# Small-molecule regulated safety switch for improved safety of CAR cell therapies in the brain

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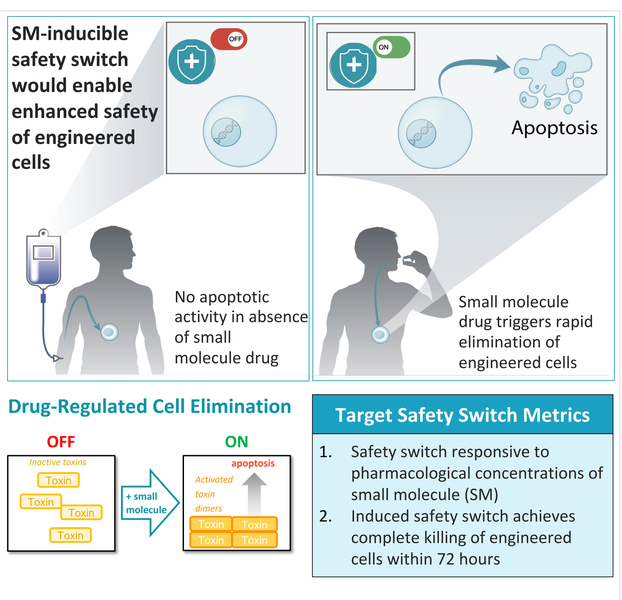
Abstract #24

## Initial Design of Safety Switches Regulated by FDA Approved Small Molecules

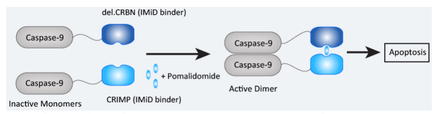



**Target Safety Switch Metrics**

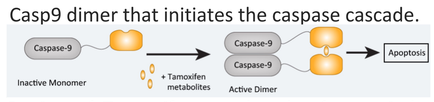
1. Safety switch responsive to pharmacological concentrations of small molecule (SM)
2. Induced safety switch achieves complete killing of engineered cells within 72 hours

| Available SM-based switches | FDA-approved & Convenient mode of delivery | Beneficial pharmacokinetics | Crosses Blood-Brain-Barrier (BBB) (BlueRock Requested) | |
|---|---|---|---|---|
| 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* |
| ★ Tamoxifen | ✓ | ✓ | ✓ | *Gallinari, P, et al. Chem Biol 2005* |

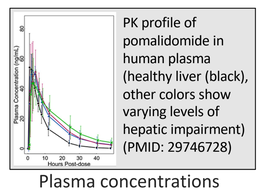
**Characteristics of existing small molecule regulated switches.** Many SM-sensor platforms have been developed, but few are suitable for a universal suicide switch. Tamoxifen and IMiDs were focused on for further development because of their unique ability to cross the BBB, a requirement of our BlueRock collaborators.




**Design of IMiD regulated safety switch.** Inactive monomers of Caspase-9 (Casp9) are fused to IMiD SM binding domains: CRIMP domain and a modified Cereblon domain (del.CRBN – prevents complexing with the E3 ubiquitin ligase complex). Apoptosis is induced in the presence of IMiDs via heterodimerization of the IMiD co-binders and formation of active Casp9 dimer that initiates the caspase cascade.



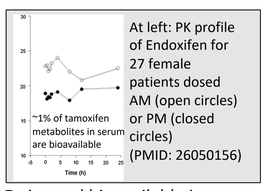

**Design of Tamoxifen regulated safety switch.** Inactive monomers of Caspase-9 (Casp9) are fused to ERT2, a modified ER1α that is insensitive to estradiol but maintains affinity to tamoxifen metabolites. Apoptosis is induced in the presence of tamoxifen metabolites via homodimerization of ERT2 resulting in the formation of an active Casp9 dimer.




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

Plasma concentrations estimated ~220 nM - ~12 nM; in rats, 39% of pomalidomide crosses the BBB (PMID: 23940785).

