H2020-MSCA-ITN-2020

Allostery in Drug Discovery

Allosteric Ligand Identification and Optimization – a covalent approach

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Abstract: This workflow outlines a comprehensive and adaptable strategy for the discovery and optimization of allosteric ligands, with a focus on covalent modulators targeting reactive residues such as cysteines and lysines. Integrating computational modeling with experimental validation, the approach supports systematic ligand development across diverse protein targets. The process begins with the assembly and assessment of a covalent fragment library, followed by experimental site mapping through mass spectrometry and structural modeling. Computational techniques—including docking, molecular dynamics, and water network analysis—guide the identification of interaction hotspots and inform warhead placement. Functional biochemical assays validate allosteric inhibition, while iterative design and synthesis refine ligand efficacy. Mass spectrometry confirms binding specificity and stoichiometry, feeding back into structure-activity relationship (SAR) optimization. Developed within the ALLODD network, this workflow has been applied to targets such as SHP2, FBW7, and HDAC8, demonstrating its general utility in covalent allosteric drug discovery.

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Allosteric Ligand Identification and Optimization – a covalent approach

Generalizable Workflow based on the contribution: Development of an Integrated Platform for Allosteric Modulator Detection and Optimization (ESR7 / HUN-REN RCNS)

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1. Overview

This workflow presents a generalizable strategy for the identification and optimization of allosteric ligands with an emphasis on covalent modulators. It integrates computational modelling with experimental methodologies and is applicable to diverse protein targets. This strategy reflects the approaches developed within the ALLODD network, with applications demonstrated for SHP2, FBW7, and HDAC8.

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2. Workflow Summary

Step 0: Library compilation, stability, and biological feasibility of selected warheads

- Compilation of a library of covalent fragments supposed to engage proteins with targets:
    - Same-*core fragments:* Offer insight into covalent warhead reactivity and selectivity. Usually targeting Cys or Lys with a diverse set of warheads that can also be tailored to the reactivity of the amino acid.
    - Different-*core fragments:* Aid in discovering cryptic or alternative binding pockets and assist in final drug development, but may be more challenging to interpret. Usually, photoaffinity probes where the fragment core is diverse and the reactive tag is the same.
- Evaluate selected warheads for stability in the buffer to be used for the targeted protein.
- Evaluate the warhead reactivity and stability, and feasibility within a biological environment through surrogate assay testing (i.e., GSH assay for cysteine-reactive warheads).

Step 1: Target Assessment and Experimental Site Mapping

Objective: Identify potential allosteric sites and reactive residues (e.g., lysine, cysteine) suitable for covalent ligand engagement.

Methodology:

- **Covalent Fragment Screening:** Employ electrophilic fragments or photoaffinity probes targeting nucleophilic or other amino acid residues from the previous step.
- **Mass Spectrometry (MS and MS/MS):**

  - Perform intact protein MS and MS/MS post-digestion to precisely localize labelling sites and inform the design of derivatives with improved potency/selectivity.

- **Protein Modelling:**
  - Evaluate residue accessibility and structural context around labelled residues using crystal structures or homology models to assess modification feasibility and guide design.

Step 2: Computational Modelling and Simulation

Objective: Understand binding dynamics, allosteric mechanisms, and interaction networks to inform ligand design.

Techniques:

- **Docking (Non-Covalent and Covalent) & RMSD calculations** (for bonded and non-bonded structures)**:**
  - Explore binding poses for both covalent and non-covalent ligands and ligand states (for covalent).
  - Perform RMSD comparisons with crystal structures (if any) or analogues to assess alignment and mimicry of key interactions.
  - Torsional analysis may be considered to provide further insights into the feasibility of the bound state for covalent and non-covalent ligands.
- **Dynamic Undocking:**
  - Examine binding reversibility, key stabilizing interactions, and dissociation routes.
  - Use these dynamic data to identify key interactions (e.g., H-bonds, π-stacking, covalent anchoring sites).
- **Water network analysis** (e.g., WaterFlap)
  - Examine the role of water positions and dynamics in ligand binding

Key Outputs:

- Identification of key residues and interactions.
- Warhead placement opportunities guided by reactive site proximity and favourable interactions.

