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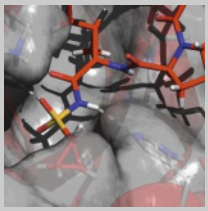

# Medicinal Chemistry and Chemical Biology Highlights

Division of Medicinal Chemistry and Chemical Biology
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## Covalency-enabled Drug Discovery is Redefining Druggability

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The connectivity between covalency and drug discovery has been established for a long time – though often these links only became obvious through a serendipitous finding, or by way of a post-hoc understanding of the mechanism of a drug.[1] Concerns of inadequate specificity and commensurate toxicity, together with a dearth of systematic approaches for drugging targets using covalent mechanisms of action, have limited the use of electrophilic compounds in drug discovery settings. This situation has changed during the last two decades, driven by advances in a number of drug discovery technologies ranging from mass spectrometry[2] to bio-orthogonal chemistry[3] and thereafter to rational drug design principles, all of which have led practitioners to reconsider a place for covalency in their toolbox.[4,5] Covalency now serves both as an advantageous modality to further harness low molecular weight (LMW) ligands (<600 Da) and as the critical principle under-pinning the interrogation of ligandability across the human proteome. The willingness to use covalency in drug discovery has changed. As a prime example, a recent retrospective analysis of the occurrence of common functional groups (FGs) in drugs and bioactive compounds highlighted a marked increase in cysteine-reactive electrophiles over the last 10 years.[6] The consequential advantages foreseen for covalent drugs include: increased potency, mitigation of resistance due to mutations within a binding site, and the potential change in pharmacokinetic (PK) requirements of a compound (as a result of pharmacodynamics and efficacy being linked to labeling efficiency and target occupancy and not simply to a sustained free drug concentration).[7] Furthermore, the 'irreversible' permanent modification of the target results in the initial protein function only being restored through protein re-synthesis.[8]

Two strategies for covalent drug optimization are available: *ligand-first*, wherein a reversible target-binding ligand has been identified and the electrophile is then added, or (more recently) an *electrophile-first* approach, where electrophilic (fragment-like) compound libraries can be used in initial screening.[9] This has led to a scenario where medicinal chemists and chemical biologists are now reassessing the druggability of a protein at a molecular level: how details of binding sites or pockets can be delineated, and how functional determinants illuminate the most appropriate drug discovery pathway (Fig. 1).[10]

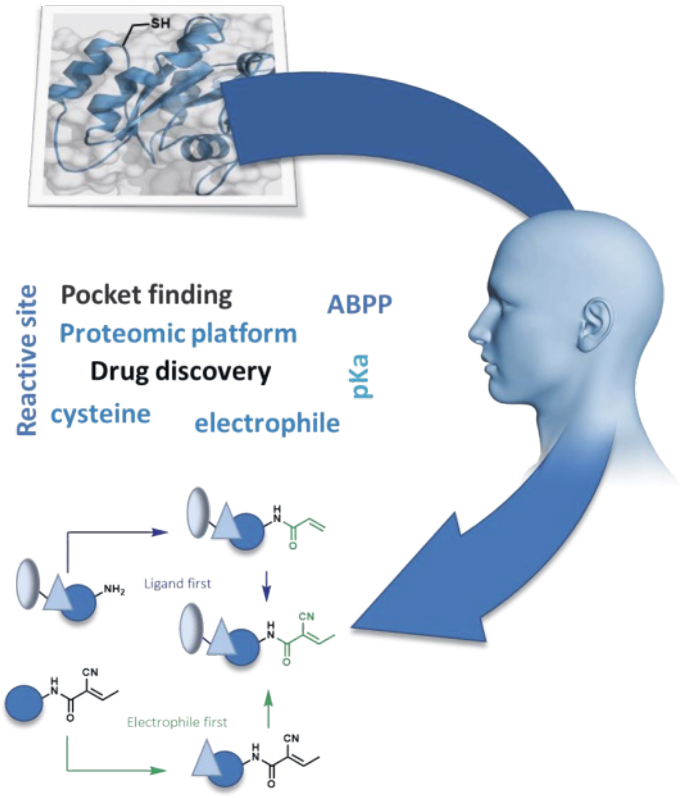


Fig. 1. Evolving opportunities for medicinal chemists through covalency. *Ligand-first*, where the reversible ligand is identified and the electrophile is then added, or *electrophile-first* where electrophilic fragment-like compound libraries can be used.

From the medicinal chemistry perspective, the molecular recognition between ligand and target is required in both *electrophile-first* and *ligand-first* approaches. The ligand efficiency (LE or LipE)[11–13] for each strategy may be similar, yet the molecular scaffolds will be entirely different in complexity. The requirements for an electrophile incorporated within a drug substance/LMW ligand are as follows:

1. That the reactive handle is optimally positioned within its target to allow bioconjugation;
2. That it confers further specificity with concomitant increased potency to the desired target;
3. That the electrophilic nature of the warhead is attenuated, thus mitigating reactions with bulk proteins within the body.

