Universiteit
Leiden
The Netherlands

Alkynes in covalent enzyme inhibitors: down the kinetic rabbit hole

Mons, E.

Citation
Mons, E. (2024, April 11). *Alkynes in covalent enzyme inhibitors: down the kinetic rabbit hole*. Retrieved from https://hdl.handle.net/1887/3734191

Version:	Publisher's Version
License:	Licence agreement concerning inclusion of doctoral thesis in the Institutional Repository of the University of Leiden
Downloaded from:	https://hdl.handle.net/1887/3734191

Note: To cite this publication please use the final published version (if applicable).

IC50
Ub
Kinact

Chapter 1

Once Upon a Time: Serendipitous Discovery of Alkynes as Electrophiles

1. Resurgence of Covalent Drugs

Enzymes are involved in all biochemical processes, ranging from (proteolytic) degradation of macromolecules to the installation and removal of post-translational modifications (PTMs). PTMs can affect protein affinity and/or function, but can also function as 'messenger tags' that facilitate communication among cellular components. Receptor kinases phosphorylate downstream effector proteins to relay extracellular growth signals, initiating a signaling cascade that affects gene expression by relocation of transcription factors to the nucleus, effectively enabling the cell to respond to changes in the extracellular environment.[1] This process is tightly regulated by phosphatases that reverse phosphorylation.[2] Histone methyltransferases (HMTs) and histone acetyltransferases (HATs) respectively methylate or acetylate histones thus affecting gene transcription. These epigenic alterations are reversed by histone demethylases (HDMs) and histone deacetylases (HDACs).[3-4] Ligases install ubiquitin (Ub) chains onto (misfolded) proteins to mark them for proteasomal degradation, which is counteracted by proteases that cleave Ub from the protein.[5] Interference with enzymatic activity has proven to be a viable drug development strategy as the pathophysiology of many diseases is associated with enzyme deficiency or overexpression, aberrant activity, and/or incorrect enzyme function.[6-7] Enzyme inhibitors have been approved for treatment of various pathological conditions including metabolic and degenerative diseases, viral/bacterial infections, cancer, and inflammation. Small molecules that interfere with enzyme activity have always been popular, but the past decade marked the rise of highly effective targeted covalent inhibitors (TCIs) – designed to interact with their target through the formation of a covalent adduct.[8]

Conventional small molecule inhibitors (<500 Da) interfere with protein function as long as they are bound to their protein target.[6, 9-10] The noncovalent interactions with the protein target are reversible, and protein function will be regained when unbound protein is released upon inhibitor dissociation. The drug target engagement can be prolonged by covalent modifiers that harbor a – strategically placed – electrophilic moiety (commonly referred to as the 'warhead') to form a covalent bond with a nucleophilic amino acid residue in the protein (e.g. cysteine, serine, threonine).[8, 11-12] The resulting protein–drug adduct is linked through a(n) (ir)reversible covalent bond that is much stronger than typical noncovalent interactions. Irreversible inhibition – typically defined as a drug residence time exceeding the normal lifespan of the target protein[7, 13] – has clear therapeutic advantages: systemic drug exposure is minimized as protein function can only be restored by *de novo* protein synthesis. Consequently, therapeutic effect is maintained long after the compound has been cleared from circulation (PK-PD decoupling).[10-11, 14-15]

Irreversible covalent modifiers were actively avoided in pharmaceutical drug development programs: the ability to covalently modify the target protein raised concerns about promiscuous reactivity with off-target proteins.[8, 16-19] Reactive electrophilic moieties have been implied as a risk factor for idiosyncratic adverse drug reactions (IADRs) and hepatotoxicity,[20-25] though it must be mentioned that the majority of this research was based on the unintentional formation of highly reactive, highly electrophilic metabolites.[26] The complex underlying mechanism of IADRs – the possibly life-threatening toxicity affecting a small subset of susceptible patients – is largely unclear but may be immune-mediated, making them difficult to predict.[23, 27-29]

Furthermore, even if the covalent inhibitor is perfectly target-selective, covalent modification may lead to hapten formation – immune activation by a covalently modified protein (fragment) that, in case of an irreversible binding mode, persists after protein degradation.[29-30] Reversible covalent drugs were perceived as a safer alternative,[31-33] as they will eventually dissociate from their protein target and have a lower propensity to form a proteolysis-stable hapten.

From avoided liabilities and accidental discoveries...

Approved drugs that act through irreversible covalent modification of their (protein) target are more prevalent than would be expected based on the efforts of pharmaceutical companies to eliminate compounds with potentially reactive functionalities.[8, 12] Ironically, there are many examples of effective covalent drugs with satisfying toxicity profiles among the most-prescribed drugs worldwide,[11] and many can be found on the WHO (World Health Organization) Essential Medicines Lists.[34] Their covalent mechanism of action was often discovered after their clinical utility had been well established, typically years after their first synthesis and sometimes long after they hit the market (**Figure 1**).[35] The most well-known unintended covalent inhibitor is **acetylsalicylic acid** (aspirin), the pharmaceutically active component in ancient medicinal consumption of willow bark.[36] Marketed in 1899 as a pain reliever and anti-inflammatory agent, aspirin is without doubt the most used drug worldwide: approximately 40,000 tons are produced annually – good for >111 billion tablets of 325 mg. Its mechanism of action remained elusive until the 1970s,[37-38] when aspirin and other non-steroidal anti-inflammatory drugs (NSAIDs) were found to block biosynthetic production of prostaglandins causing inflammation, a discovery awarded with the 1982 Nobel Prize in Physiology or Medicine. The exact molecular mechanism was elucidated decades after its first clinical use: aspirin acetylates Ser530 of the cyclooxygenase (COX) enzymes (isoforms COX-1 and COX-2), thereby irreversibly inhibiting the biosynthetic transformation of arachidonic acid to prostaglandins.[36, 39-41]

Pharmaceutical companies remained reluctant to include irreversible covalent modifiers in their drug development efforts, despite numerous examples of efficient and safe breakthrough therapies that were later found to have a covalent mode of action such as β-lactam **penicillin** antibiotics,[42] proton pump inhibitor **(es)omeprazole** (Prilosec, Nexium) for treatment of esophageal reflux and heartburn,[43-44] and antiplatelet agent **clopidogrel** (Plavix) to prevent thrombosis events.[45-46] These covalent (pro)drugs are successfully used as long-term therapies and have shown to be safe in millions of patients.[11] Their bad reputation is not helped by the irreversible covalent binding mode of chemical warfare agents such as nerve gas sarin (a fluorophosphonate that phosphonylates the catalytic Ser203 of acetylcholinesterase AChE)[47] and blister agent **mustard gas** (a class of sulfur mustards – bearing a 2-chloroethyl sulfide warhead – that alkylate the N7 guanidine in DNA).[48] Controversially, mustard gas sparked the development of cancer chemotherapy: victim autopsy revealed leucopenia and affected bone marrow function,[49] which resulted in the development of the less volatile nitrogen mustard DNA alkylating agents (e.g. **mechlorethamine** (Mustargen), **chlorambucil** (Leukeran)) that became the first cytotoxic chemotherapeutics for treatment of lymphoma.[50-53] This is not the only class of covalent chemotherapy drugs developed in the previous century: DNA cross-linking agent **cisplatin** (Platinol, cis-diamminedichloroplatinum(II)), proteasome inhibitor **carfilzomib** (Kyprolis, PX-171-007), antimetabolites **fluorouracil** (Adrucil, 5-FU)

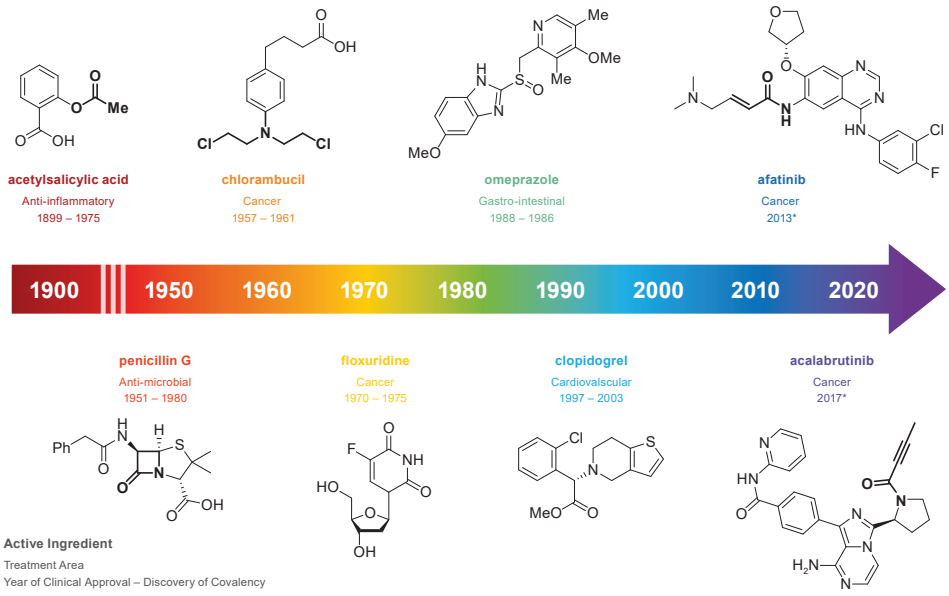


Figure 1 | Development timeline of (ir)reversible covalent drugs. Shown are the chemical structure, name of the active ingredient, the year of first clinical approval, and the year that the irreversible covalent binding mechanism was reported. The covalent warhead is shown in bold. Asterix marks targeted covalent inhibitors (TCIs) that were designed to have a covalent binding mode.

and **floxuridine** (Fudr),[54-55] and many more chemotherapeutic agents have an (unintentional) irreversible covalent binding mode.[12]

... To desired modalities

A shift in paradigm was initiated in the 1990s, when the pharmaceutical industry was presented with pharmacological kinase targets that required complete and sustained inhibition.[10, 56-58] Noncovalent screening hits could only suppress EGFR signaling of the acquired $EGFR^{T790M}$ mutant for a short period, and prolonged inhibition was required to improve *in vivo* antitumor activity. Computational analysis revealed a nonconserved cysteine residue near the inhibitor binding site,[59] which could be covalently targeted by incorporation of a strategically placed electrophilic acrylamide warhead. This led to the development of multiple irreversible covalent clinical drug candidates[60-61] that showed no remarkable toxicity and were able to overcome (acquired) T790M-mediated resistance to noncovalent EGFR inhibitors **gefitinib** (Iressa, ZD1839) and **erlotinib** (Tarceva, CP-358774) with 'pretty spectacular' antitumor activity in patients suffering from non-small cell lung carcinoma (NSCLC).[56-57] In 2013, the first two irreversible targeted covalent inhibitors (TCIs) – inhibitors designed to have a covalent binding mode – were approved for clinical use:[8, 35] **afatinib** (Gilotrif, BIBW 2992) for treatment of gefitinib-resistant NSCLC and **ibrutinib** (Imbruvica, PCI-32765) for treatment of B-cell malignancies. Coincidentally, both teams chose to incorporate an acrylamide warhead – a cysteine-reactive Michael acceptor that covalently modifies the target protein and shows

moderate intrinsic reactivity with nontargeted thiols.[62] Lack of promiscuous reactivity with nontargeted thiols is a desirable feature: cross-reactivity with nontargeted nucleophiles not only increases the risk of aforementioned idiosyncratic adverse effects, but adduct formation with biologically relevant thiols – such as glutathione (GSH) present in serum and cytochrome P450 (CYP) enzymes in human liver microsomes (HLM) – also renders the inhibitor susceptible to rapid depletion and (extrahepatic) metabolic inactivation.[21, 63-65] Nowadays, pharmaceutical drug discovery programs still favor the acrylamide warhead because it balances on-target reactivity with acceptable selectivity: in 2022, nine out of ten clinically approved TCIs feature an acrylamide warhead.[12, 66-67] There is a clear need for novel cysteine-targeting electrophiles extending beyond these Michael acceptors to further improve the reactivity and safety profiles of irreversible TCIs.[8, 62, 68]

2. Acetylenes in Drug Development and Chemical Tools

The acetylene group is a privileged structural element that has been featured in clinical compounds targeting various therapeutic areas.[69] In these noncovalent drugs, the alkyne is used as an isostere for many functional groups to improve potency or modulate the drug metabolism pharmacokinetic (DMPK) profile. Covalent reactions of nonactivated alkynes with cellular nucleophiles are either metal-catalyzed or radical-mediated (e.g. thiol–yne coupling (TYC)[70-73]), or are enabled by (metabolic) conversion to form a reactive intermediate. Inactivation of enzymes in the CYP family has been reported for nonactivated acetylenes: metabolic oxidation of alkynes generates ketene or alkynone intermediates, and these electrophilic intermediates can form a covalent adduct with nucleophilic residues in CYP enzymes (**Figure 2A**).[74-76] An exception are clinically approved Parkinson inhibitors **selegiline** (Eldepryl, E-250) and **rasagiline** (Azilect, VP-1012) that form a covalent adduct with monoamine oxidase B (MAO-B) through a nonactivated propargylamine moiety (**Figure 2B**).[77] However, a more electrophilic ynimine/allenamine intermediate is likely responsible for the observed covalent adduct with the flavin adenine dinucleotide (FAD) co-factor N5 nitrogen.

