Accelerating PROTAC Drug Discovery: Relationship Between Ubiquitination and Degradation of Target Proteins
TANDEM UBIQUITIN BINDING ENTITIES (TUBEs)

- Isolation of poly-ubiquitinated substrates from cell lysates
- Superior to antibodies, detection by Western blot
- E3 ligase and DUB assays
- In situ detection with fluorescence
- Ubiquitin mass spec proteomics bypassing SILAC
HTS\textit{-in vitro} PROTAC Screening Platform
Efficient Large-Scale Screening Tool
HTS-\textit{in vitro} PROTAC Screening Platform

- How to differentiate multiple PROTAC variants in a HT fashion
- Rapid ubiquitination kinetics and dose response of \textit{native} targets
- Guiding Med Chem to establish rapid SAR

\textit{“Ub_{Max}” A better way to measure potency of PROTACs}
CRBN based Bromodomain PROTAC®

Effect of Linker Length on ubiquitination of BET proteins

CRBN-BRD4 dBET1 PROTAC
- Excess BRD4 inhibitor JQ1 + CRBN-BRD4 PROTAC

Ub$_{\text{Max}}$: 0.3 µM

CRBN-BRD4 dBET6 PROTAC
- Excess BRD4 inhibitor JQ1 + CRBN-BRD4 PROTAC

Ub$_{\text{Max}}$: 0.03 µM

CRBN-based Bromodomain PROTAC®

Effect of Linker Length on ubiquitination of BET proteins

CRBN-BRD4 dBET1 PROTAC – DC$_{50}$: 1.0 µM

CRBN-BRD4 dBET6 PROTAC
DC$_{50}$: 0.03 – 0.1 µM

LifeSensors www.lifesensors.com
## Summary – *In vitro* vs Cellular assays

<table>
<thead>
<tr>
<th>PROTAC</th>
<th>In vitro vs Cellular degradation</th>
<th>In vitro vs Cellular degradation</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Ub\textsubscript{Max} (<em>In vitro</em>), µM</td>
<td>DC\textsubscript{50} (Cell), µM</td>
</tr>
<tr>
<td>Multi-Kinase PROTAC</td>
<td>0.1-0.3</td>
<td>N/A</td>
</tr>
<tr>
<td>dBET1</td>
<td>0.3</td>
<td>1.0</td>
</tr>
<tr>
<td>dBET6</td>
<td>0.03</td>
<td>0.03</td>
</tr>
<tr>
<td>BETd-24-6</td>
<td>0.03</td>
<td>0.1</td>
</tr>
</tbody>
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**Table.** Comparison and correlation of Ub\textsubscript{Max} and Hook effect from *In vitro* assays to cellular degradation assays.
VHL based Bromodomain PROTAC®

HTS In vitro Screening with BET proteins

<table>
<thead>
<tr>
<th>PROTAC</th>
<th>In vitro Ubiquitination vs Binding Affinity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ub_{Max} (In vitro), μM</td>
</tr>
<tr>
<td>AT1</td>
<td>0.1-0.03 (3-fold Peak)</td>
</tr>
<tr>
<td>MZP54</td>
<td>0.03 (10-fold Peak)</td>
</tr>
<tr>
<td>NE987</td>
<td>0.03 (8-fold Peak)</td>
</tr>
</tbody>
</table>
**PROTAC® mediated ubiquitination of KRAS G12C**

**HTS In vitro Screening**

**Figure A**
- VHL G12C KRAS LC2
- CREN G12C KRAS D1
- VHL G12C KRAS LC2 - E1

**Figure B**
- VHL WT KRAS LC2
- VHL G12D KRAS LC2

**KRAS in vitro ubiquitination assays:** recombinant G12C KRAS ubiquitination was monitored as function of cereblon/VHL PROTAC dose response (A) comparison of KRAS G12C ubiquitination using VHL and Cereblon PROTACs. (B) KRAS selectivity assays for G12D and WT using G12C VHL PROTACs.
**PROTAC® mediated ubiquitination of KRAS G12C**

**Gel based Assay**

**KRAS in vitro ubiquitination assays:** Western blot based KRAS ubiquitination-validation to demonstrate PROTAC mediated ubiquitination.
Pathway to PROTAC Drug Discovery

**IN VITRO ASSAY DEVELOPMENT**
- E3 ligase and substrate purification

**EC50 & RANK ORDER POTENCY**
- Identify high value PROTACs for cell-based assays

**CELL-BASED ASSAY DEVELOPMENT**
- Substrate ubiquitylation and degradation

**MASS SPEC & PROTAC SELECTIVITY**
- TUBE-based to establish total ubiquitome changes

**ASSAY VALIDATION & COMPOUND SCREENING**
- HTS to monitor substrate ubiquitylation

**COUNTER SCREENS & VALIDATION**
- Western blotting to establish substrate ubiquitylation

**VALIDATION & ESTABLISH DC50**
- Validate ubiquitylation via WBs and Ubilfet

**METHOD TRANSFER**
- Transfer technology for further validation studies