Small-molecule tools to study human cysteine enzymes SENPs and PARK7

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Chapter 1

General introduction and thesis outline

Small-molecule inhibitors, chemical compounds with a molecular weight in the range of 0.1-1 kDa, are indispensable chemical tools to study the biological function of enzymes, elucidate protein-protein interactions, and are used as therapeutic reagents to inhibit disease-related enzyme activity.[1-3] Notably, small-molecule inhibitors occupy 90% of all approved pharmaceutical drugs, and well-known examples include Penicillin and Omeprazole. Due to their small size, small-molecule inhibitors have several advantages:[4] firstly, they can pass through cell membranes easily to interact with their target; secondly, they can be easily synthesized by chemical reactions; thirdly, they can also be designed to engage biological targets by various mechanisms of action. Based on the interaction mechanism, small-molecule inhibitors can be classified into two types: non-covalent inhibitors and covalent inhibitors. Non-covalent inhibitors are designed to bind with the target proteins by their intrinsic binding affinity in a reversible manner, while covalent inhibitors can form a stronger complex through a covalent bond.[5] Compared to non-covalent inhibitors, covalent inhibitors have been demonstrated to own a series of advantages, such as high potency, prolonged duration of interaction, and less-frequent dosing.[6, 7] As a result, covalent inhibitors can be converted into activity-based probes (ABPs) with broad applications, e.g., monitoring enzyme activity, identifying inhibitors of enzymes, and further investigating the selectivity and target occupancy of inhibitors *in vitro* and *in vivo*.[6-8]

The work described in this thesis is mainly focused on the development of covalent small-molecule inhibitors and chemical reagents for enzymes that harbor an active site cysteine, more specifically for the SUMO proteases SENPs and protein/nucleotide deglycase PARK7/DJ1. Given that **Chapter 2** and **Chapter 4** provide a detailed overview of the target proteins that are studied in this thesis, this introduction provides a concise outline of covalent inhibitors, strategies to develop covalent inhibitors, as well as the scope of this thesis.

1. Small-molecule covalent inhibitors

Covalent inhibitors have emerged as an important class of small-molecule inhibitors and played a pivotal role in drug discovery since Aspirin, the first covalent drug, was approved in the late 19th century.[6, 9] The past two centuries have witnessed remarkable advances in this field. Currently, almost 30% of marketed drugs are covalent inhibitors against various targets.[6, 10] The advantages of covalent inhibitors counteract the disadvantage of the potential off-target toxicity.[6, 7] Here, an overview of the mechanism of covalent inhibitors, the advantages and disadvantages of covalent inhibitors, and cysteine-targeting covalent inhibitors is given below.

1.1 Mechanism of action

Traditional drug designs are based on the equilibrium binding between the small

molecules and the protein targets (Figure 1A). A non-covalent inhibitor binds to its target enzyme in a rapid fashion, where the equilibrium between the bound and unbound states of the complex depends on their intrinsic affinity, thus affecting the duration of action.[5] The interaction between them is indicated by the inhibitory constant K_i, which is a calculated ratio of k_{on} (inhibitor association constant)/k_{off} (inhibitor disassociation constant). In contrast, covalent inhibitors are designed to increase these non-covalent molecular interactions via a much stronger covalent bond.[6, 11] In general, covalent inhibitors bind to their target in a two-step process (Figure 1B).[12] First, the inhibitor binds reversibly with its target enzyme, introducing a reactive moiety (often referred to as 'warhead') of the inhibitor in the proximity of a nucleophilic residue on the protein. The potency of this step is described by the binding constant K_i. Next, a reaction occurs within the reversible complex to form a covalent bond between inhibitor and protein, and this is defined by the first-order rate constant k_{inact}.