Numerous laboratories have been working to achieve these desires: those working to modify warheads (the electrophilic moiety) and determine their reactivities,[14,15] those showing that fragment-like electrophiles can be specific,[16] and finally those groups demonstrating that the rational addition of electrophiles to reversible ligands is not only achievable but worth the invest

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ment.[17] The result is a renewed research field with a broad scope that looks at multiple implications of electrophilicity – from labeling efficiency to clearance, from novel chemistry to new targets unveiled by proteomic platforms.

Although the historical landscape of covalent inhibitors through the 20th century had focused upon antibiotics (and the consequential use of serine (Ser) as the nucleophilic amino acid), the advent of targeted covalent inhibitors (TCIs) in the early 2000s brought the utility of non-catalytic cysteines into focus in drug discovery, fortuitously coinciding with landmark advances in bioinformatics.[18–20] These purposefully designed covalent molecules positioned weak electrophiles within proximity of rare cysteine (Cys) residues (cysteine is only expressed to ~2% within the human proteome) creating unique molecules with enhanced pharmacological profiles. These advances have even necessitated the development of a new nomenclature.[21]

Cysteine controls numerous cellular processes. It is involved in structural stabilisation of proteins *via* disulphide bridges, in redox chemistry, and in metal coordination. Critical to these functions is the pKa of the individual Cys residue. On average this pKa is 8.5, meaning at physiological pH of 7.4 only about 8% of the cysteine side chains will be in their thiolate form and hence more reactive. Importantly, the pKa of various Cys residues can be perturbed several orders of magnitude by their local environment and hence render them very reactive or quite inert.[22] Cysteine is a 'soft' nucleophile – a categorisation resulting from the sulfur's polarisability and large atomic radius. As a result, Cys residues will react with soft electrophiles. *In silico* predictions of Cys pKa are possible, albeit complex, and this strategy is often preferred to a physical determination.[23] In addition to the continually growing body of protein co-structures from experimental X-ray crystallography and Cryo-EM efforts, the advent of AlphaFold2 has firmly put both sequences and 3-dimensional topologies of proteins and the structural surroundings of Cys residues in the hands of bioinformaticians and structural biologists.[24,25] There are, however, shortcomings in current *in silico* workflows regarding accurate pKa calculations that need to be resolved before *a priori* predictions of covalent targeting can be achieved.[26]

*In silico* calculation limits aside, exploiting Cys reactivity alongside the aforementioned advances in the field of mass-spectrometry (MS)-based proteomics has recently led to the increased popularity of covalent chemoproteomics. This indispensable tool is being developed in pursuit of global profiling of reactive Cys residues or determining specificity of advanced ligands. Activity-based protein profiling (ABPP), pioneered by Cravatt and Bogyo,[27–29] in one of its newer variants can be considered as the combination of covalent fragment-like molecules working in tandem with proteomics, and is primed to unlock previously undruggable spaces.[30] This methodology can be applied to cell lysates or live cell systems, where proteins exist in their native state and their respective complexes can be interrogated. As a consequence, molecular and structural biology groups no longer need to produce the necessary recombinant protein, which can often only be done with truncated constructs when it is possible at all. The progression seen within this area has been transformative, moving from investigating orthosteric active sites to the detection of ligandable allosteric sites. As the screening is an unbiased approach, allowing the inspection of the entire human proteome in any given cell-type, where ~20,000 proteins in their native state may exist, it has created a wealth of opportunities. The necessary ABPP workflow requires a pan-Cys reactive probe, consisting of a highly reactive electrophile (often an iodo-acetamide) and an enrichment handle (*i.e.* an alkyne, fluorophore, biotin, desthiobiotin) (Fig. 2) to facilitate the detection of Cys-containing peptides and enable a competition-based assay. Desthiobiotin-iodoacetamide (DBIA), with its attenuated avidity, has also been used to great effect as the probe to increase speed without affecting quantitation.[31] Furthermore, the recent progress in quantitative MS approaches, often utilising the introduction of isotopic labeling *via* the probe (isoTOP ABPP) or *e.g.* the amine-reactive tandem mass tags (TMT), have enabled more accurate and precise quantitation as well as increased throughput *via* multiplexing.[32,33] The resultant processes are a high capacity system capable of rapidly quantifying ligand interactions and their sites of modification, determining proteome-wide selectivity of covalent ligands and lastly, identifying 'ligand hotspots' from ligands that have evoked specific phenotypes in directed cellular screens. These hotspots can be in catalytic active sites, be solvent exposed, exist in allosteric pockets, or be sites of post-translational modification.