The most prominent application of terminal alkynes is in chemical biology, where they are frequently used as bioorthogonal Click handles.[69] These reagents are unreactive toward biological functionalities (bioorthogonal) while participating in simple and high yielding reactions that are compatible with mild (aqueous) conditions and – aside from the desired product – only generate unoffensive byproducts (Click reaction).[78-79] Terminal alkynes have a low propensity of spontaneous engagement in covalent adducts with cellular components but can selectively form a triazole adduct in the Copper-catalyzed azide–alkyne cycloaddition (CuAAC) (**Figure 2C**).[80-81] The CuAAC is extensively used in various protein labeling strategies such as the popular activity-based protein profiling (ABPP):[67, 82] proteome incubation with a residue-selective reagent bearing an alkyne handle is followed by treatment with a tagged azide and a Cu(I) catalyst, after which the labeled proteins can be enriched and/or visualized, depending on the detection tag on the azide reagent (**Figure 2C**). The toxic copper catalyst in the CuAAC is not compatible with living cells and organisms, which was overcome by the development of a strain-promoted azide–alkyne cycloaddition (SPAAC) that employs a cyclooctyne derivatives of the alkynyl motif (**Figure 2D**).[81] The drawback of the SPAAC is

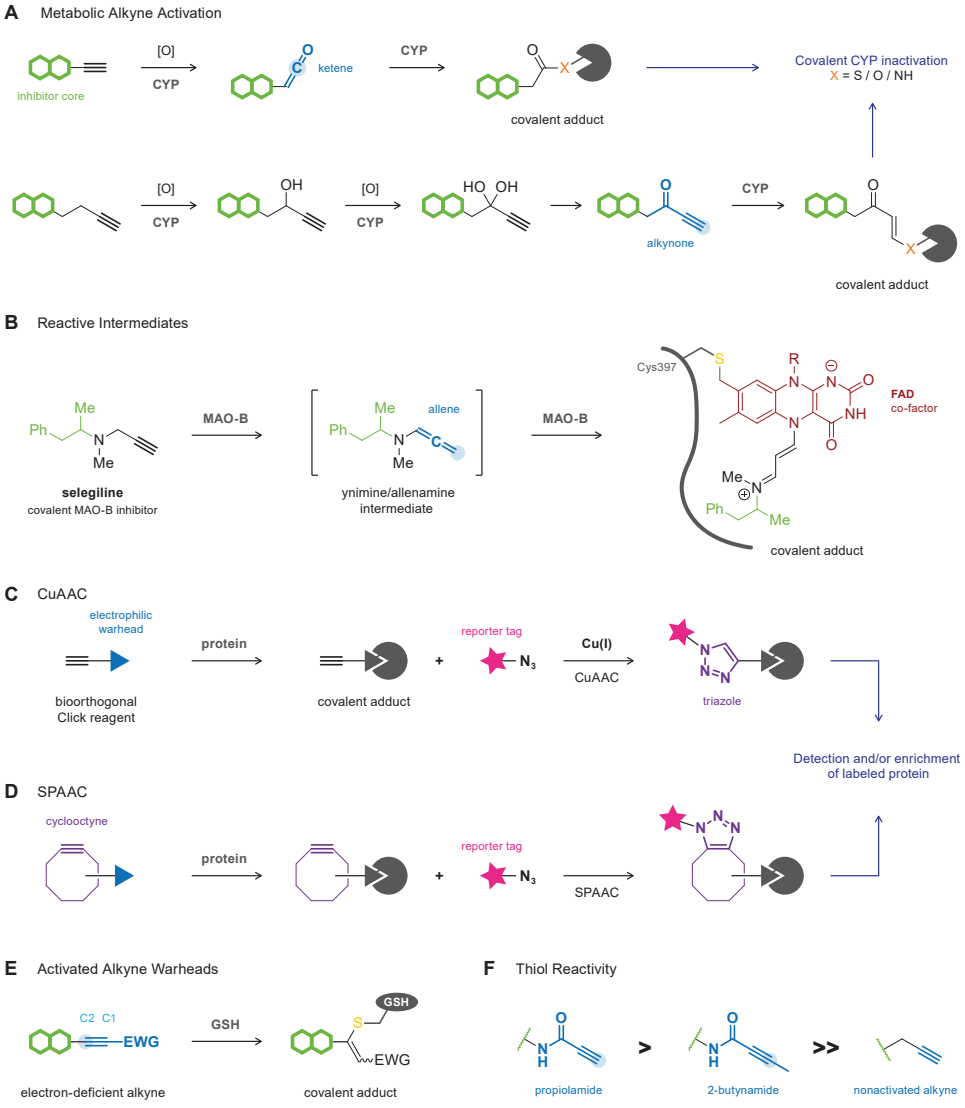


Figure 2 | The acetylene group in covalent drug development and chemical biology tools. Electrophilic warheads are shown in blue, with the reactive carbon marked with a blue circle. **(A)** Metabolic activation of acetylenes to form reactive ketene or alkynone intermediates can result in undesired inactivation of cytochrome P450 (CYP) isoforms. **(B)** Covalent MAO-B inhibitors **selegiline** (Eldepryl, E-250) and **rasagiline** (Azilect, VP-1012) form a covalent adduct with the FAD (flavin adenine dinucleotide) co-factor through a propargylamine group. An allenic intermediate is responsible for the observed reactivity. **(C)** Terminal nonactivated alkynes as bioorthogonal Click handles in chemical biology reagents. The proteome is incubated with alkyne-tagged protein-reactive reagent followed by Cu(I)-catalyzed coupling of the azide-labeled reporter tag in a copper-catalyzed azide–alkyne cycloaddition (CuAAC) to visualize protein labeling. **(D)** Cyclooctynes as bioorthogonal Click handles. The strain-promoted azide–alkyne cycloaddition (SPAAC) does not require toxic Cu(I)-catalyst. **(E)** Activated alkynes form adducts with (biological) thiols such as glutathione (GSH). Introduction of an electron-withdrawing group on the C1 carbon generates a thiol-reactive electron-deficient alkyne warhead. **(F)** Electron-deficient ynamide warheads used in chemical tools and TCIs have a higher (indiscriminate) thiol reactivity with GSH than nonactivated alkynes.

azide-independent labeling of biological functionalities, as the ring-strain and/or increased hydrophobicity of a strained alkyne also enhances undesired reactivity with cysteine thiols.[83] The importance of bioorthogonal and Click chemistry was recognized by the 2022 Nobel Prize in Chemistry.[84]

An electron-rich acetylene motif is unlikely to spontaneously engage in covalent adduct formation with biological thiols, but so-called electron-deficient alkynes are an upcoming class of cysteine-targeting warheads. Electron-deficient alkynes are generated by introduction of an electron-withdrawing group (EWG) onto the alkyne C1 or C3 carbon that increases the electrophilicity, while introduction of an electron-donating group (EDG) has the opposite effect (**Figure 2E**).[85] The intrinsic thiol reactivity of activated (electron-deficient) alkynes has been employed in chemical tools for chemoselective cysteine modification – specifically alkynoic amides/esters and alkynones,[86] ethynyl-triazolyl-phosphinates (ETPs),[87] and arylpropiolonitriles (APNs).[68, 88] Moreover, electron-deficient ynamide warheads have been prominently featured in various drug candidates, including clinical covalent BTK inhibitors **acalabrutinib** (Calquence, ACP-196)[89] and **tirabrutinib** (Velexbru, ONO/GS-4059).[90] The increased electrophilicity of the 2-butyanamide and propynamide/propiolamide warheads compared to nonactivated alkynes comes at the cost of promiscuous adduct formation with nontargeted cellular thiols (**Figure 2F**),[85, 91] though the 2-butyanamide in **acalabrutinib** was still less reactive towards GSH than the corresponding acrylamide.[89] Increased promiscuous thiol reactivity is also observed for the class of alkynyl-substituted heteroarenes[68] – (electron-deficient) heteroaryl moieties modified with an alkynyl group – including but not limited to the 2-alkynylthiazoles,[92] alkynyl benzoxazines,[93] alkynylpyrimidines,[94] ethynylthienopyrimidines,[95] and ethynylpurines.[96]

Nonactivated acetylenes were considered 'inert' towards proteins under physiological conditions until 2013, when two research groups – both active in the field of chemical biology to study the ubiquitin-proteasome system – independently discovered that nonactivated terminal alkynes can covalently modify catalytic cysteines.[97-98]

3. On Terminal Alkynes that React with Catalytic Cysteines

Ubiquitination is a post-translational modification (PTM) that involves installation of ubiquitin (Ub) – a 76-amino acid protein – onto a lysine residue of the target protein by the E1-E2-E3 ligase cascade enzymes.[99-100] The target protein can be monoubiquitinated on multiple residues but commonly Ub chains are formed by conjugating one of the ubiquitin lysine amines or the N-terminal amine to the C-terminus of another Ub (**Figure 3**). Which linkage is formed is driven by the E2-E3 ligase combination, and chain topology impacts the destiny of the ubiquitinated protein: K48 chains enhance proteasomal degradation of the ubiquitinated protein, while K63 chains have a role in inflammatory signaling.[100-102] The process of ubiquitination is reversed by deubiquitinating enzymes (DUBs) – proteases that cleave the native isopeptide bond between the C-terminus of the distal Ub and the Lys residue in the target protein or a Lys residue or the N-terminus of M1 in the proximal Ub (**Figure 3A**).[103-104] Human DUBs are divided into classes: there is one class of zinc-dependent metalloDUBs (JAMM) and six known classes