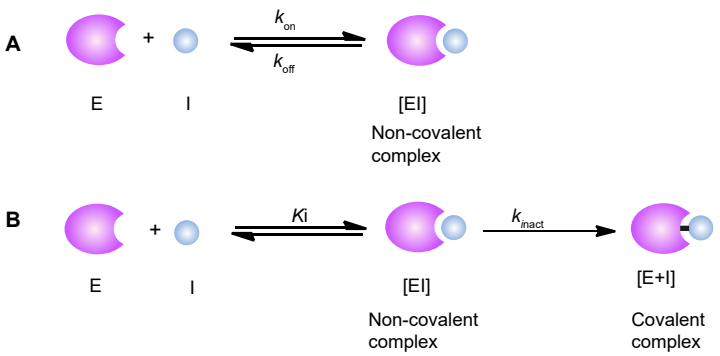

Figure 1. (A) An inhibitor interacts with an enzyme in a non-covalent manner. (B) A covalent inhibitor binds with an enzyme in two steps.

Depending on the warhead and the type of enzyme, covalent inhibitors can be classified as reversible and irreversible covalent inhibitors.[13] Reversible covalent inhibitors enable covalent binding of the inhibitor with the enzyme and assist the inhibition, while the enzyme activity is subsequently recovered because the reversible covalent inhibitor dissociates from the inhibitor-protein complex.[9, 13] Although the covalent bond formation increases the affinity between them, subsequent dissociation will decrease the duration of inhibition. On the other hand, irreversible covalent inhibitors do not dissociate from their target proteins.[7, 9, 13] Once the irreversible covalent bond is formed the enzyme activity can only be recovered by protein re-synthesis.[9, 13] Different effective strategies are currently available to determine the reversibility of an identified covalent inhibitor, such as surface plasmon resonance (SPR), isothermal titration calorimetry (ITC), jump dilution assay, and gel-based competition assay.[14-16] In this

thesis, jump-dilution assay, and gel-based competition assay have been employed to assess the reversibility of covalent inhibitors for SENPs and PARK7. In a jump dilution experiment, the enzyme and inhibitor can form a complex to achieve equilibrium at saturated concentrations. The complex is further diluted into assay buffer and the recovery of the enzyme activity is measured as the inhibitor dissociates.[15] In a gel-based competition experiment, the enzyme and a covalent inhibitor are incubated to form a complex, followed by incubation with a relevant ABP. A reversible covalent inhibitor can be displaced by an ABP due to the irreversible nature of the binding between the probe and enzyme.[16]

1.2 Advantages and disadvantages of covalent inhibitors

Covalent inhibitors have several advantages over non-covalent inhibitors that are summarized below.[5-7, 12] 1) Increased potency. The non-equilibrium binding of irreversible covalent inhibitors can hamper the endogenous substrate to bind at the same target site, thus, the desired inhibitory effect is expected to be achieved at lower drug doses. 2) Extended duration of the interaction. Once the enzyme is inactivated by irreversible covalent inhibitors, its activity can only be recovered by the re-synthesis of a new protein. Provided that the protein turnover is not too fast, only enough drug exposure to inactivate the target is required. 3) Low dose and less frequent dosing due to prolonged duration of action. On the other hand, covalent inhibitors also display several disadvantages.[5-7, 11] 1) Higher risk and potential toxicity. If the covalent inhibitor lacks selectivity, it can covalently and irreversibly modify off-target proteins. In some cases, covalent inhibitors lead to an idiosyncratic adverse drug reaction (IDAR) when they are highly reactive.[17] 2) Unsuitable for the target proteins with rapid protein turnover. As mentioned above, covalent inhibitors own prolonged duration of interaction. If the target protein turnover is too fast, they will not benefit from irreversible covalent inhibitors. Fortunately, the emerging technologies of activity-based protein profiling (ABPP) and proteomics mass spectrometry allow investigation of the selectivity of covalent inhibitors *in vitro* and *in vivo*, enabling the identification of non-selective covalent inhibitors.[8] In addition, the advanced knowledge to understand the binding mode and reactivity of different types of covalent warheads will favor minimizing the disadvantages.[10, 18]