These advances have meant that one can use electrophilic libraries in a *ligand-first* approach to determine novel and functional interaction maps for each compound. Though the initial electrophilic library concept was an extension of fragment-based ligand discovery (FBLD), these strategies are now being applied to mature ligands of high molecular and stereochemical complexity (*vide infra*). ABPP and its complementary workflows have been used widely, creating novel insights into protein ligandability and these efforts have required the necessary validation of function at the individual sites of modification *via* siRNA, CRISPR, and disease-relevant cell line analysis.[34]

The emerging modality of targeted protein degradation (TPD)[35] has created much excitement in the drug discovery

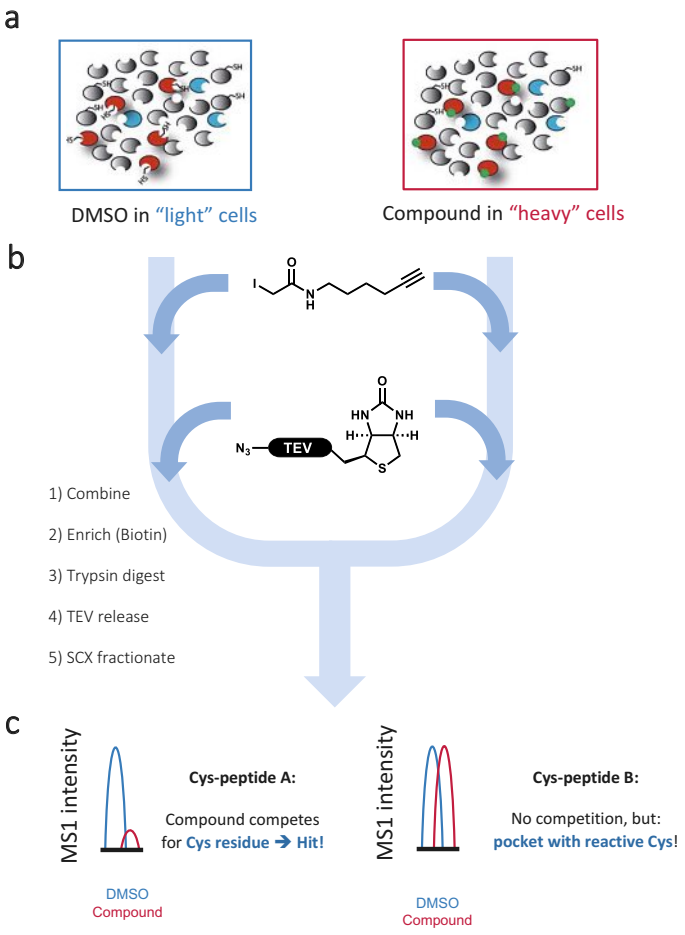


Fig. 2. isoTOP ABPP workflow, where a) isotopically labelled (heavy) live cells are treated with an unmodified electrophilic Cys-reactive compound, unlabeled (light) cells with a DMSO control and then the cells are lysed (non-denaturing). b) The remaining free Cys are then labeled with pan-Cys probe (iodo-acetamide-alkyne). c) The identification and quantification of Cys labeling in combined cell lysates is undertaken to determine the site of modification and the specificity of compound labeling, all by LCMS.

field. The simultaneous disclosures from the laboratories of Cravatt and Nomura detailed differing approaches harnessing ABPP to identify new ligases that can support TPD when engaged by electrophilic heterobifunctional proteolysis targeting chimeras (PROTACs).[36,37] TPD ligands have the potential to act catalytically, reducing the drug concentrations required to produce a pharmacological effect. There is also great opportunity for specificity due to restricted tissue distributions of the necessary E3 ligases. In one study, Cravatt and co-workers employed a non-selective electrophile in tandem with FKBP12-selective ligands as a tool system. They identified DCAF16 as an E3 ligase that could support the degradation. The key validation was achieved using a PROTAC derived from their scout ligand and the potent and selective BRD4 ligand JQ1 to achieve protein degradation of BRD4. Most interestingly this work highlighted how covalent modification of the E3 ligase reduced this system to a pseudo-binary complex meaning that sub-stochiometric engagement was now sufficient for effective degradation. Nomura and co-workers using nimbolide, an electrophilic natural product, also discovered a link to E3-ligase biology. The team found that nimbolide reacts with Cys8 located within an intrinsically disordered region of RNF114 and associated this modification of the E3 ligase with resultant anti-proliferative activity. Thereafter, nimbolide was harnessed as the chaperone motif to recruit RNF114 for TPD, again using BRD4 degradation as a proof of concept. As a result of these endeavours, DCAF16 and RNF114 have been added to the short list of ligases that have been co-opted to induce protein degradation of neo-substrates through induced proximity (Fig. 3). Other ligases such as RNF4,[38] FEM1B[39] and DCAF11 have been shown to be useful in TPD subsequently.[40,41] Most recently, the Gray, Ebert and Fischer groups showed that JQ-1 could be employed as a ligand to develop a novel class of degraders, again harnessing DCAF16. Critical to this effort was the use of a *ligand-first* approach, to develop what they termed 'template-assisted covalent modification'.[42]