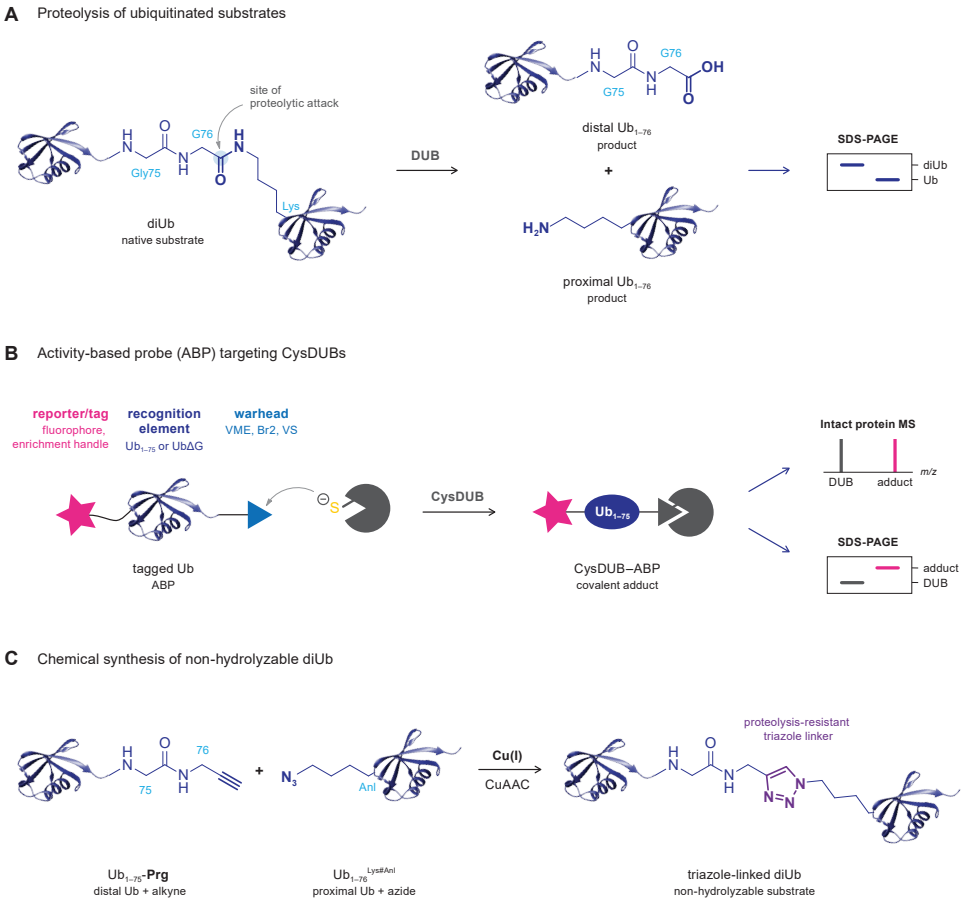


Figure 3 | Chemical tools to study deubiquitinase (DUB) activity. **(A)** DUB-mediated proteolysis of native diUb chains. **(B)** General design of CysDUB activity-based probes (ABPs). The reactive carbon in the electrophilic warhead is aligned with the carbonyl in native ubiquitinated substrates. Covalent adduct is typically visualized by an increase in deconvoluted mass (intact protein MS) or a band shift after gel electrophoresis with detection by protein staining, in-gel fluorescence, or immunoblotting. **(C)** Chemical synthesis of triazole-linked non-hydrolyzable diUb substrates.

of human cysteine DUBs (CysDUBs) based on the catalytic domain (USP, OTU, UCH, MJD, MINDY, and ZUFSP).[99] Some DUBs indiscriminately cleave all linkage types (e.g. USP21) [103] while others exhibit a specificity or preference for proteolytic cleavage of a certain diUb linkage (e.g., OTULIN for M1, OTUB1 for K48).[104] The full 'ubiquitin code' is much more complex, with mixed linkages, branched chains, phosphorylated or acetylated Ub, and incorporation of ubiquitin-like (Ubl) modifiers SUMO, Nedd8, ISG15, or UFM.[100-101]

Chemical tools to study *in vitro* DUB and Ubl protease activity have a key role in our current understanding of the Ub(l) system.[104-106] Proteolytic DUB activity can be evaluated using a ubiquitinated model substrate (e.g. diUb), with resolution of the substrate (diUb) and the

smaller product (monoUb) by gel electrophoresis, but this is restricted to recombinant purified proteases. Early on, activity-based probes (ABPs) with a monoUb recognition element enabled identification of novel CysDUBs and concurrent assessment of DUB activity profiles in cell lysates (**Figure 3B**).[5, 105] On the C-terminus of the recognition element (Ub_{1-75} or UbΔG), an electrophilic warhead (e.g. **VME**, **VS**, **Br2**) is installed with careful positioning of the reactive center in alignment with a native isopeptide bond, and a detection element (e.g. a fluorophore such as Rho, TMR, or Cy5, or an epitope/reporter tag such as biotin or HA) is usually placed at the N-terminus.[105-106] Nucleophilic attack of the catalytic Cys residue (CysDUB) to the warhead (ABP) generates a covalent adduct that can be detected by protein resolution using SDS-PAGE followed by immunoblotting (reporter tag) or direct in-gel fluorescence scanning (fluorophore).

Nowadays, the molecular toolbox to monitor proteolytic activity of DUBs and Ubl proteases contains a variety of assay reagents based on (chemically) modified Ub(l).[5, 106-107] Advances in the chemical Ub(l) synthesis have been instrumental in the efficient synthesis of Ub(l)-based assay reagents.[108-110] Linear chemical synthesis of Ub(l) by solid phase peptide synthesis (SPPS)[111] conveniently enables introduction of (fluorescent) detection tags,[104, 112] synthesis of Ub(l) variants,[113-115] and selective modification of a single amino acid residue – essential features in the preparation of assay tools to interrogate DUB activity/specificity towards specific linkage types.[109] The cellular role and binding affinity of various Ub(l) chains can be studied with non-hydrolyzable Ub(l) conjugates [116] – synthetic conjugates linked by an enzymatically stable amide isostere that mimics the native isopeptide bond but is resistant to DUB cleavage (e.g. triazole [116-117] or oxime [118]) (**Figure 3C**). The distal building block Ub-**Prg** is obtained by coupling propargylamine to chemically synthesized Ub_{1-75}, thus replacing the C-terminal Gly76 to mimic the alignment of native diUb. In the proximal building block, an azidonorleucine (Anl) residue replaces the lysine residue that will be ubiquitinated. Finally, the proteolytically stable triazole-linked diUb is obtained by Click chemistry.

Serendipitous discovery of the *in Situ* thiol–alkyne addition

To study the inhibitory potency of various diUb linkages spanning the active site of UCHL3, Ekkebus and co-workers from the Ovaa group prepared nonhydrolyzable diUbs to prevent premature proteolytic degradation of the inhibitory diUb (**Figure 3C**).[97] Surprisingly, building block Ub-**Prg** inhibited the UCHL3 proteolytic activity by itself with unprecedented potency (IC_{50} < 40 pM) (**Figure 4A**). Intact protein analysis of recombinant purified UCHL3 incubated with Ub-**Prg** revealed an increased deconvoluted mass, corresponding to covalent addition of a single Ub-**Prg** (**Figure 4B**). SDS-PAGE analysis indicated quantitative formation of a stable covalent adduct within one minute, which was resistant to reducing agents (BME, DTT) and denaturing conditions (heating to 94 °C) (**Figure 4C**). Preincubation of UCHL3 with thiol alkylating agents (NEM, IAc) abolished adduct formation, indicative of cysteine modification. Adduct formation in MelJuSo cells expressing wild-type or catalytic CS mutant CysDUBs upon incubation with TMR-Ub-**Prg** – a Ub-**Prg** analogue modified with the fluorophore 5-carboxytetramethylrhodamine (TMR) on the N-terminus – was in line with specific modification of the catalytic cysteine residue. Covalent adduct with (TMR-)Ub-**Prg** was detected for members of all four CysDUB families known at that time (UCH, USP, OTU, and MJD), including notoriously unreactive members of the OTU DUB family that could not be

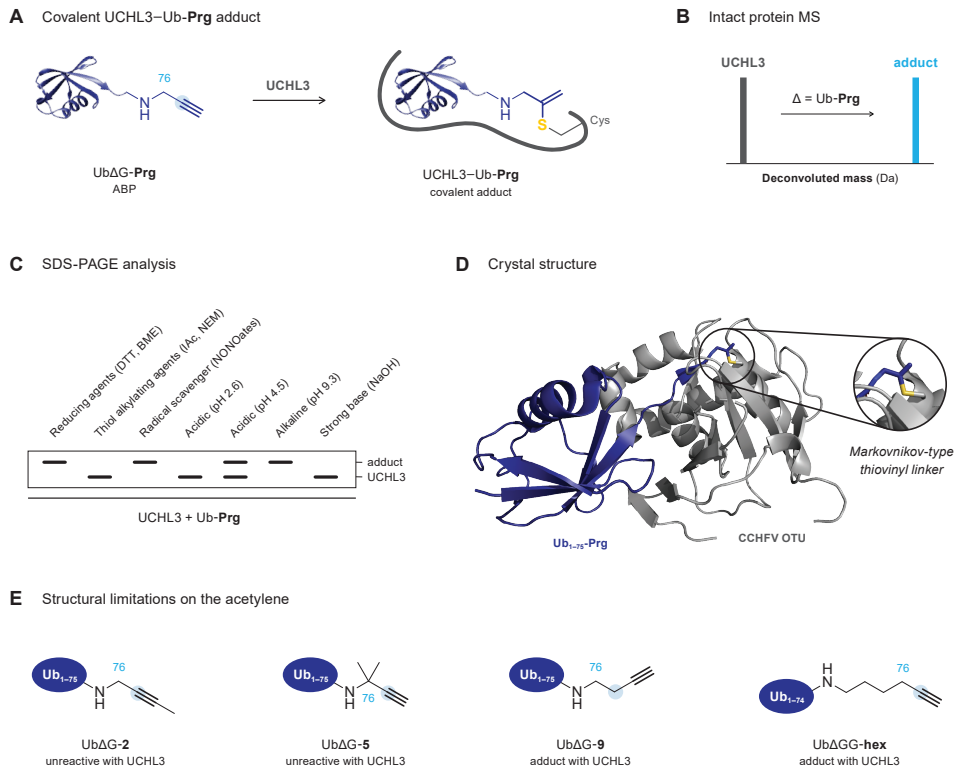


Figure 4 | Serendipitous discovery of terminal alkynes that react with cysteine deubiquitinases (CysDUBs) by Ekkebus *et al.*[97] **(A)** Building block Ub-**Prg** (synthetic $Ub_{1–75}$ or UbΔG modified with a propargyl warhead on the C-terminus) inhibits proteolytic UCHL3 activity through covalent modification of the catalytic cysteine residue. The reactive carbon is aligned with the carbonyl in native ubiquitinated substrates. **(B)** Intact protein MS of covalent UCHL3–Ub-**Prg** adduct reveals an increase in deconvoluted mass corresponding with addition of a single Ub-**Prg**. **(C)** SDS-PAGE gel analysis of recombinant UCHL3 incubated with Ub-**Prg** under different reaction conditions. Visualization by Coomassie protein staining. **(D)** Protein crystallography of Ub-**Prg** bound to CCHFV (Crimean Congo Hemorrhagic Fever Virus) OTU domain reveals a covalent Markovnikov-type thiovinyl adduct (PDB: 3ZNH). **(E)** Covalent adduct formation of propargylamide analogues with recombinant UCHL3, as detected by SDS-PAGE analysis. Alignment with site of cysteine attack in native substrate (see **Figure 3A**) is marked with blue.

probed with other Ub-ABPs. This reactivity was combined with an excellent target selectivity: Ub-**Prg** adduct was not observed with the nontargeted cysteines in cysteine proteases of other classes (e.g. SENP6, UBE1) nor with cysteine-rich BSA.

The covalent UCHL3–Ub-**Prg** adduct was stable to mild acid but was labile to strong acid, as is to be expected for a thiovinyl linker. However, preincubation of UCHL3 with NONOates (producing the water-soluble radical scavenger NO) or galvinoxyl free radical did not impair adduct formation (**Figure 4C**), contradicting a radical-mediated thiol–yne mechanism. Finally, the crystal structure of Ub-**Prg** bound to the vOTU (viral ovarian tumor DUB) of CCHFV (Crimean Congo hemorrhagic fever virus) unambiguously revealed a covalent adduct

with a Markovnikov-type vinyl thioether between the catalytic cysteine thiol and the internal alkyne carbon (**Figure 4D**). UCHL3–ABP adduct formation with Ub(ΔG)-**Prg** analogues provided insight on the structural limitations of the terminal alkyne warhead (**Figure 4E**). Terminal methylation of the alkyne disrupted adduct formation with Ub(ΔG)-**2**, suggesting the terminal CH proton has an important role. The backbone amide was found to be unimportant as covalent adduct was still observed with but-3-ynyl analogue Ub(ΔG)-**9** as well as with Ub(ΔG)-**Prg** isostere Ub(ΔGG)-**Hex**. Geminal dimethylation of the internal carbon impaired adduct formation with Ub(ΔG)-**5**, so it was not possible to exclude formation of an allenic intermediate at the enzyme active site.

