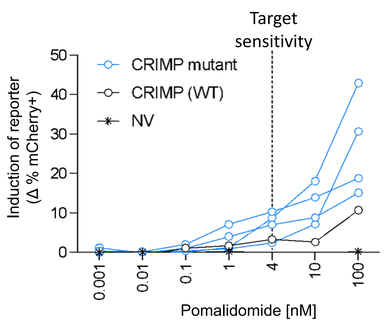
Mutant del.CRBN - pomalidomide
$\Delta\Delta G_{bind} = \Delta G_{bind}(mutant) - \Delta G_{bind}(WT)$

Variants predicted to increase affinity were further validated in vitro

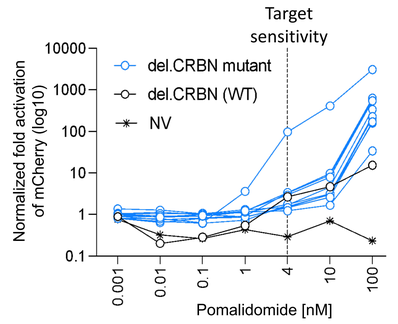


Reporter system for *in vitro* validation of selected variant IMiD co-binders. The CRIMP and del.CRBN variants were each fused to one half of a split synthetic transcription factor (SynTF). In the absence of pomalidomide, the split synTF remains non-functional and there is no expression of the mCherry reporter. Addition of pomalidomide allows the IMiD co-binders to dimerize, resulting in a functional synTF that induces mCherry expression. The variants were evaluated for responsiveness to drug in this system at a range of pomalidomide concentrations.

CRIMP domain

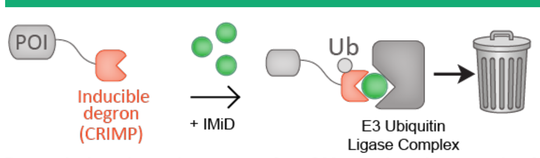


del.CRBN domain



Lead del.CRBN mutants and CRIMP mutants demonstrated activity below target sensitivity of 4 nM pomalidomide. *In vitro* validation of selected candidates from the CRIMP and del.CRBN library identified several variants with improved sensitivity to pomalidomide compared to their wildtype counterparts.

Degradation-based Switches Regulated by FDA Approved Small Molecules

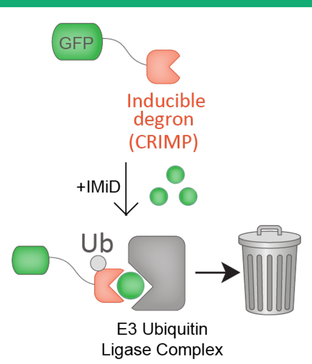


Degradation based system for SM-regulated control of protein levels. The CRIMP domain can be fused to any protein of interest (POI) and upon addition of IMiDs such as pomalidomide, it will be recruited to the E3 Ubiquitin Ligase Complex via binding to the Cereblon domain and Ubiquitinated for degradation.

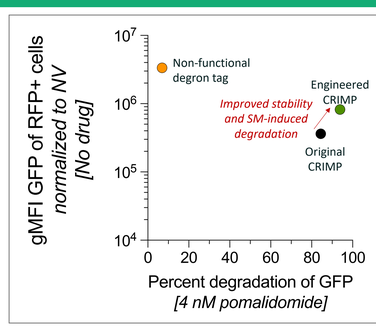
Flow-seq performed to identify candidate CRIMP mutations that degrade at greater sensitivity to pomalidomide

Library of single CRIMP mutations
↓
Sequential sorting for library members that degrade at decreasing concentrations of pomalidomide
↓
Identification of CRIMP mutants enriched in sorted pools

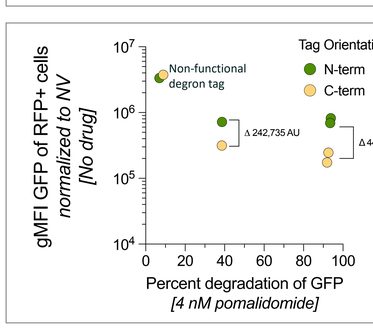
Top enriched CRIMP mutants were selected for in vitro validation in degradation assay



Degradation system for *in vitro* validation of selected CRIMP variants. The CRIMP mutants were fused to GFP upstream of 2A-mCherry (CRIMP-GFP-2A-mCherry). These constructs were stably expressed in a model cell line via viral transduction. The mCherry enables gating on transduced cells while GFP will be used to quantify degradation in the presence of pomalidomide. The CRIMP mutants were evaluated in this system at physiologically relevant concentration of 4 nM pomalidomide.



Engineered CRIMP demonstrates improved performance at target drug concentration. Original CRIMP degron achieved ~80% degradation of GFP when induced by 4 nM pomalidomide. The tag also destabilized GFP compared to the non-functional control (orange). One of the engineered CRIMP mutant demonstrates improved stability and degradation of tagged GFP.



N-terminal tagging of GFP results in overall higher stability of POI. Several engineered CRIMP variants were tagged either the N or C-terminal end of GFP and evaluated for stability and degradation in +/- 4 nM pomalidomide. Tag orientation does not result in change of drug inducible degradation of GFP.

Conclusions & Next Steps

Conclusions

- Optimization IMiD-responsive binding domains by computational design and high throughput screening yielded transcriptional switches that induce payload expression *in vitro* at SM concentrations expected in the serum and brain of patients following an FDA-approved dosing regimen.
- Engineered CRIMP domain demonstrates improved tagged protein stability while maintaining IMiD-induced degradation.

Next Steps

- Combine CRIMP and del.CRBN mutants to increase responsiveness of the IMiD-regulated transcriptional switch.
- Generate combinatorial library from lead del.CRBN and CRIMP variants for improved sensitivity to pomalidomide.
- Identify CRIMP variants that do not degrade in the presence of pomalidomide to further enhance transcriptional switch performance.
- Generate combinatorial library from lead single CRIMP mutations and screen for CRIMP variants that further improve degradation.

Acknowledgments:

This work was done in collaboration with BlueRock Therapeutics and on behalf of Senti Biosciences