Integrated platform for rapid PROTACs discovery: Case Study Targeting BRD4

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Introduction

Proteolysis-targeting chimeras (PROTACs) are heterobifunctional molecules designed to bring the protein of interest into close proximity with E3 ubiquitin ligase, facilitating its selective ubiquitination and proteasomal degradation. This approach is a promising therapeutic strategy to target proteins that are traditionally considered "undruggable" by conventional small-molecule inhibitors.

Although the technology has a bright future in drug development, it also has many challenges due to the complex molecular structures and properties. Thus, multidisciplinary approaches need to be implemented for both the rational design of PROTAC molecules and the evaluation of their biological effects.

Aim

The aim was to establish an integrated platform by combining virtual screening (2D QSAR, machine learning (ML) and protein-protein interaction (PPI) docking), synthetic chemistry and *in vitro* screening (TR-FRET, SPR) expertise for novel PROTACs discovery.

Research Background

This collaborative project builds on earlier work at Enamine that led to the discovery and publication of novel BRD4 ligands. Further evaluation identified two potential BRD4 binders suitable for PROTACs development. For this purpose, known CRBN binders – Thalidomide, Lenalidomide, and compound 164 – were selected as the E3 ligase-recruiting components of the PROTACs.

Chemspace carried out an *in silico* study combining 2D QSAR, machine learning (ML), and protein-protein interaction (PPI) docking. This approach identified 182 potential PROTAC candidates from a virtual library of 1,742 molecules, derived from three CRBN and two BRD4 binders, featuring diverse linker variations in length (from 3 to 18 atoms), composition (CARBA-, PEG-, and mixed chains), and attachment point geometry.

Of these, 135 PROTACs were successfully synthesized by Enamine using commercially available CRBN Ligand-Linkers Conjugate Kits from Enamine Protein Degradation Toolbox. These synthesized compounds were then evaluated *in vitro* at Enamine, which is the focus of the current work.

Methods

In vitro evaluation was divided into four stages to confirm the ability of these compounds to form CRBN:PROTAC:BRD4 complexes and induce proteasomal degradation, with following assays used each stage:

Stage 1. Time-resolved fluorescence energy transfer (TR-FRET) to assess binary complex formation with CRBN,
Stage 2. Surface Plasmon Resonance (SPR) to evaluate binary complex formation with BRD4,
Stage 3. TR-FRET for ternary complex formation (TCF) assessment,
Stage 4. Cell-based TR-FRET to measure BRD4 degradation.

In Stages 1-3 FLAG-CRBN/DDB1 or/and Biotin-Avi-BRD4(BD1) were used to assess the formation of complexes of interest. Stage 4 was performed using HEK293T cell line.

Results

Identification of 2 novel BRD4 binders based on previously published results[3] and selection of CRBN ligands for PROTACs development

Identification of 182 perspective PROTACs via *in silico* study by Chemspace (2 weeks)

Synthesis of 135 PROTAC molecules by Enamine (7 weeks)

In vitro evaluation

Stage 1. (1 week) **Binary complex formation with CRBN** TR-FRET assay

129 PROTACs

Stage 2. (1 week) **Binary complex formation with BRD4** SPR assay

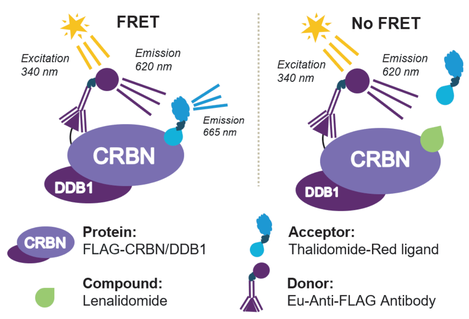
56 PROTACs

Stage 3. (1 week) **Ternary complex formation** TR-FRET assay

47 PROTACs

Stage 4. (1 week) **Protein degradation** Cell-based TR-FRET BRD4 degradation assay

3 PROTACs



Out of the 135 PROTACs tested at a single concentration (11 μM), 129 exhibited displacement greater than 80% under the tested conditions, confirming the formation of CRBN-PROTAC complexes (Figure 1).

Consequently, these 129 compounds were selected for further assessment in Stage 2.

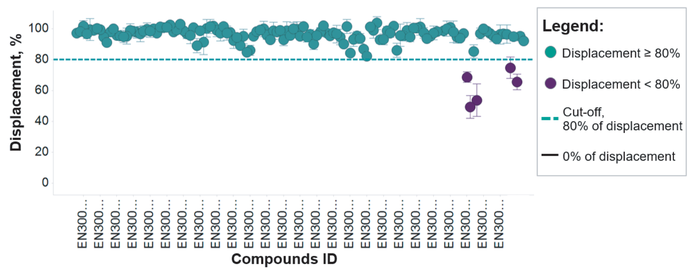
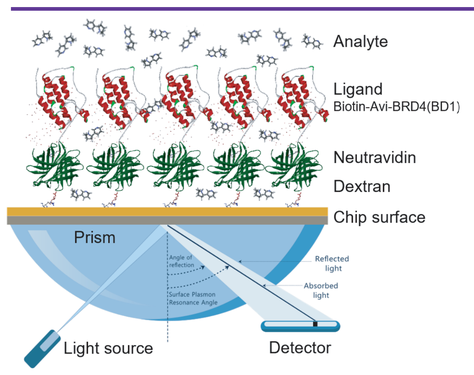


Figure 1. Displacement Screen for 135 PROTACs in TR-FRET assay.