1.3 Cysteine-targeting covalent inhibitors

Over the years, covalent inhibitors have gained a gradually increasing interest in pharmaceutical research. They have been designed to target various classes of enzymes, such as serine hydrolases,[19] kinases,[20] and the proteasome.[21] Since the work described in this thesis is mainly focused on developing covalent inhibitors for cysteine enzymes SENPs and PARK7, here a highlight of covalent inhibitors targeting cysteine enzymes is provided. Remarkably, there are several covalent

inhibitors targeting cysteine enzymes that have been approved as market drugs, such as EGFR inhibitor Dacimitib, and BTK inhibitors Ibrutinib and Zanubrutinib.[10] In addition, some cysteine targeting-covalent inhibitors in pre-clinical stage have been used as valuable tools in advancing the biological research of therapeutically interesting targets, e.g., kinase inhibitors,[19] and deubiquitinases (DUBs) inhibitors.[22] Furthermore, various reactive warheads have been developed to target cysteine residue, including Michael acceptors (acrylamide, propionamide, fumaric acid ester, etc.), non-Michael addition acceptors (cyanimide, isothiocyanate, etc.), SN2-substitution warheads (α-haloacetamides, epoxides, haloalkane, etc.), and disulfides, providing broad options to develop covalent inhibitors.[23] It is important to select the appropriate warhead to facilitate the design of a potent and specific cysteine-targeting covalent inhibitor. For example, the warhead should not bind with nucleophilic amino acids other than cysteine.[10] In addition, it should avoid using too reactive warheads that would cause promiscuous activity and potential toxicity.[24] To address this issue regarding the high reactivity, several assays have been developed to assess the reactivity of cysteine-targeting warheads. For example, the GSH-based assay is commonly used to determine the reactivity of cysteine electrophiles by measuring the half-life ($t_{1/2}$) of the consumption of glutathione (GSH).[25] In addition, London *et al.* have developed a high-throughput thiol-reactivity assay that enables the assessment of the reactivity of warheads by measuring the absorbance of TNB^{2-}(λ_{max}: 412 nm), reduced DTNB (Ellman's reagent, 5,5-dithio-bis-2-nitrobenzoic acid).[26] The observed absorbance will decrease after alkylation by a cysteine warhead. In this thesis, covalent inhibitors with a cyanimide warhead have been developed to target the cysteine residue of PARK7 to form a thiourea linkage, and covalent fragments with diverse warheads have been identified for SENPs.

2. Strategies to develop and investigate covalent enzyme inhibitors

To date, several strategies have been applied to discover covalent enzyme inhibitors, including structure-based design, covalent virtual screens, and high-throughput screening (HTS).[26] Structure-based drug design is a widespread method that involves the installment of an electrophilic warhead onto a known reversible inhibitor, and this method has proven to be successful in the discovery of kinase inhibitors.[20] This approach requires a deep understanding of how reversible inhibitors interact with the protein structure. In addition, covalent virtual screens have gained popularity in discovering covalent binders, but a crystal structure or a high-quality model of the target protein is necessary.[27] HTS focuses on developing covalent inhibitors for the target protein in a short time with a suitable assay and compound library, especially in cases where a reversible inhibitor of the target proteins and the crystal structure of the target proteins are unavailable.[28, 29] Often, once the hits are identified from HTS, the covalent binding between inhibitors and their target is first confirmed by X-ray crystallography and mass spectrometry, followed by additional medicinal chemistry

effort to optimize the inhibitors.[20] The inhibitor discovery that is mainly employed in this thesis is the HTS strategy, for which a general introduction is provided below.