Another serendipitous discovery of covalent adduct formation with Ub(l)-Prg

Around the same time, Sommer and co-workers [98] independently discovered that SUMO2-**Prg** can form a covalent adduct with SENP1, a human SUMO-specific cysteine protease (**Figure 5A**). In agreement with the findings of Ekkebus *et al.*,[97] SDS-PAGE analysis revealed an mass increase corresponding with a covalent SENP1–SUMO2-**Prg** adduct that was stable to denaturing and reducing conditions, and its formation was unaffected by strict exclusion of light, presence of radical scavenger sodium ascorbate or mildly acidic conditions.[98] Mutagenesis studies of key catalytic residues provided valuable insight into the reaction mechanism (**Figure 5B**). SENP1 has a catalytic triad that consists of Cys603, His533 and Asp550, in which Cys603 acts as the nucleophile after His533 deprotonates the thiol to form the active thiolate (**Figure 5C**).[119] The

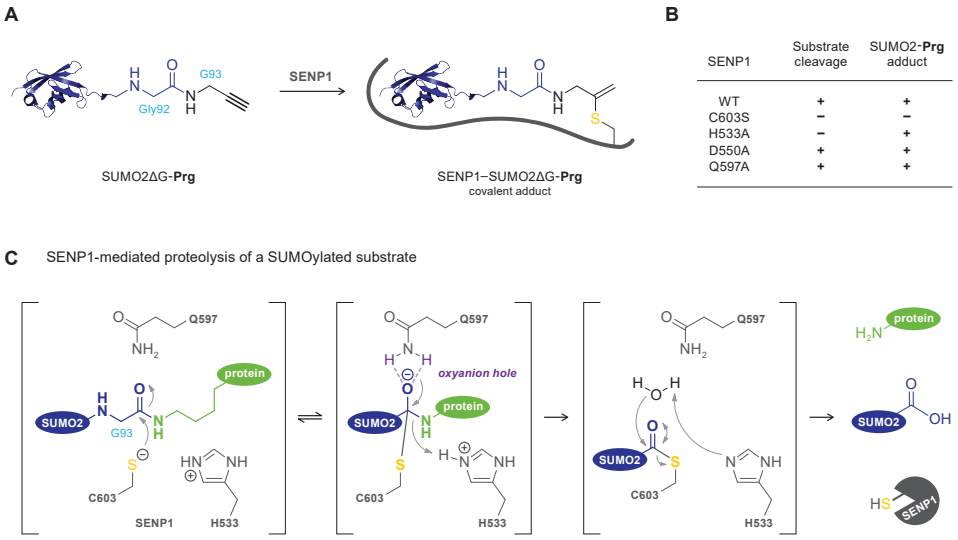


Figure 5 | Serendipitous discovery of covalent adduct formation of SUMO2-**Prg** with SENP1 by Sommer *et al.*[98] Structural SUMO2 representation based on noncovalent SENP1–SUMO2 complex (PDB: 2CKH). **(A)** Formation of a covalent SENP1–SUMO2-**Prg** adduct. **(B)** Mutagenesis studies with (mutant) recombinant SENP1. Proteolytic activity against a SUMOylated model substrate and covalent adduct formation with SUMO2-**Prg** were detected by gel analysis. **(C)** Simplified reaction mechanism for SENP1-mediated proteolysis of SUMOylated protein substrates, adapted from the general mechanism for CysDUBs.[120] The stepwise reaction involves stabilization of the anionic tetrahedral intermediate in the oxyanion hole, *via* stabilizing interactions with the Gln597 residue.

SENP1^{C603S} and SENP1^{H533A} mutants were proteolytically inactive and were no longer able to process the SUMOylated model protein (**Figure 5B**). Covalent adduct with ABPs SUMO2-**Prg** and SUMO2-**VS** was not observed for the C603S mutant but the H533A mutation did not affect adduct formation with either ABP, indicating the thiol–alkyne reaction may not require formation of an active thiolate. SENP1-catalyzed proteolysis of native SUMOylated substrates involves stabilization of an anionic tetrahedral intermediate in the oxyanion hole, through interaction with the polar Gln597 residue (**Figure 5C**).[119] Based on the maintained covalent adduct formation with the SENP1^{Q597A} mutant, the authors proposed an *in situ* proximity-driven reaction mechanism that does not involve stabilization in the oxyanion hole, though the role of stabilizing interactions with backbone amides cannot be excluded.

Mechanism of covalent thiol–alkyne addition

The serendipitous discoveries that Ub(l)-alkyne ABPs can form a Markovnikov-type thiovinyl adduct with the catalytic cysteine thiol of cysteine proteases prompted investigations into the reaction mechanism of this novel and unexpected reaction (**Scheme 1**). The proposed mechanisms can be divided into four general classes: radical-mediated addition to the alkyne (**Scheme 1A**), nucleophilic concerted thiolate addition to the alkyne (**Scheme 1B**), nucleophilic/radical addition to a more reactive allenic intermediate (**Scheme 1C**), and nucleophilic stepwise thiolate addition to the alkyne (**Scheme 1D**).

One of the best-known thiol–alkyne reactions forming a thiovinyl product is the radical-mediated thiol–yne coupling (TYC) (*mechanism A1* in **Scheme 1A**).[121] Here, the sulfonyl radical attacks at the terminal C1 carbon forming an anti-Markovnikov-type thiovinyl product.[122] This mechanism was quickly excluded after the crystal structure of the vOTU–Ub-**Prg** adduct revealed a Markovnikov-type vinyl thioether adduct (**Figure 4D**). Ekkebus and co-workers[97] comment that existence of an alkyne radical in solution seems unlikely in presence of radical scavengers, but they argue that the potential existence of radical species at the enzyme active site cannot be eliminated. They proposed another radical-mediated mechanism that does generate the correct Markovnikov-type adduct (*mechanism A2* in **Scheme 1A**) but this thiyl radical addition to the more substituted C2 carbon contradicts the established reactivity in radical alkyne reactions: acetylenes undergo radical addition on the least substituted carbon – the terminal C1 carbon of propargylamine.[123]

Ekkebus[97] and Sommer[98] both proposed a concerted proximity-driven *in situ* thiol(ate)–alkyne addition mechanism (*mechanism B* in **Scheme 1B**). Here, the Markovnikov-type thiovinyl adduct is formed *via* direct nucleophilic attack of the catalytic cysteine thiol(ate) to the quaternary C2 carbon of the alkyne, with concurrent protonation of the terminal C1 carbon.

An alternative explanation to the observed thiol–alkyne addition provided in the work of Ekkebus *et al.*[97] is that the unreactive alkyne is in equilibrium with a more reactive allenic intermediate at the enzyme active site (**Scheme 1C**).[124] Ynamine and ynamide groups are known to undergo base-mediated isomerization to form the more electrophilic allenamines/allenamides[125-126] that exhibit reactivity towards cysteine residues.[127] Moreover, allenamides are bioisosteres of the popular acrylamide warhead.[68, 128] It is unlikely that the thiol–alkyne addition proceeds *via* base-mediated formation of an allenic Ub-**Prg** intermediate in solution,

prior to enzyme binding, since covalent adduct proceeded to form in acidic buffers (**Figure 4C**). However, formation of an allenic intermediate at the enzyme active site cannot be ruled out based on the current data. Nucleophilic attack of a thiolate to the internal C2 carbon of the allenimide warhead generates a Markovnikov-type thiovinyl product (*mechanism C1* in **Scheme 1C**).[127] Alternatively, radical intermediates are known to be involved in thiol addition of cysteine proteases to allenyl esters/amides (*mechanism C2* in **Scheme 1C**).[129] Thiyl radical addition to terminal allenes is possible at the terminal C1 and the quaternary C2 carbon and, contrary to radical attack on propargylamine (**Scheme 1A**), is expected to form the observed Markovnikov-type thiovinyl product.[123, 130]

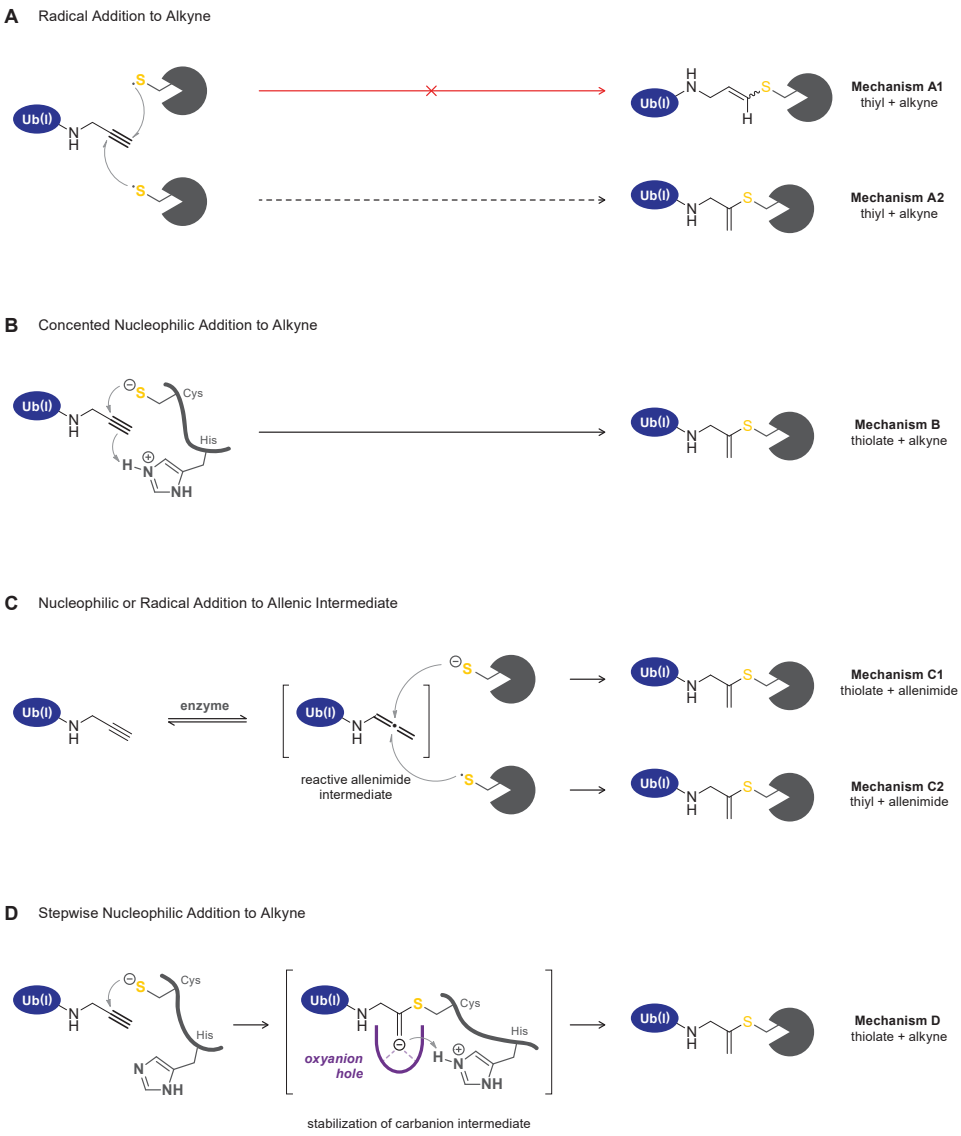
Finally, an alternative nucleophilic mechanism is suggested by Arkona and Rademann.[131] They propose an enzyme-templated stepwise reaction, with stabilization of a secondary carbanion intermediate in the protease oxyanion hole (*mechanism D* in **Scheme 1D**). This stepwise mechanism resembles the mechanism of cysteine/serine protease-mediated proteolysis of native amide bonds (**Figure 5C**): proteolysis involves stabilization of an anionic intermediate in the oxyanion hole, *via* interactions with polar residues such as glutamine.[120, 132] Covalent adduct formation of SUMO2-**Prg** with the SENP1^{Q597A} mutant does not support this mechanism (**Figure 5B**), though the role of stabilizing interactions with backbone amides cannot be excluded.