Step 3: Biochemical Assays for Functional Validation

Objective: Evaluate potency, mechanism, and allosteric inhibition.

- Perform functional inhibition assays (e.g., DiFMUP-based phosphatase assay for SHP2) to determine enzyme inhibition/residual activity and IC_{50} values and validate inhibition and SAR.

- Optimize assay conditions to support covalent targeting (e.g., omit interfering reducing agents like DTT where necessary).

Step 4: Ligand Design and Synthesis

Objective: Develop warhead-equipped ligands or lead-like molecules targeting **mapped** allosteric pockets.

Strategies:

- Utilise SAR data (if available), MD, docking, and dynamic undocking insights to preserve key interactions while integrating covalent moieties.
- Evaluate the possibility of incorporating linkers before the warheads to enhance proximity and flexibility, simplify synthesis, or modulate reactivity.

Step 5: MS-Based Validation of Ligand Binding & SAR Refinement

Objective: Confirm binding mode, site, SAR, and stoichiometry and refine structure-activity hypotheses.

Methodology:

- Perform intact MS to validate adduct formation with full-length protein.
- Digest and analyse via MS/MS to pinpoint covalent modification residues and map residue selectivity.
- Correlate MS findings with activity assays to validate ligand-target engagement and refine SAR.
- Combine biochemical and MS data to define critical interactions and refine inhibitor design.

Step 6: Iterative Optimization

Objective: Advance compound potency, selectivity, and mechanistic specificity.

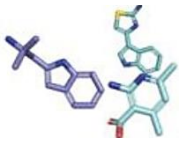
Approach:

- Generate reports integrating structural, biochemical, MS, and computational findings for knowledge-based design.
- Redesign ligands or fragments based on binding efficiency and site reactivity.
- Perform round-based synthesis and evaluation.

File Package

- **General Workflow Diagram:**

Allosteric Ligand Identification and Optimization – a covalent approach



Step 0: Library compilation, stability and biological feasibility

- **Compilation of covalent fragments library** (with *same and different-cores*) –for broader tackling of the target
- Evaluation of selected warheads **stability in buffer**, and **feasibility with relevant surrogates**

STEP 1: Target Assessment and Site Mapping

Identify potential allosteric sites & reactive residues

- **Employ the library** on the protein target & perform intact MS and MS/MS post-digestion
- **Assess structural context** and solvent accessible environment around targeted residue

STEP 2: Computational Modelling

Understand mechanism of action and guide design techniques

- **Docking** (cov. and non-cov.) vs original ligand (e.g. *RMSD*) and **Dynamic Undoking**, for key interactions and determine feasible modifications.
- **Water network analysis** (e.g. *WaterFlap*) examine the role of water and dynamics in ligand binding

STEP 3: Biochemical assays for functional validation

Evaluate potency, mechanism, and allosteric inhibition

- Conduct biochemical assays, validate **inhibition**, determine **IC50 and SAR**
- **Optimise assay** conditions to support covalent targeting
- **Combine biochemical and MS** data to retrieve and interpret interactions

STEP 4: Ligand Design and Syntheses

Strategic develop warhead-equipped ligands or lead-like molecules targeting mapped residues

- Use available SAR data, Undocking, MD, and docking insights and **maintain key interactions when introducing reactive moieties** at validated positions

STEP 5: MS-Based Validation of Ligand Binding & SAR refinement

Confirm binding mode, site, SAR and stoichiometry

- **Generate reports** that lead to confirmation mechanism, MS mapping and computational findings
- **Re-design ligands or fragments** with optimised binding efficiency site reactivity

3. General Workflow for Fragment Screening Campaign

1. **Initial Screening – Reactivity & Stability**
    - Discard highly reactive, unstable fragments:
        1. Incubate fragments in PBS or aqueous buffer at physiological pH (7.4), 37 °C.
        2. Evaluate intrinsic stability and surrogates (i.e., GSH, N-acetyl Lysine) reactivity using HPLC-MS.
    - Prioritize fragments with moderate reactivity and acceptable aqueous stability.
2. **Selectivity Evaluation**
    - Confirm the fragment binds **site-specifically** to the desired amino acid on the target protein.