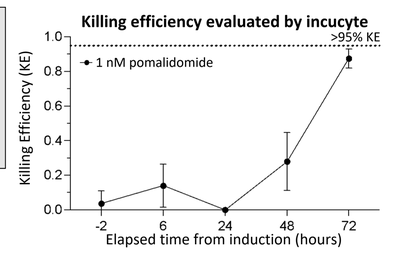
**Target concentration: 4 nM**



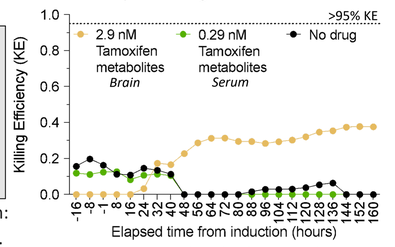

At left: PK profile of Endoxifen for 27 female patients dosed AM (open circles) or PM (closed circles) (PMID: 26050156)

Estimated bioavailable in serum: 0.22-0.29 nM free metabolites. Tamoxifen metabolites are 10x concentrated in tissue, organs

**Target concentration: 2.9 nM**




Existing IMiD regulated safety switch does not achieve complete killing within 72 hours of induction



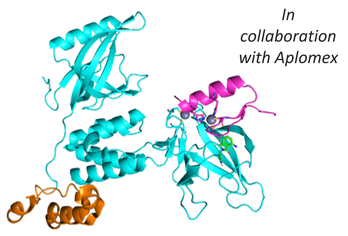

Existing Tam regulated safety switch does not achieve complete killing within 72 hours of induction

***Highlights need for improved SM-regulated safety switches***

## Improving Sensitivity of Small-Molecule Binding Domain

**Safety Switches Responsive to Therapeutic Concentrations of SM in HEK293T Cells**

### IMiD regulated safety switch



*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

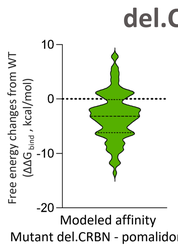
**Flow-seq performed to identify candidate CRIMP mutations with improved sensitivity to pomalidomide**

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

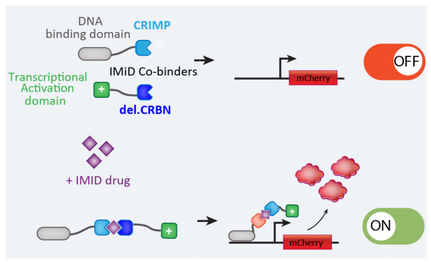



**Modeled binding affinity of del.CRBN variants, with single residue mutations, to pomalidomide.** Computational modeling of del.CRBN variants in complex with pomalidomide was performed in collaboration with Aplomex.

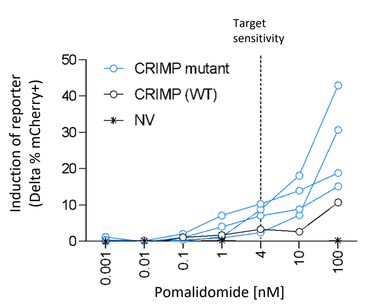
$\Delta\Delta G_{bind} = \Delta G_{bind(mutant)} - \Delta G_{bind(WT)}$

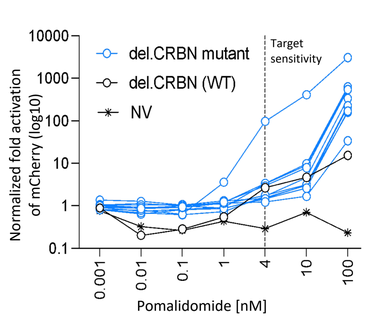
*Variants predicted to increase affinity were further validated* in vitro

### *In vitro* validation




**Reporter system for *in vitro* validation of selected variant IMiD co-binders.** The CRIMP and del.CRBN variants were fused to one half of a split synthetic transcription factor (SynTF). In the absence of pomalidomide, the synTF remained split and resulted in 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 in this system at a range of pomalidomide concentrations.