4. Scope of this Dissertation

In this dissertation, the scope and versatility of the thiol–alkyne addition to covalently modify targeted cysteine residues with nonactivated alkynes is further evaluated. The nonactivated terminal alkynes have the potential to be the perfect electrophile for irreversible covalent drug development: alkynes exhibit an unprecedented target reactivity with excellent thiol selectivity, thereby outperforming cysteine-reactive electrophilic moieties currently used in targeted covalent inhibitors (TCIs).

An essential step in covalent drug development is experimental detection of the covalent adduct to validate the covalent binding mode. In the first part of this thesis, we elaborate on the theoretical framework for evaluation of (ir)reversible covalent inhibitors. The wide array of technologies that have been employed in (recent) drug discovery are reviewed in ***Chapter 2***. These technologies strictly discriminate between a noncovalent protein–drug complexes and protein and drug engaged in a covalent protein–drug adduct. Next, as a covalent binding mode affects the relevant kinetic parameters to assess the structure-activity relationship (SAR) of an inhibitor, the theoretical background on kinetic evaluation of (ir)reversible covalent inhibitors is provided in ***Chapter 3***. Here we illustrate how reaction conditions affect the read-out and what assumptions are embedded in the algebraic equations to fit kinetic data. The theory is accompanied by kinetic simulations, step-wise protocols for experimental enzymatic activity assays and subsequent data analysis tailored to various covalent binding modes.

In the second part, the potential of the nonactivated alkyne as latent electrophile in small molecule covalent inhibitors is explored. Ekkebus[97] and Sommer[98] both used relatively large recognition elements (>8 kDa). Preliminary evaluations with small molecule CatS/Casp1



Scheme 1 | Proposed reaction mechanisms for Markovnikov-type thiovinyl adduct formation between a catalytic cysteine thiol(ate) and an Ub(I)-**Prg** ABP. **(A)** Direct addition of thiyl radical to the terminal alkyne. **(B)** Proximity-driven *in situ* thiol–alkyne addition with concerted nucleophilic attack and protonation. **(C)** Tautomerization of the terminal alkyne moiety to a thiol-reactive allenic intermediate at the enzyme active site prior to nucleophilic *(top)* or radical *(bottom)* addition. **(D)** Stepwise enzyme-templated thiol(ate)–alkyne addition *via* stabilization of a secondary carbanion intermediate in the enzyme oxyanion hole.

inhibitors (<1.8 kDa) – replacing the aldehyde warhead with an alkyne – were unsuccessful. In ***Chapter 4***, we first evaluate whether replacing an isoelectric nitrile warhead with an alkyne is a successful approach, as this is more likely to correctly place the internal alkyne

carbon in juxtaposition to the catalytic cysteine residue. As a proof-of-principle, we designed several alkyne derivatives of odanacatib (ODN), a reversible covalent inhibitor of cysteine protease cathepsin K (CatK) with a nitrile warhead. Potency, reversibility and covalent adduct formation of the alkyne analogues are evaluated on recombinant CatK as well as cultures of human osteoclast cells. Finally, protein crystallography is employed to validate formation of a Markovnikov-type covalent thiovinyl linker. In ***Chapter 5***, the scope is extended to noncatalytic cysteines – less nucleophilic (nonconserved) cysteine residues that are targeted by covalent kinase inhibitors. Aside from compatibility with kinases – the most popular protein class for irreversible drug development – adduct formation with a noncatalytic cysteine residue could also provide mechanistic insight as kinases do not have an oxyanion hole to stabilize anionic intermediates (**Scheme 1D**). The irreversible covalent acrylamide warhead in dual EGFR/HER2 inhibitor neratinib is replaced by an alkyne warhead, and preliminary results on covalent adduct formation with the tyrosine kinase domain of EGFR are reported.

The third part focuses on the versatility and mechanism of the *in situ* thiol–alkyne reaction. In ***Chapter 6***, the impact of substituents on the alkyne warhead is explored. Covalent adduct formation with a panel of ubiquitin-based ABPs bearing substituents on the internal and terminal position of the propargylamide warhead is evaluated in cellular lysates and on recombinant DUBs. Moreover, MS evaluation of a covalent adduct with a deuterated propargylamide analogue provides evidence on the existence of an allenic intermediate (**Scheme 1C**).

Finally, the most important findings are summarized in ***Chapter 7***. The potential impact of the *in situ* thiol–alkyne reaction is placed in the context of covalent drug discovery and an outlook will be provided on the future prospects of this work.

5. References

1. Lemmon, M.A.; Schlessinger, J. Cell Signaling by Receptor Tyrosine Kinases. *Cell* **2010**, *141*, 1117-1134. doi: 10.1016/j.cell.2010.06.011.
2. Turdo, A.; D'Accardo, C.; Glaviano, A.; Porcelli, G.; Colarossi, C.; Colarossi, L.; Mare, M.; Faldetta, N.; Modica, C.; Pistone, G.; Bongiorno, M.R.; Todaro, M.; Stassi, G. Targeting Phosphatases and Kinases: How to Checkmate Cancer. *Front. Cell Dev. Biol.* **2021**, *9*, 690306. doi: 10.3389/fcell.2021.690306.
3. Hyun, K.; Jeon, J.; Park, K.; Kim, J. Writing, Erasing and Reading Histone Lysine Methylations. *Exp. Mol. Med.* **2017**, *49*, e324. doi: 10.1038/emm.2017.11.
4. Fallah, M.S.; Szarics, D.; Robson, C.M.; Eubanks, J.H. Impaired Regulation of Histone Methylation and Acetylation Underlies Specific Neurodevelopmental Disorders. *Front. Genet.* **2021**, *11*, 613098. doi: 10.3389/fgene.2020.613098.
5. Hameed, D.S.; Sapmaz, A.; Ovaa, H. How Chemical Synthesis of Ubiquitin Conjugates Helps To Understand Ubiquitin Signal Transduction. *Bioconjugate Chem.* **2017**, *28*, 805-815. doi: 10.1021/acs.bioconjchem.6b00140.
6. Copeland, R.A. *Evaluation of Enzyme Inhibitors in Drug Discovery: A Guide for Medicinal Chemists and Pharmacologists*. Second ed.; John Wiley & Sons, Inc.: Hoboken, New Jersey, 2013. doi: 10.1002/9781118540398.
7. Holdgate, G.A.; Meek, T.D.; Grimley, R.L. Mechanistic Enzymology in Drug Discovery: A Fresh Perspective. *Nat. Rev. Drug Discov.* **2017**, *17*, 115. doi: 10.1038/nrd.2017.219.
8. Singh, J. The Ascension of Targeted Covalent Inhibitors. *J. Med. Chem.* **2022**, *65*, 5886-5901. doi: 10.1021/acs.jmedchem.1c02134.
9. Copeland, R.A. The Drug–Target Residence Time Model: A 10-Year Retrospective. *Nat. Rev. Drug Discov.* **2016**, *15*, 87-95. doi: 10.1038/nrd.2015.18.
10. Barf, T.; Kaptein, A. Irreversible Protein Kinase Inhibitors: Balancing the Benefits and Risks. *J. Med. Chem.* **2012**, *55*, 6243-6262. doi: 10.1021/jm3003203.
11. Singh, J.; Petter, R.C.; Baillie, T.A.; Whitty, A. The Resurgence of Covalent Drugs. *Nat. Rev. Drug Discov.* **2011**, *10*, 307-317. doi: 10.1038/nrd3410.
12. De Vita, E. 10 Years into the Resurgence of Covalent Drugs. *Future Med. Chem.* **2021**, *13*, 193-210. doi: 10.4155/fmc-2020-0236.
13. Mons, E.; Roet, S.; Kim, R.Q.; Mulder, M.P.C. A Comprehensive Guide for Assessing Covalent Inhibition in Enzymatic Assays Illustrated with Kinetic Simulations. *Curr. Protoc.* **2022**, *2*, e419. doi: 10.1002/cpz1.419.
14. Abdeldayem, A.; Raouf, Y.S.; Constantinescu, S.N.; Moriggl, R.; Gunning, P.T. Advances in Covalent Kinase Inhibitors. *Chem. Soc. Rev.* **2020**, *49*, 2617-2687. doi: 10.1039/C9CS00720B.
15. Kim, H.; Hwang, Y.S.; Kim, M.; Park, S.B. Recent Advances in the Development of Covalent Inhibitors. *RSC Med. Chem.* **2021**, *12*, 1037-1045. doi: 10.1039/D1MD00068C.