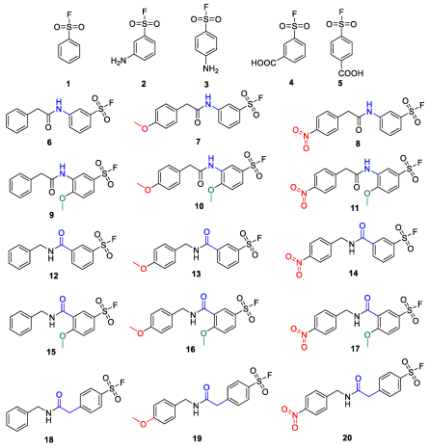
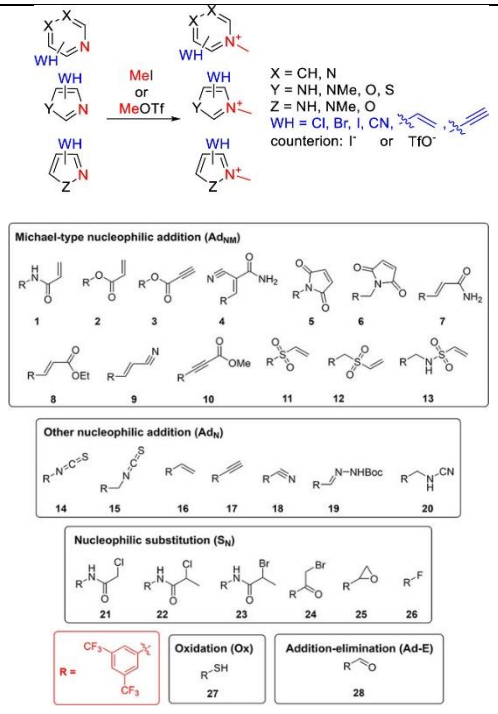
      Approach:

        1. Screen against **thiol- or amine-containing model peptides** (for Cys or Lys, respectively).
        2. Confirm covalent adducts via LC-MS/MS mapping after proteolytic digestion.
    - Confirm site of action via LC-MS/MS mapping.
3. **Functional Validation**
    - Confirm biochemical inhibition of the target through covalent binding, conducting biochemical activity assays (e.g., enzymatic inhibition, IC_{50} determination).
    - Evaluate the possibility of testing time-dependent inhibition (TDI).
4. **Mechanism Confirmation**
    - Determine K_I (affinity) and $\mathbf{k_{inact}}$ (inactivation rate) using multiple timepoints and concentrations.
    - Perform proteomics, **chemoproteomics, or shotgun MS/MS** to compare on-target vs. off-target labeling.
5. **Cellular Validation**
    - Test cytotoxicity and target modulation.
    - Validate through immunoblots or pathway-specific assays.
6. **SAR and Optimization**
    - Redesign based on MS site mapping and biochemical data. Improve potency, selectivity, and drug-likeness through iterative design.
    - Retest refined analogues through the same workflow to confirm improvements.
    - **Examples:**

1. Add warheads at mapped attachment points or optimize linker geometry to improve **fragment disposition toward the reactive residue**.
2. Tune reactivity by modifying the electronic or steric environment of the warhead.

Key Property	Assay Type	Readout/Output
Stability	PBS Incubation + HPLC-MS	k_deg, $t_1/_2$
Reactivity	Surrogate Reactivity Assay	k_surrogate
Specificity	MS/MS Peptide/Protein Mapping	Site of modification
Functional Inhibition	Biochemical Activity Assay	IC_{50}, K_I, k_{inact}
Cellular Activity	Viability, Western	IC_{50}, pathway inhibition
SAR Refinement	Combine MS + Biochem	Fragment optimization

Type of fragments explored:

Lysine reactive:	Cysteine reactive:
	

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4. Notes for Adaptation

- The workflow is generalizable to other targets by adapting pocket detection criteria, fragment libraries, and assay formats according to the structural and functional properties of the protein.
- Covalent vs non-covalent strategies can be prioritized depending on target tractability.

5. Acknowledgment

This workflow builds on Deliverables 3.5, 3.6, 3.7, and 3.8 of the ALLODD project, with specific contributions from ESR7 and HUN-REN RCNS.