2.1 High-throughput screening

HTS has emerged as a key technology to identify hit and lead compounds within the pharmaceutical industry and academia since the 1990s.[30] It involves the use of robotic-assisted sample handling equipment, sensitive detection systems, dedicated software, and data processing to rapidly screen large libraries of small molecules to identify compounds against a biological target or in a more complex assay system, such as a cell-based assay.[30] The success of HTS campaigns requires a high-quality assay read-out which should be simple, robust, sensitive, and affordable.[31] Fluorescence is the most commonly used assay readout for HTS, and many fluorescence-based assay reagents have been developed, such as fluorogenic assay reagents that detect a simple fluorescent intensity (FI) output (Figure 2A) and fluorescence polarization (FP) assay reagents that monitor the change in FP value (Figure 2B).[32] In this thesis, SUMO2-RhoMorpholine is used as a substrate for SENPs in HTS efforts. This substrate is recognized and processed by SENPs thereby releasing the Rho-Morpholine dye. Its fluorescent signal can be used to monitor the activity of SENPs and thus make it possible to screen inhibitors for SENPs. However, in some cases, a substrate to directly monitor an enzyme activity does not exist and alternative ways must be developed. In case a ligand for the target enzyme is known, a fluopol-ABPP reagent can be developed and applied as a complementary assay reagent for HTS.[33] In this thesis, such kind of assay reagent was developed

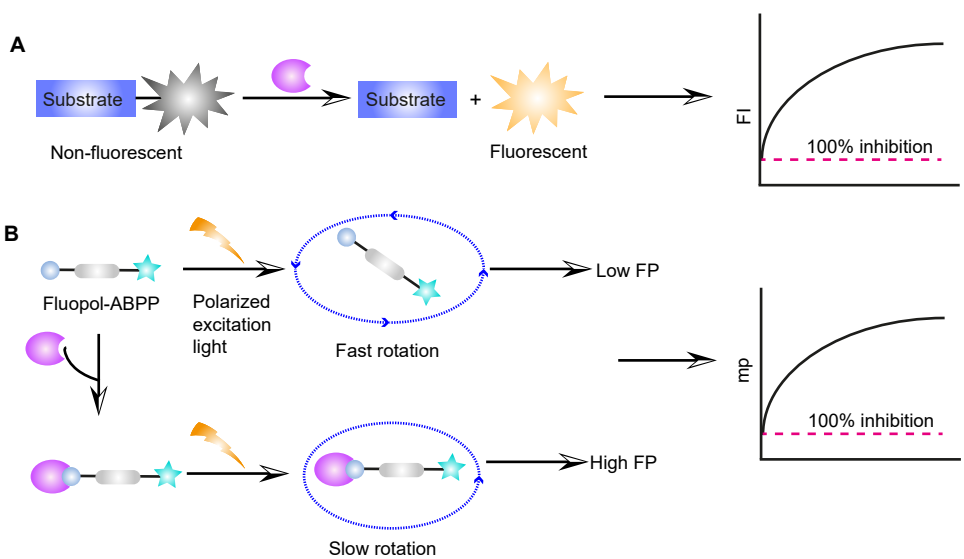

Figure 2. (A) Schematic illustration of the fluorogenic assay. (B) Schematic illustration of fluorescence polarization assay.

and used to perform HTS for PARK7. A summary of activity-based probes (ABPs), activity-based protein profiling (ABPP), and Fluopol-ABPP is given below.

2.2 Activity-based probes (ABPs)

Activity-based probes (ABPs) comprise three basic components (Figure 3A):[34, 35] 1) a binding or affinity group that specifically recognizes the target enzymes, which could be for example a substrate-derived peptide or protein, or a small molecule inhibitor. 2) A reactive group, normally an electrophile, that covalently modifies nucleophilic residues in the enzyme. 3) A reporter group, commonly a fluorescent dye or an affinity tag, that enables the detection and visualization of the probe-bound enzymes. ABPs based on small-molecule inhibitors have gained widespread popularity since they are generally cell-permeable and can be adapted chemically to become selective, which is helpful to study the localization and biological function of enzymes in a cellular context.[34-37] For example, a fluorescent small-molecule UCHL1 probe **8RK59** was used to selectively label UCHL1 DUB activity in cells and zebrafish.[37] A biotinylated version of the probe allowed for the rapid identification of potential off-targets,