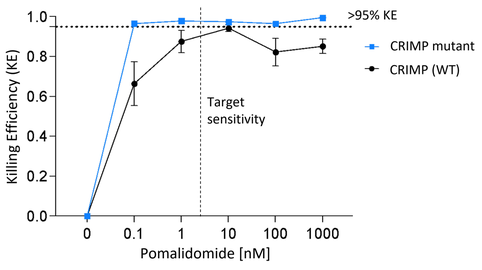




**Lead del.CRBN mutants and CRIMP mutants demonstrated activity below target sensitivity of 4 nM pomalidomide.** *In vitro* validation of selected candidates from the CRIMP and del.CRBN library identified several variants with improved sensitivity to pomalidomide compared to their wildtype counterparts.

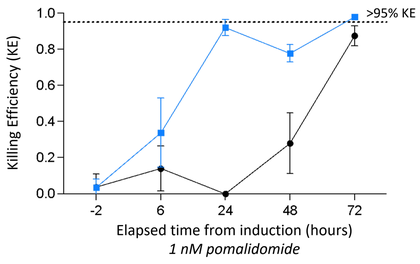
Lead CRIMP mutation was integrated into IMiD-responsive safety switch for evaluation

Combinatorial variants of the lead del.CRBN mutations were created for further testing *in vitro*

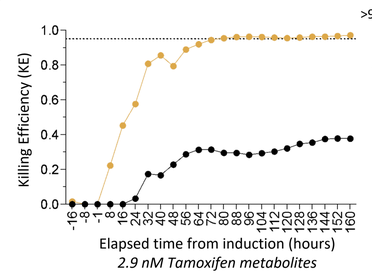
**Engineered switch has enhanced sensitivity to pomalidomide**




**Engineered switch has improved killing speed**



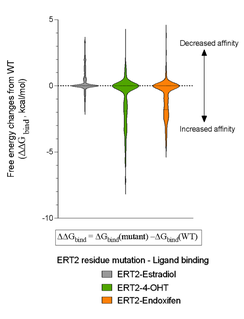

**Engineered switch has enhanced killing speed and is responsive to target concentration of tamoxifen metabolites in the brain**




**Engineered SM binding domains improve the function of SM-regulated safety switches in HEK293T cells.** Engineered switches are responsive to pharmacological concentrations of SM estimated in the brain and can achieve complete killing within 72 hours of induction.

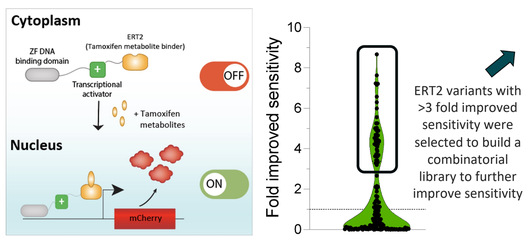
### Tamoxifen regulated safety switch

#### Computational ID of mutations




**Modeled binding affinity of ERT2 variants with single residue mutations to tamoxifen metabolites.** The predicted impacts of mutations to ERT2 domain on the binding affinity were calculated in relation to the binding affinity of WT ERT2 to the specified Tamoxifen metabolites in collaboration with Aplomex.

#### *In vitro* validation




ERT2 variants with >3 fold improved sensitivity were selected to build a combinatorial library to further improve sensitivity

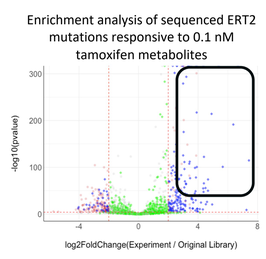
**Reporter system for *in vitro* validation of ERT2 variants.** Model cell line was co-transduced with an mCherry reporter construct and a tamoxifen-responsive SynTF. In the absence of drug, the SynTF is sequestered in the cytoplasm via HSP90 binding to ERT2. In the presence of tamoxifen metabolites, ERT2 domain will release from HSP90 and the synTF will translocate to the nuclease where it can bind upstream of a minimal promoter via ZF DBD and initiate the expression of mCherry.