16. Baillie, T.A. Drug–Protein Adducts: Past, Present, and Future. *Med. Chem. Res.* **2020**, *29*, 1093-1104. doi: 10.1007/s00044-020-02567-8.
17. Park, B.K.; Boobis, A.; Clarke, S.; Goldring, C.E.P.; Jones, D.; Kenna, J.G.; Lambert, C.; Laverty, H.G.; Naisbitt, D.J.; Nelson, S.; Nicoll-Griffith, D.A.; Obach, R.S.; Routledge, P.; Smith, D.A.; Tweedie, D.J.; Vermeulen, N.; Williams, D.P.; Wilson, I.D.; Baillie, T.A. Managing the Challenge of Chemically Reactive Metabolites in Drug Development. *Nat. Rev. Drug Discov.* **2011**, *10*, 292-306. doi: 10.1038/nrd3408.
18. Baillie, T.A. Future of Toxicology–Metabolic Activation and Drug Design: Challenges and Opportunities in Chemical Toxicology. *Chem. Res. Toxicol.* **2006**, *19*, 889-893. doi: 10.1021/tx060062o.
19. Potashman, M.H.; Duggan, M.E. Covalent Modifiers: An Orthogonal Approach to Drug Design. *J. Med. Chem.* **2009**, *52*, 1231-1246. doi: 10.1021/jm8008597.
20. Schwöbel, J.A.H.; Koleva, Y.K.; Enoch, S.J.; Bajot, F.; Hewitt, M.; Madden, J.C.; Roberts, D.W.; Schultz, T.W.; Cronin, M.T.D. Measurement and Estimation of Electrophilic Reactivity for Predictive Toxicology. *Chem. Rev.* **2011**, *111*, 2562-2596. doi: 10.1021/cr100098n.21. Dahal, U.P.; Obach, R.S.; Gilbert, A.M. Benchmarking *In Vitro* Covalent Binding Burden As a Tool To Assess Potential Toxicity Caused by Nonspecific Covalent Binding of Covalent Drugs. *Chem. Res. Toxicol.* **2013**, *26*, 1739-1745. doi: 10.1021/tx400301q.
22. Nakayama, S.; Atsumi, R.; Takakusa, H.; Kobayashi, Y.; Kurihara, A.; Nagai, Y.; Nakai, D.; Okazaki, O. A Zone Classification System for Risk Assessment of Idiosyncratic Drug Toxicity Using Daily Dose and Covalent Binding. *Drug Metab. Dispos.* **2009**, *37*, 1970. doi: 10.1124/dmd.109.027797.
23. Mosedale, M.; Watkins, P.B. Understanding Idiosyncratic Toxicity: Lessons Learned from Drug-Induced Liver Injury. *J. Med. Chem.* **2020**, *63*, 6436-6461. doi: 10.1021/acs.jmedchem.9b01297.
24. Gan, J.; Ruan, Q.; He, B.; Zhu, M.; Shyu, W.C.; Humphreys, W.G. In Vitro Screening of 50 Highly Prescribed Drugs for Thiol Adduct Formation—Comparison of Potential for Drug-Induced Toxicity and Extent of Adduct Formation. *Chem. Res. Toxicol.* **2009**, *22*, 690-698. doi: 10.1021/tx800368n.
25. Gan, J.; Zhang, H.; Humphreys, W.G. Drug–Protein Adducts: Chemistry, Mechanisms of Toxicity, and Methods of Characterization. *Chem. Res. Toxicol.* **2016**, *29*, 2040-2057. doi: 10.1021/acs.chemrestox.6b00274.
26. Miller, J.A. The Metabolism of Xenobiotics to Reactive Electrophiles in Chemical Carcinogenesis and Mutagenesis: A Collaboration with Elizabeth Cavert Miller and our Associates. *Drug Metab. Rev.* **1998**, *30*, 645-674. doi: 10.3109/03602539808996326.
27. Liebler, D.C.; Guengerich, F.P. Elucidating Mechanisms of Drug-Induced Toxicity. *Nat. Rev. Drug Discov.* **2005**, *4*, 410-420. doi: 10.1038/nrd1720.
28. Ulrich, R.G. Idiosyncratic Toxicity: A Convergence of Risk Factors. *Annu. Rev. Med.* **2007**, *58*, 17-34. doi: 10.1146/annurev.med.58.072905.160823.
29. Uetrecht, J. Immune-Mediated Adverse Drug Reactions. *Chem. Res. Toxicol.* **2009**, *22*, 24-34. doi: 10.1021/tx800389u.
30. Kalgutkar, A.S.; Dalvie, D.K. Drug Discovery for a New Generation of Covalent Drugs. *Expert Opin. Drug Discov.* **2012**, *7*, 561-581. doi: 10.1517/17460441.2012.688744.
31. Lee, C.-U.; Grossmann, T.N. Reversible Covalent Inhibition of a Protein Target. *Angew. Chem. Int. Ed.* **2012**, *51*, 8699-8700. doi: 10.1002/anie.201203341.
32. Serafimova, I.M.; Pufall, M.A.; Krishnan, S.; Duda, K.; Cohen, M.S.; Maglathlin, R.L.; McFarland, J.M.; Miller, R.M.; Frödin, M.; Taunton, J. Reversible Targeting of Noncatalytic Cysteines with Chemically Tuned Electrophiles. *Nat. Chem. Biol.* **2012**, *8*, 471-476. doi: 10.1038/nchembio.925.
33. Faridoon; Ng, R.; Zhang, G.; Li, J.J. An Update on the Discovery and Development of Reversible Covalent Inhibitors. *Med. Chem. Res.* **2023**, *32*, 1039-1062. doi: 10.1007/s00044-023-03065-3.
34. World Health Organization. *Model Lists of Essential Medicines.* https://www.who.int/groups/expert-committee-on-selection-and-use-of-essential-medicines/essential-medicines-lists (accessed 2023-05-21).
35. Ghosh, A.K.; Samanta, I.; Mondal, A.; Liu, W.R. Covalent Inhibition in Drug Discovery. *ChemMedChem* **2019**, *14*, 889-906. doi: 10.1002/cmdc.201900107.
36. Fuster, V.; Sweeny, J.M. Aspirin: A Historical and Contemporary Therapeutic Overview. *Circulation* **2011**, *123*, 768-778. doi: 10.1161/CIRCULATIONAHA.110.963843.
37. Vane, J.R. Inhibition of Prostaglandin Synthesis as a Mechanism of Action for Aspirin-like Drugs. *Nature New Biol.* **1971**, *231*, 232-235. doi: 10.1038/newbio231232a0.
38. Roth, G.J.; Majerus, P.W. The Mechanism of the Effect of Aspirin on Human Platelets. I. Acetylation of a Particulate Fraction Protein. *J. Clin. Invest.* **1975**, *56*, 624-632. doi: 10.1172/JCI108132.
39. Vane, J.R.; Botting, R.M. The Mechanism of Action of Aspirin. *Thromb. Res.* **2003**, *110*, 255-258. doi: 10.1016/S0049-3848(03)00379-7.
40. Roth, G.J.; Stanford, N.; Majerus, P.W. Acetylation of Prostaglandin Synthase by Aspirin. *Proc. Natl. Acad. Sci.* **1975**, *72*, 3073-3076. doi: 10.1073/pnas.72.8.3073.
41. Blobaum, A.L.; Marnett, L.J. Structural and Functional Basis of Cyclooxygenase Inhibition. *J. Med. Chem.* **2007**, *50*, 1425-1441. doi: 10.1021/jm0613166.
42. Yocum, R.R.; Rasmussen, J.R.; Strominger, J.L. The Mechanism of Action of Penicillin. Penicillin Acylates the Active Site of Bacillus Stearothermophilus D-Alanine Carboxypeptidase. *J. Biol. Chem.* **1980**, *255*, 3977-3986. doi: 10.1016/S0021-9258(19)85621-1.
43. Olbe, L.; Carlsson, E.; Lindberg, P. A Proton-Pump Inhibitor Expedition: the Case Histories of Omeprazole and Esomeprazole. *Nat. Rev. Drug Discov.* **2003**, *2*, 132-139. doi: 10.1038/nrd1010.
44. Lindberg, P.; Nordberg, P.; Alminger, T.; Braendstroem, A.; Wallmark, B. The Mechanism of Action of the Antisecretory Agent Omeprazole. *J. Med. Chem.* **1986**, *29*, 1327-1329. doi: 10.1021/jm00158a001.
45. Ding, Z.; Kim, S.; Dorsam, R.T.; Jin, J.; Kunapuli, S.P. Inactivation of the Human P2Y12 Receptor by Thiol Reagents Requires Interaction with Both Extracellular Cysteine Residues, Cys17 and Cys270. *Blood* **2003**, *101*, 3908-3914. doi: 10.1182/blood-2002-10-3027.
46. Maffrand, J.-P. The Story of Clopidogrel and Its Predecessor, Ticlopidine: Could These Major Antiplatelet and Antithrombotic Drugs be Discovered and Developed Today? *C. R. Chim.* **2012**, *15*, 737-743. doi: 10.1016/j.crci.2012.05.006.
47. Allgardsson, A.; Berg, L.; Akfur, C.; Hörnberg, A.; Worek, F.; Linusson, A.; Ekström, F.J. Structure of a Prereaction Complex Between the Nerve Agent Sarin, Its Biological Target Acetylcholinesterase, and the Antidote HI-6. *Proc. Natl. Acad. Sci.* **2016**, *113*, 5514-5519. doi: 10.1073/pnas.1523362113.
48. Shakarjian, M.P.; Heck, D.E.; Gray, J.P.; Sinko, P.J.; Gordon, M.K.; Casillas, R.P.; Heindel, N.D.; Gerecke, D.R.; Laskin, D.L.; Laskin, J.D. Mechanisms Mediating the Vesicant Actions of Sulfur Mustard after Cutaneous Exposure. *Toxicol. Sci.* **2010**, *114*, 5-19. doi: 10.1093/toxsci/kfp253.
49. Krumbhaar, E.B.; Krumbhaar, H.D. The Blood and Bone Marrow in Yellow Cross Gas (Mustard Gas) Poisoning: Changes Produced in the Bone Marrow of Fatal Cases. *J. Med. Res.* **1919**, *40*, 497-508.3.
50. DeVita, V.T., Jr.; Chu, E.A History of Cancer Chemotherapy. *Cancer Res.* **2008**, *68*, 8643-8653. doi: 10.1158/0008-5472.Can-07-6611.