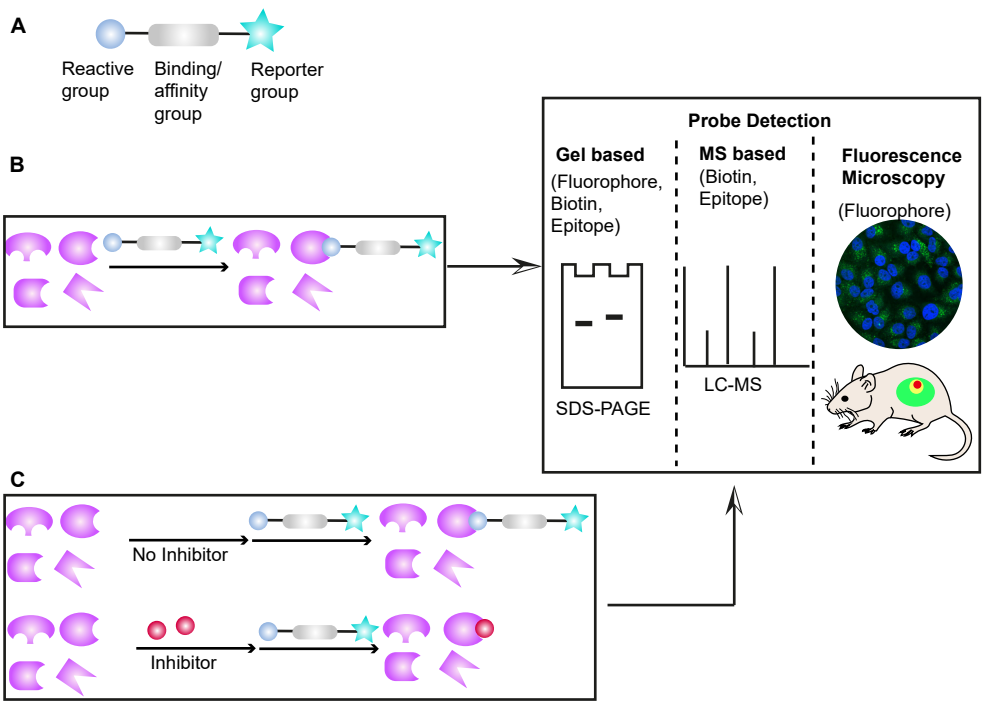

Figure 3. (A) Representative illustration of an active-based probe. (B) Workflow of ABPP. (C) Workflow of competitive ABPP.

providing critical insight for inhibitors optimization and drug discovery.[35, 37] In most cases, covalent inhibitors can be converted into ABPs by attaching a reporter group, however, it should be noted that improper installment of reporter groups can affect the cell permeability and activity of the ABPs, which will limit their application.[34]

2.3 Activity-based protein profiling

ABPP, a powerful chemical proteomic technique, employs ABPs to study the functional state of enzymes in various systems, such as purified enzymes, cell lysates, cells, and tissues.[34, 35] Gel-based ABPP (Figure 3B) is used to detect probe-labeled proteins by SDS-PAGE and fluorescence scanning (fluorescent reporter tag) or western blotting (epitope or biotin reporter tag).[8, 34] MS-based ABPP (Figure 3B) makes use of antibody/streptavidin enrichment and liquid chromatography-mass spectrometry (LC-MS) to enrich, identify, and quantify probe-labeled (epitope and biotin reporter tag) proteins from a complex proteome.[8, 34] Fluorescence microscopy (Figure 3B) enables visualization of enzyme activity in cells and *in vivo*.[34, 35] To broaden the application of ABPP, some advanced technologies have been developed so far,[34, 35] e.g., CC-ABPP (click chemistry ABPP), fluopol-ABPP, iso-top ABPP (isotopic tandem orthogonal proteolysis-ABPP), SLC-ABPP (streamlined cysteine ABPP). All the techniques can be used for competitive ABPP, which will be discussed in the following section.