#### Library construction and screen

Combinatorial Library of selected ERT2 mutations

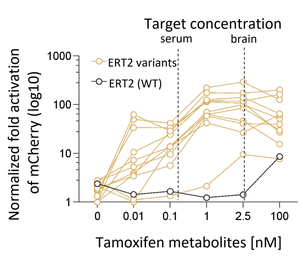
Sequential sorting for library members with sensitivity to decreasing concentrations of tamoxifen metabolites using reporter cell line

Identification of ERT2 mutants in sorted pools utilizing PacBio Long-read sequencing




**Flow-seq of combinatorial library identifies ~250 ERT2 variants enriched in population responsive to 0.1 nM tamoxifen metabolites.** Combinatorial library was screened using the mCherry reporter system and sorted for members that had sensitivity to decreasing concentrations of tamoxifen metabolites.

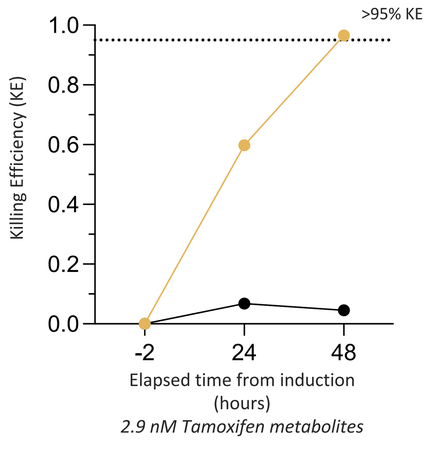
#### *In vitro* validation




**ERT2 variants have improved sensitivity to Tamoxifen metabolites.** Variants were evaluated *in vitro* using the previously described mCherry based reporter system.

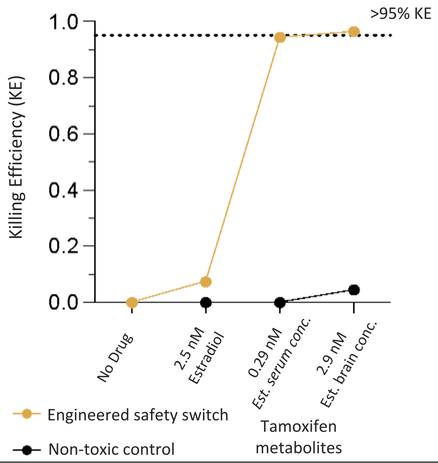
## Tamoxifen Safety Switch Successfully Induces Death in Primary T Cells

Engineered switch killing efficiency over time




**Engineered safety switch achieves complete killing within 48 hours of induction.** Primary T cells transduced with engineered safety switch or non-toxic control were induced with indicated drug condition for 48 hours. Engineered switch achieves complete killing at estimated concentrations of tamoxifen metabolites in both the brain and serum while maintaining insensitivity to high levels of estradiol.

Killing efficiency at 48 hours of induction




**Engineered safety switch achieves complete killing of primary T Cells at pharmacologically relevant concentrations of tamoxifen metabolites estimated in the brain and serum**. The engineered switch was also tested for maintained insensitivity to estradiol. Estradiol concentrations fluctuate throughout the 28 day menstrual cycle, peaking between 1.3 nM and 2.2 nM (Sluss, et al., Clinica Chimica Avct, 2008; Verdonk, SS. Et al., Clinica Chimica At. 2019). We evaluated our switch at a supraphysiological concentration of 2.5 nM.

## Conclusions & Next Steps

### Conclusions

- Optimization of tamoxifen-responsive and IMiD-responsive binding domains by computational design and high throughput screening yielded safety switches that trigger cell death *in vitro* at SM concentrations expected in the serum and brain of patients following an FDA-approved dosing regiment
- Tamoxifen-regulated safety switch achieves complete killing at pharmacologically relevant concentrations of tamoxifen metabolites in primary T cells – enabling potential use in cell therapies for enhanced safety.

### Next Steps

- Combine CRIMP and del.CRBN variants to increase speed of killing of IMiD-regulated safety switch
- *in vivo* testing