51. Singh, R.K.; Kumar, S.; Prasad, D.N.; Bhardwaj, T.R. Therapeutic Journery of Nitrogen Mustard as Alkylating Anticancer Agents: Historic to Future Perspectives. *Eur. J. Med. Chem.* **2018**, *151*, 401-433. doi: 10.1016/j.ejmech.2018.04.001.
52. Brookes, P.; Lawley, P. The Reaction of Mono- and Di-Functional Alkylating Agents with Nucleic Acids. *Biochem. J.* **1961**, *80*, 496-503. doi: 10.1042/bj0800496.
53. Millard, J.T.; Raucher, S.; Hopkins, P.B. Mechlorethamine Cross-Links Deoxyguanosine Residues at 5'-GNC Sequences in Duplex DNA Fragments. *J. Am. Chem. Soc.* **1990**, *112*, 2459-2460. doi: 10.1021/ja00162a079.
54. Moore, M.A.; Ahmed, F.; Dunlap, R.B. Evidence for the Existence of Covalent Nucleotide-Thymidylate Synthase Complexes, Identification of Site of Attachment, and Enhancement by Folates. *J. Biol. Chem.* **1986**, *261*, 12745-12749. doi: 10.1016/S0021-9258(18)67155-8.
55. Myers, C.E.; Young, R.C.; Chabner, B.A. Biochemical Determinants of 5-Fluorouracil Response In Vivo. The Role of Deoxyuridylate Pool Expansion. *J. Clin. Invest.* **1975**, *56*, 1231-1238. doi: 10.1172/JCI108199.
56. Guterman, L. Covalent Drugs Form Long-lived Ties. *Chem. Eng. News Archive* **2011**, *89*, 19-26. doi: 10.1021/cen-v089n036.p019.
57. Bauer, R.A. Covalent Inhibitors in Drug Discovery: From Accidental Discoveries To Avoided Liabilities and Designed Therapies. *Drug Discov. Today* **2015**, *20*, 1061-1073. doi: 10.1016/j.drudis.2015.05.005.
58. Roskoski, R. Orally Effective FDA-Approved Protein Kinase Targeted Covalent Inhibitors (TCIs). *Pharmacol. Res.* **2021**, *165*, 105422. doi: 10.1016/j.phrs.2021.105422.
59. Singh, J.; Dobrusin, E.M.; Fry, D.W.; Haske, T.; Whitty, A.; McNamara, D.J. Structure-Based Design of a Potent, Selective, and Irreversible Inhibitor of the Catalytic Domain of the erbB Receptor Subfamily of Protein Tyrosine Kinases. *J. Med. Chem.* **1997**, *40*, 1130-1135. doi: 10.1021/jm960380s.
60. Li, D.; Ambrogio, L.; Shimamura, T.; Kubo, S.; Takahashi, M.; Chirieac, L.R.; Padera, R.F.; Shapiro, G.I.; Baum, A.; Himmelsbach, F.; Rettig, W.J.; Meyerson, M.; Solca, F.; Greulich, H.; Wong, K.K. BIBW2992, an Irreversible EGFR/HER2 Inhibitor Highly Effective in Preclinical Lung Cancer Models. *Oncogene* **2008**, *27*, 4702-4711. doi: 10.1038/onc.2008.109.
61. Engelman, J.A.; Zejnullahu, K.; Gale, C.-M.; Lifshits, E.; Gonzales, A.J.; Shimamura, T.; Zhao, F.; Vincent, P.W.; Naumov, G.N.; Bradner, J.E.; Althaus, I.W.; Gandhi, L.; Shapiro, G.I.; Nelson, J.M.; Heymach, J.V.; Meyerson, M.; Wong, K.-K.; Jänne, P.A. PF00299804, an Irreversible Pan-ERBB Inhibitor, Is Effective in Lung Cancer Models with *EGFR* and *ERBB2* Mutations that Are Resistant to Gefitinib. *Cancer Res.* **2007**, *67*, 11924-11932. doi: 10.1158/0008-5472.Can-07-1885.
62. McAulay, K.; Bilsland, A.; Bon, M. Reactivity of Covalent Fragments and Their Role in Fragment Based Drug Discovery. *Pharmaceuticals* **2022**, *15*, 1366. doi: 10.3390/ph15111366.
63. Baillie, T.A. Targeted Covalent Inhibitors for Drug Design. *Angew. Chem. Int. Ed.* **2016**, *55*, 13408-13421. doi: 10.1002/anie.201601091.
64. Jianyao, W.; Xiao Xian, L.-C.; Jim, A.; Lin, D.; Robert, E.; Linning, Y.; Peter, H.; Steven, R.; Susan, L.; Syed, A.; Appavu, C.; Aram, O.; JoAnn, S.; Abdul, M.; Rasmy, T. Characterization of HKI-272 Covalent Binding to Human Serum Albumin. *Drug Metab. Dispos.* **2010**, *38*, 1083. doi: 10.1124/dmd.110.032292.
65. Shibata, Y.; Chiba, M. The Role of Extrahepatic Metabolism in the Pharmacokinetics of the Targeted Covalent Inhibitors Afatinib, Ibrutinib, and Neratinib. *Drug Metab. Dispos.* **2015**, *43*, 375.
66. Yver, A. Osimertinib (AZD9291)—A Science-Driven, Collaborative Approach to Rapid Drug Design and Development. *Ann. Oncol.* **2016**, *27*, 1165-1170. doi: 10.1093/annonc/mdw129.
67. Mons, E.; Kim, R.Q.; Mulder, M.P.C. Technologies for Direct Detection of Covalent Protein–Drug Adducts. *Pharmaceuticals* **2023**, *16*, 547. doi: 10.3390/ph16040547.
68. Gehringer, M.; Laufer, S.A. Emerging and Re-Emerging Warheads for Targeted Covalent Inhibitors: Applications in Medicinal Chemistry and Chemical Biology. *J. Med. Chem.* **2019**, *62*, 5673-5724. doi: 10.1021/acs.jmedchem.8b01153.
69. Talele, T.T. Acetylene Group, Friend or Foe in Medicinal Chemistry. *J. Med. Chem.* **2020**, *63*, 5625-5663. doi: 10.1021/acs.jmedchem.9b01617.
70. Lowe, A.B. Thiol-Yne 'Click'/Coupling Chemistry and Recent Applications in Polymer and Materials Synthesis and Modification. *Polymer* **2014**, *55*, 5517-5549. doi: 10.1016/j.polymer.2014.08.015.
71. Orlov, N.V. Metal Catalysis in Thiolation and Selenation Reactions of Alkynes Leading to Chalcogen-Substituted Alkenes and Dienes. *ChemistryOpen* **2015**, *4*, 682-697. doi: 10.1002/open.201500137.
72. Castarlenas, R.; Di Giuseppe, A.; Pérez-Torrente, J.J.; Oro, L.A. The Emergence of Transition-Metal-Mediated Hydrothiolation of Unsaturated Carbon–Carbon Bonds: A Mechanistic Outlook. *Angew. Chem. Int. Ed.* **2012**, *52*, 211-222. doi: 10.1002/anie.201205468.
73. Müller, T.E.; Hultzsch, K.C.; Yus, M.; Foubelo, F.; Tada, M. Hydroamination: Direct Addition of Amines to Alkenes and Alkynes. *Chem. Rev.* **2008**, *108*, 3795-3892. doi: 10.1021/cr0306788.
74. Hollenberg, P.F.; Kent, U.M.; Bumpus, N.N. Mechanism-Based Inactivation of Human Cytochromes P450s: Experimental Characterization, Reactive Intermediates, and Clinical Implications. *Chem. Res. Toxicol.* **2008**, *21*, 189-205. doi: 10.1021/tx7002504.
75. Ortiz de Montellano, P.R. Acetylenes: Cytochrome P450 Oxidation and Mechanism-Based Enzyme Inactivation. *Drug Metab. Rev.* **2019**, *51*, 162-177. doi: 10.1080/03602532.2019.1632891.
76. Wright, A.T.; Song, J.D.; Cravatt, B.F. A Suite of Activity-Based Probes for Human Cytochrome P450 Enzymes. *J. Am. Chem. Soc.* **2009**, *131*, 10692-10700. doi: 10.1021/ja9037609.
77. Tandarić, T.; Vianello, R. Computational Insight into the Mechanism of the Irreversible Inhibition of Monoamine Oxidase Enzymes by the Anti-Parkinsonian Propargylamine Inhibitors Rasagiline and Selegiline. *ACS Chem. Neurosci.* **2019**, *10*, 3532-3542. doi: 10.1021/acschemneuro.9b00147.
78. Parker, C.G.; Pratt, M.R. Click Chemistry in Proteomic Investigations. *Cell* **2020**, *180*, 605-632. doi: 10.1016/j.cell.2020.01.025.
79. Kolb, H.C.; Finn, M.G.; Sharpless, K.B. Click Chemistry: Diverse Chemical Function from a Few Good Reactions. *Angew. Chem. Int. Ed.* **2001**, *40*, 2004-2021. doi: 10.1002/1521-3773(20010601)40:11<2004::AID-ANIE2004>3.0.CO;2-5.
80. Bird, R.E.; Lemmel, S.A.; Yu, X.; Zhou, Q.A. Bioorthogonal Chemistry and Its Applications. *Bioconjugate Chem.* **2021**, *32*, 2457-2479. doi: 10.1021/acs.bioconjchem.1c00461.
81. Agard, N.J.; Baskin, J.M.; Prescher, J.A.; Lo, A.; Bertozzi, C.R. A Comparative Study of Bioorthogonal Reactions with Azides. *ACS Chem. Biol.* **2006**, *1*, 644-648. doi: 10.1021/cb6003228.
82. Cravatt, B.F.; Hsu, K.-L.; Weerapana, E. Activity-Based Protein Profiling. *Curr. Top. Microbiol. Immunol.* **2019**, *420*, 1-417. doi: 10.1007/978-3-030-11143-4.
83. van Geel, R.; Pruijn, G.J.M.; van Delft, F.L.; Boelens, W.C. Preventing Thiol-Yne Addition Improves the Specificity of Strain-Promoted Azide–Alkyne Cycloaddition. *Bioconjugate Chem.* **2012**, *23*, 392-398. doi: 10.1021/bc200365k.
84. Ramström, O. *Click Chemistry and Bioorthogonal Chemistry*; The Royal Swedish Academy of Sciences: NobelPrize.org, 5 October, 2022; pp 1-18. https://www.nobelprize.org/uploads/2022/10/advanced-chemistryprize2022-2.pdf.