2.4 Competitive ABPP

Competitive ABPP is a powerful tool to identify (covalent) inhibitors of target proteins.[35] Briefly, a proteome is pre-treated with inhibitors, followed by profiling with the appropriate ABP, and effective covalent inhibition is reflected by a decreased or abrogated labeling by the ABP, which can be determined using SDS-PAGE or LC-MS analysis (Figure 3C).[16] However, it can only be applied in a low-throughput manner. To address this limitation, Cravatt and co-workers have developed a fluorescence polarization technology for competitive ABPP (fluopol-ABPP), that enables the identification of inhibitors in a high-throughput manner.[37, 38] Basically, when a small fluorescent probe is excited by polarized light it will emit largely depolarized light due to its fast rotation. When bound to a big molecule, such as a protein, the emitted light remains polarized. The change in polarization is a quantitative measure of the probe binding to the enzyme and this can be disrupted by inhibitor binding (Figure 2B).[37] This technology has successfully been applied to screen different targets, such as serine hydrolase RBBP9,[37] nonlysosomal glucosylceramidase GBA2,[39]

protein arginine methyl-transferases (PRMTs),[40] and Golgi mannosidase GMII.[41] In this thesis, fluopol-ABPP is used to discover covalent inhibitors for PARK7 in a high-throughput manner (**Chapter 5** and **6**).

In addition, adapted from competitive ABPP, iso-top ABPP, and SLC-ABPP have become standard approaches to identify protein targets and evaluate the selectivity of enzyme (covalent) inhibitors.[42, 43] In this thesis, SLC-ABPP has been employed to determine the selectivity of PARK7 covalent inhibitors in **Chapter 6**. In an SLC-ABPP experiment, a proteome is pre-incubated with a cysteine-targeting covalent inhibitor, prior to incubation with a desthiobiotin iodoacetamide (DBIA) probe to profile and enrich reactive cysteine sites that are not bound to an inhibitor.

Scope of the thesis

SENPs are attractive drug candidates due to their involvement with various diseases, especially cancers.[44] Although several inhibitors are currently available for SENPs, they lack potency and selectivity, hampering the biological research of SENPs.[45] Therefore, one of the main objectives of this thesis is to develop more selective and potent inhibitors for SENPs to investigate the function of SENPs in a cellular context and explore new therapeutics.

PARK7 is a small multifunctional protein that regulates a broad of cellular events, and it is well-known that dysregulation of PARK7 is related to various diseases, e.g., Parkinson's disease,[46] and cancer.[47] Although it has been extensively studied for over two decades, a dedicated chemical toolbox for PARK7 is still missing, such as potent and well-characterized inhibitors, activity-based probes, and assay reagents.[48] Thus, another main objective of the thesis is to develop such kind of chemical tools to advance the understanding of PARK7 and explore the therapeutic possibilities towards cancer and neurodegenerative diseases.

Chapter 2 provides an overview of the available chemical reagents and biochemical assays of SENPs.

Chapter 3 provides an overview of the screening results of SENPs with an in-house cyanimide library (23,000 molecules) and a commercially available covalent fragment library (8,000 molecules) using a fluorogenic assay. The yielded compounds from HTS could serve as starting points for further optimization to develop selective and potent inhibitors of SENPs.

Chapter 4 describes the biological functions of PARK7 and provides a comprehensive overview of the available chemical reagents and biochemical assays to study PARK7.

Chapter 5 describes the development of a small-molecule covalent inhibitor equipped with a cyanimide warhead against PARK7, showing the target engagement in a cell lysate. Installment of different dyes onto the inhibitor resulted in two PARK7 probes. The Rhodamine110 probe provides an HTS-compatible FP assay, showcased by

screening a compound library (8,000 molecules). The SulfoCy5-equipped probe is a valuable tool to assess the effect of PARK7 inhibitors in a cell lysate.

Chapter 6 describes the characterization of two potent, cell-permeable, and specific inhibitors against PARK7 using the HTS technique developed in chapter 5. Conjugating a SulfoCy5 dye to these inhibitors enables monitoring PARK7 or oxidized PARK7 activity in cells. In addition, a potent and selective PARK7 degrader based on these inhibitors is reported.

Chapter 7 summarizes the research results and provides future perspectives of this thesis.

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