Screening of 129 PROTACs at a single concentration (50 μM) identified 56 promising candidates that exhibited binding activity greater than 95% of the response of the reference ligand JQ-1 at a saturated concentration. These compounds also demonstrated good binding behavior, such as absence of non-specific binding to the reference flow cell and no stickiness (Figure 2).

Identified 56 compounds were selected for further assessment in Stage 3.

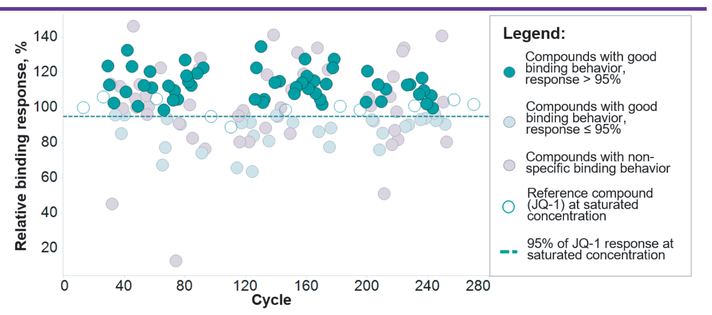
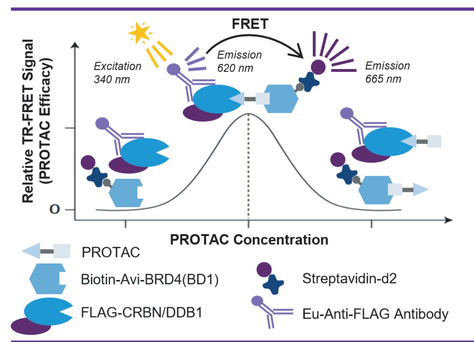


Figure 2. Binding Screen for 129 PROTACs in SPR assay. The relative binding response is presented after blank subtraction, molecular weight adjustment, and normalization to the JQ-1 response at saturated concentration.



To assess TCF for the 56 selected PROTACs, they were tested at 12 concentrations using a 3-fold dilution series starting from 30 μM. TCF was initiated by 47 PROTACs, with 17 exhibiting ECmax values below 500 nM (Figure 3).

All 47 PROTACs with confirmed ternary complex formation were selected for further assessment in Stage 4.

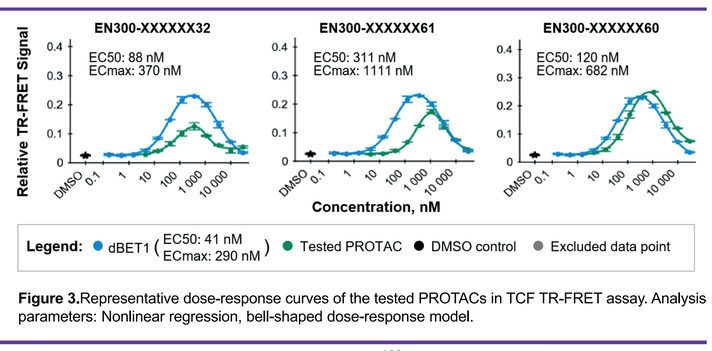
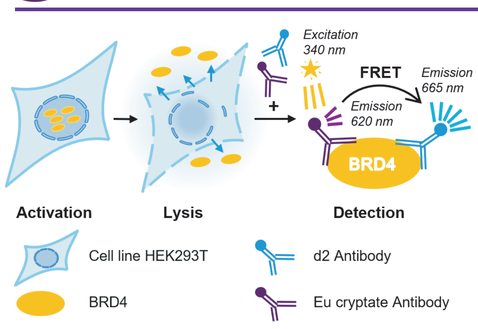


Figure 3. Representative dose-response curves of the tested PROTACs in TCF TR-FRET assay. Analysis parameters: Nonlinear regression, bell-shaped dose-response model.



Screening of 47 PROTACs at a single concentration (30 μM) showed degradation activity by three PROTACs, with BRD4 degradation levels of 80%, 53%, and 23% under tested conditions (Figure 4A).

These three compounds were further assessed in a dose-response format (3-fold dilution series starting from 30 μM, 10 points) where DC50 value was determined for one PROTAC (775 nM) (Figure 4B).

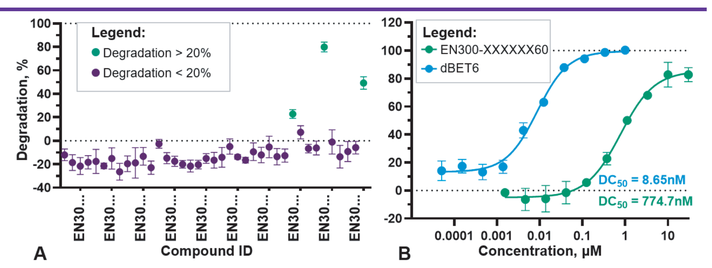


Figure 4. A. Degradation screen of 47 PROTACs at a single concentration in cell-based BRD4 degradation TR-FRET assay. B. Representative DRC of the tested PROTAC (EN300-XXXXXX60) in cell-based BRD4 degradation TR-FRET assay. Analysis parameters: Nonlinear regression, Sigmoidal 4PL model.

Conclusions

Three CRBN-recruiting BRD4-targeting PROTAC candidates were identified and characterized within 3 months, demonstrating the efficiency of the integrated approach and Emamine resources in accelerating the PROTAC discovery.

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References

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