85. Hermann, M.R.; Tautermann, C.S.; Sieger, P.; Grundl, M.A.; Weber, A. BIreactive: Expanding the Scope of Reactivity Predictions to Propynamides. *Pharmaceuticals* **2023**, *16*, 116. doi: 10.3390/ph16010116.
86. Shiu, H.-Y.; Chan, T.-C.; Ho, C.-M.; Liu, Y.; Wong, M.-K.; Che, C.-M. Electron-Deficient Alkynes as Cleavable Reagents for the Modification of Cysteine-Containing Peptides in Aqueous Medium. *Chem. Eur. J.* **2009**, *15*, 3839-3850. doi: 10.1002/chem.200800669.
87. Stieger, C.E.; Park, Y.; de Geus, M.A.R.; Kim, D.; Huhn, C.; Slenczka, J.S.; Ochtrop, P.; Müchler, J.M.; Süssmuth, R.D.; Broichhagen, J.; Baik, M.-H.; Hackenberger, C.P.R. DFT-Guided Discovery of Ethynyl-Triazolyl-Phosphinates as Modular Electrophiles for Chemoselective Cysteine Bioconjugation and Profiling. *Angew. Chem. Int. Ed.* **2022**, *61*, e202205348. doi: 10.1002/anie.202205348.
88. Koniev, O.; Leriche, G.; Nothisen, M.; Remy, J.-S.; Strub, J.-M.; Schaeffer-Reiss, C.; Van Dorsselaer, A.; Baati, R.; Wagner, A. Selective Irreversible Chemical Tagging of Cysteine with 3-Arylpropiolonitriles. *Bioconjugate Chem.* **2014**, *25*, 202-206. doi: 10.1021/bc400469d.
89. Barf, T.; Covey, T.; Izumi, R.; van de Kar, B.; Gulrajani, M.; van Lith, B.; van Hoek, M.; de Zwart, E.; Mittag, D.; Demont, D.; Verkaik, S.; Krantz, F.; Pearson, P.G.; Ulrich, R.; Kaptein, A. Acalabrutinib (ACP-196): A Covalent Bruton Tyrosine Kinase Inhibitor with a Differentiated Selectivity and In Vivo Potency Profile. *J. Pharmacol. Exp. Ther.* **2017**, *363*, 240. doi: 10.1124/jpet.117.242909.
90. Liclican, A.; Serafini, L.; Xing, W.; Czerwieniec, G.; Steiner, B.; Wang, T.; Brendza, K.M.; Lutz, J.D.; Keegan, K.S.; Ray, A.S.; Schultz, B.E.; Sakowicz, R.; Feng, J.Y. Biochemical Characterization of Tirabrutinib and Other Irreversible Inhibitors of Bruton's Tyrosine Kinase Reveals Differences in On - and Off - Target Inhibition. *Biochim. Biophys. Acta, Gen. Subj.* **2020**, *1864*, 129531. doi: 10.1016/j.bbagen.2020.129531.
91. Cordon, M.B.; Jacobsen, K.M.; Nielsen, C.S.; Hjerrild, P.; Poulsen, T.B. Forward Chemical Genetic Screen for Oxygen-Dependent Cytotoxins Uncovers New Covalent Fragments that Target GPX4. *ChemBioChem* **2022**, *23*, e202100253. doi: 10.1002/cbic.202100253.
92. Karaj, E.; Sindi, S.H.; Kuganesan, N.; Perera, L.; Taylor, W.; Tillekeratne, L.M.V. Tunable Cysteine-Targeting Electrophilic Heteroaromatic Warheads Induce Ferroptosis. *J. Med. Chem.* **2022**, *65*, 11788-11817. doi: 10.1021/acs.jmedchem.2c00909.
93. McAulay, K.; Hoyt, E.A.; Thomas, M.; Schimpl, M.; Bodnarchuk, M.S.; Lewis, H.J.; Barratt, D.; Bhavsar, D.; Robinson, D.M.; Deery, M.J.; Ogg, D.J.; Bernardes, G.J.L.; Ward, R.A.; Waring, M.J.; Kettle, J.G. Alkynyl Benzoxazines and Dihydroquinazolines as Cysteine Targeting Covalent Warheads and Their Application in Identification of Selective Irreversible Kinase Inhibitors. *J. Am. Chem. Soc.* **2020**, *142*, 10358-10372. doi: 10.1021/jacs.9b13391.
94. Al-Khawaldeh, I.; Al Yasiri, M.J.; Aldred, G.G.; Basmadjian, C.; Bordoni, C.; Harnor, S.J.; Heptinstall, A.B.; Hobson, S.J.; Jennings, C.E.; Khalifa, S., et al. An Alkynylpyrimidine-Based Covalent Inhibitor That Targets a Unique Cysteine in NF-κB-Inducing Kinase. *J. Med. Chem.* **2021**, *64*, 10001-10018. doi: 10.1021/acs.jmedchem.0c01249.
95. Wood, E.R.; Shewchuk, L.M.; Ellis, B.; Brignola, P.; Brashear, R.L.; Caferro, T.R.; Dickerson, S.H.; Dickson, H.D.; Donaldson, K.H.; Gaul, M., et al. 6-Ethynylthieno[3,2-d]- and 6-Ethynylthieno[2,3-d]pyrimidin-4-anilines as Tunable Covalent Modifiers of ErbB Kinases. *Proc. Natl. Acad. Sci.* **2008**, *105*, 2773. doi: 10.1073/pnas.0708281105.
96. Matheson, C.J.; Coxon, C.R.; Bayliss, R.; Boxall, K.; Carbain, B.; Fry, A.M.; Hardcastle, I.R.; Harnor, S.J.; Mas-Droux, C.; Newell, D.R.; Richards, M.W.; Sivaprakasam, M.; Turner, D.; Griffin, R.J.; Golding, B.T.; Cano, C. 2-Arylamino-6-ethynylpurines are Cysteine-Targeting Irreversible Inhibitors of Nek2 Kinase. *RSC Med. Chem.* **2020**, *11*, 707-731. doi: 10.1039/D0MD00074D.
97. Ekkebus, R.; van Kasteren, S.I.; Kulathu, Y.; Scholten, A.; Berlin, I.; Geurink, P.P.; de Jong, A.; Goerdayal, S.; Neefjes, J.; Heck, A.J.R.; Komander, D.; Ovaa, H. On Terminal Alkynes That Can React with Active-Site Cysteine Nucleophiles in Proteases. *J. Am. Chem. Soc.* **2013**, *135*, 2867-2870. doi: 10.1021/ja309802n.
98. Sommer, S.; Weikart, N.D.; Linne, U.; Mootz, H.D. Covalent Inhibition of SUMO and Ubiquitin-Specific Cysteine Proteases by an In Situ Thiol–Alkyne Addition. *Bioorg. Med. Chem.* **2013**, *21*, 2511-2517. doi: 10.1016/j.bmc.2013.02.039.
99. Kemp, M. Chapter Three - Recent Advances in the Discovery of Deubiquitinating Enzyme Inhibitors. *Prog. Med. Chem.* **2016**, *55*, 149-192. doi: 10.1016/bs.pmch.2015.10.002.
100. Swatek, K.N.; Komander, D. Ubiquitin Modifications. *Cell Res.* **2016**, *26*, 399-422. doi: 10.1038/cr.2016.39.
101. Komander, D.; Rape, M. The Ubiquitin Code. *Annu. Rev. Biochem.* **2012**, *81*, 203-229. doi: 10.1146/annurev-biochem-060310-170328.
102. Xu, P.; Duong, D.M.; Seyfried, N.T.; Cheng, D.; Xie, Y.; Robert, J.; Rush, J.; Hochstrasser, M.; Finley, D.; Peng, J. Quantitative Proteomics Reveals the Function of Unconventional Ubiquitin Chains in Proteasomal Degradation. *Cell* **2009**, *137*, 133-145. doi: 10.1016/j.cell.2009.01.041.
103. van Tol, B.D.M.; van Doodewaerd, B.R.; Lageveen-Kammeijer, G.S.M.; Jansen, B.C.; Talavera Ormeño, C.M.P.; Hekking, P.J.M.; Sapmaz, A.; Kim, R.Q.; Moutsiopoulou, A.; Komander, D.; Wuhrer, M.; van der Heden van Noort, G.J.; Ovaa, H.; Geurink, P.P. Neutron-Encoded Diubiquitins to Profile Linkage Selectivity of Deubiquitinating Enzymes. *Nat. Commun.* **2023**, *14*, 1661. doi: 10.1038/s41467-023-37363-6.
104. Pruneda, J.N.; Komander, D. Evaluating Enzyme Activities and Structures of DUBs. *Meth. Enzymol.* **2019**, *618*, 321-341. doi: 10.1016/bs.mie.2019.01.001.
105. Gorka, M.; Magnussen, H.M.; Kulathu, Y. Chemical Biology Tools to Study Deubiquitinases and Ubl Proteases. *Semin. Cell Dev. Biol.* **2022**, *132*, 86-96. doi: 10.1016/j.semcdb.2022.02.006.
106. Geurink, P.P.; van der Heden van Noort, G.J.; Mulder, M.P.C.; Knaap, R.C.M.; Kikkert, M.; Ovaa, H. Chapter Sixteen - Profiling DUBs and Ubl-Specific Proteases with Activity-Based Probes. *Meth. Enzymol.* **2019**, *618*, 357-387. doi: 10.1016/bs.mie.2018.12.037.
107. Ernst, A.; Avvakumov, G.; Tong, J.; Fan, Y.; Zhao, Y.; Alberts, P.; Persaud, A.; Walker, J.R.; Neculai, A.-M.; Neculai, D., et al. A Strategy for Modulation of Enzymes in the Ubiquitin System. *Science* **2013**, *339*, 590-595. doi: 10.1126/science.1230161.
108. Ekkebus, R.; Flierman, D.; Geurink, P.P.; Ovaa, H. Catching a DUB in the Act: Novel Ubiquitin-Based Active Site Directed Probes. *Curr. Opin. Chem. Biol.* **2014**, *23*, 63-70. doi: 10.1016/j.cbpa.2014.10.005.
109. de Jong, A.; Merkx, R.; Berlin, I.; Rodenko, B.; Wijdeven, R.H.M.; El Atmioui, D.; Yalçin, Z.; Robson, C.N.; Neefjes, J.J.; Ovaa, H. Ubiquitin-Based Probes Prepared by Total Synthesis To Profile the Activity of Deubiquitinating Enzymes. *ChemBioChem* **2012**, *13*, 2251-2258. doi: 10.1002/cbic.201200497.
110. Huppelschoten, Y.; van der Heden van Noort, G.J. State of the Art in (Semi-)Synthesis of Ubiquitin- and Ubiquitin-like Tools. *Semin. Cell Dev. Biol.* **2022**, *132*, 74-85. doi: 10.1016/j.semcdb.2021.11.025.
111. El Oualid, F.; Merkx, R.; Ekkebus, R.; Hameed, D.S.; Smit, J.J.; de Jong, A.; Hilkmann, H.; Sixma, T.K.; Ovaa, H. Chemical Synthesis of Ubiquitin, Ubiquitin-Based Probes, and Diubiquitin. *Angew. Chem. Int. Ed.* **2010**, *49*, 10149-10153. doi: 10.1002/anie.201005995.
112. Hewings, D.S.; Flygare, J.A.; Bogyo, M.; Wertz, I.E. Activity-Based Probes for the Ubiquitin Conjugation–Deconjugation Machinery: New Chemistries, New Tools, and New Insights. *FEBS J.* **2017**, *284*, 1555-1576. doi: 10.1111/febs.14039.
113. Gjonaj, L.; Sapmaz, A.; Flierman, D.; Janssen, G.M.C.; van Veelen, P.A.; Ovaa, H. Development of a DUB-Selective Fluorogenic Substrate. *Chem. Sci.* **2019**, *10*, 10290-10296. doi: 10.1039/C9SC02226K.
114. Gjonaj, L.; Sapmaz, A.; González-Prieto, R.; Vertegaal, A.C.O.; Flierman, D.; Ovaa, H. USP7: Combining Tools towards Selectivity. *Chem. Commun.* **2019**, *55*, 5075-5078. doi: 10.1039/C9CC00969H.

115. Caba, C.; Mohammadzadeh, A.; Tong, Y. On the Study of Deubiquitinases: Using the Right Tools for the Job. *Biomolecules* **2022**, *12*, 703. doi: 10.3390/biom12050703.
116. Weikart, N.D.; Mootz, H.D. Generation of Site-Specific and Enzymatically Stable Conjugates of Recombinant Proteins with Ubiquitin-Like Modifiers by the Cul-Catalyzed Azide–Alkyne Cycloaddition. *ChemBioChem* **2010**, *11*, 774-777. doi: 10.1002/cbic.200900738.
117. Rečnik, L.-M.; Kandioller, W.; Mindt, T.L. 1,4-Disubstituted 1,2,3-Triazoles as Amide Bond Surrogates for the Stabilisation of Linear Peptides with Biological Activity. *Molecules* **2020**, *25*, 3576. doi: 10.3390/molecules25163576.
118. Shanmugham, A.; Fish, A.; Luna-Vargas, M.P.A.; Faesen, A.C.; el Oualid, F.; Sixma, T.K.; Ovaa, H. Nonhydrolyzable Ubiquitin−Isopeptide Isosteres as Deubiquitinating Enzyme Probes. *J. Am. Chem. Soc.* **2010**, *132*, 8834-8835. doi: 10.1021/ja101803s.
119. Xu, Z.; Au, S.W.N. Mapping Residues of SUMO Precursors Essential in Differential Maturation by SUMO-Specific Protease, SENP1. *Biochem. J.* **2005**, *386*, 325-330. doi: 10.1042/bj20041210.
120. Hanpude, P.; Bhattacharya, S.; Dey, A.K.; Maiti, T.K. Deubiquitinating Enzymes in Cellular Signaling and Disease Regulation. *IUBMB Life* **2015**, *67*, 544-555. doi: 10.1002/iub.1402.
121. Fairbanks, B.D.; Sims, E.A.; Anseth, K.S.; Bowman, C.N. Reaction Rates and Mechanisms for Radical, Photoinitated Addition of Thiols to Alkynes, and Implications for Thiol−Yne Photopolymerizations and Click Reactions. *Macromolecules* **2010**, *43*, 4113-4119. doi: 10.1021/ma1002968.
122. Jayasree, E.G.; Reshma, S. A Computational Study on the Reaction Mechanism and Energetics of Markovnikov and Anti-Markovnikov Addition in Alkyne Hydrothiolation Reactions. *Comput. Theor. Chem.* **2016**, *1098*, 13-21. doi: 10.1016/j.comptc.2016.10.012.
123. Dénès, F.; Pichowicz, M.; Povie, G.; Renaud, P. Thiyl Radicals in Organic Synthesis. *Chem. Rev.* **2014**, *114*, 2587-2693. doi: 10.1021/cr400441m.
124. Hashmi, A.S.K. Synthesis of Allenes by Isomerization Reactions. *Mod. Allene Chem.* **2004**, 2-50. doi: 10.1002/9783527619573.ch1.
125. Lu, T.; Lu, Z.; Ma, Z.-X.; Zhang, Y.; Hsung, R.P. Allenamides: A Powerful and Versatile Building Block in Organic Synthesis. *Chem. Rev.* **2013**, *113*, 4862-4904. doi: 10.1021/cr400015d.
126. Cameron, A.J.; Harris, P.W.R.; Brimble, M.A. On-Resin Preparation of Allenamidyl Peptides: A Versatile Chemoselective Conjugation and Intramolecular Cyclisation Tool. *Angew. Chem. Int. Ed.* **2020**, *59*, 18054-18061. doi: 10.1002/anie.202004656.
127. Abbas, A.; Xing, B.; Loh, T.-P. Allenamides as Orthogonal Handles for Selective Modification of Cysteine in Peptides and Proteins. *Angew. Chem. Int. Ed.* **2014**, *53*, 7491-7494. doi: 10.1002/anie.201403121.
128. Chen, D.; Guo, D.; Yan, Z.; Zhao, Y. Allenamide As a Bioisostere of Acrylamide in the Design and Synthesis of Targeted Covalent Inhibitors. *MedChemComm* **2018**, *9*, 244-253. doi: 10.1039/C7MD00571G.
129. Takeuchi, Y.; Fujiwara, T.; Shimone, Y.; Miyataka, H.; Satoh, T.; Kirk, K.L.; Hori, H. Possible Involvement of Radical Intermediates in the Inhibition of Cysteine Proteases by Allenyl Esters and Amides. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 6202-6205. doi: 10.1016/j.bmcl.2008.10.007.
130. Liu, L.; Ward, R.M.; Schomaker, J.M. Mechanistic Aspects and Synthetic Applications of Radical Additions to Allenes. *Chem. Rev.* **2019**, *119*, 12422-12490. doi: 10.1021/acs.chemrev.9b00312.
131. Arkona, C.; Rademann, J. Propargyl Amides as Irreversible Inhibitors of Cysteine Proteases—A Lesson on the Biological Reactivity of Alkynes. *Angew. Chem. Int. Ed.* **2013**, *52*, 8210-8212. doi: 10.1002/anie.201303544.
132. Ménard, R.; Storer, A.C. Oxyanion Hole Interactions in Serine and Cysteine Proteases. *Biol. Chem.* **1992**, *373*, 393-400. doi: 10.1515/bchm3.1992.373.2.393.