Just as cellular machinery can be harnessed to degrade, so it can be used to stabilise. When contemplating employing covalent molecules to recruit deubiquitinase (DUB) enzymes for protein stabilisation, the clear challenge was to maintain the key thiol-mediated enzymatic activity of the DUB. Nomura and co-workers identified an allosteric, functionally silent cysteine to which to anchor an electrophilic ligand. Relying upon data mining and careful triaging of available DUBs, the authors arrived at an ABPP gel-based screening of recombinant DUB Otubain 1 (OTUB1) for identification of a suitable covalent ligand.[43] They then built that ligand into an active heterobifunctional molecule (coined as a DUBTAC) for deubiquitination of the cystic fibrosis transmembrane conductance regulator (CFTR) protein.

In a function-focused *electrophile-first* approach introduced by the Cravatt group, the combination of size-exclusion chromatography and ABPP were used to determine the net effect of electrophiles on the function of cellular complexes.[44] Here, clear functional effects in human cells were correlated to stereoselective and site-specific modification of protein–protein interactions. Disease-relevant cell types are critical to further the opportunities for ligand and pathway discovery. To this end, ABPP has been applied to human T-cells with a consequential ligandability map created across proteins in many signaling pathways and numerous transcription factors.[45]

In situations where an appropriate pan-reactive probe cannot be supplied to enable the competitive ABPP workflow, alkyne-substituted electrophilic compounds can be used. This has an added benefit of enrichment of the covalently modified target, thus allowing access to proteins of lower abundance. Similarly, photo-reactive probes such as diazirines allow an analogous and alternate strategy to globally map the interactome of fragments or mature ligands. This approach has been used to develop SLC15A4 inhibitors that suppress endosomal TLR and NOD functions in a

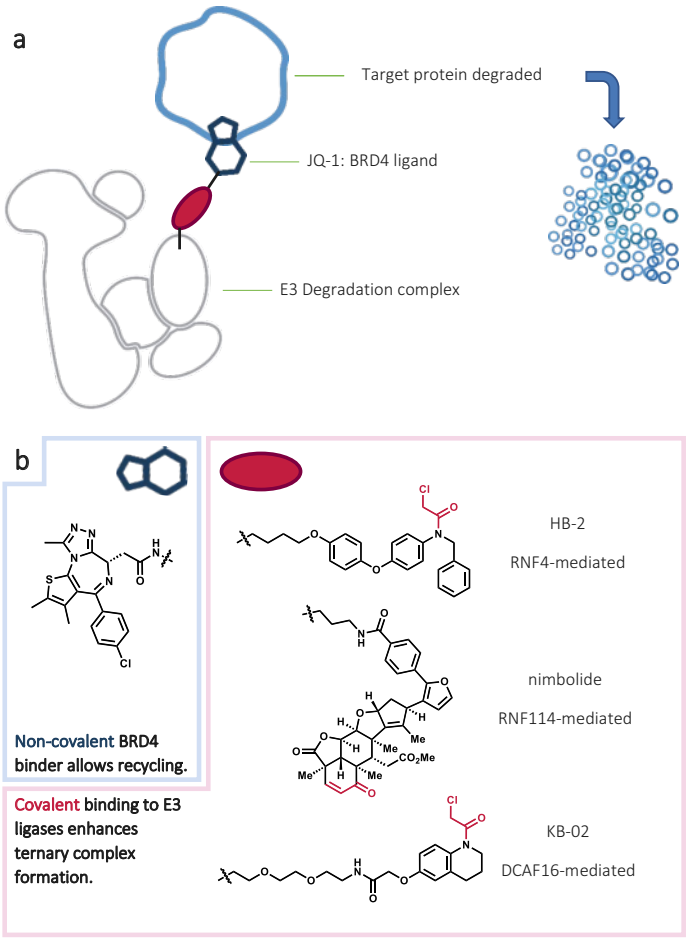


Fig. 3. a) Schematic representation of a ligase complex modified by an electrophilic heterobifunctional ligand leading to target (BRD4) degradation. b) The BRD4 ligand JQ-1, a thienotriazolodiazepine and potent inhibitor of the BET family of bromodomain proteins (blue box) and the tool heterobifunctional PROTACs developed to show that the novel E3 ligases (RNF114, DCAF16 and RNF4) can support TPD when engaged by an electrophilic mechanism.

variety of human and mouse immune cells, establishing SLC15A4 as a druggable target for the treatment of autoimmune and autoinflammatory conditions.[46,47]

Thus far the highlighted examples have been focused on irreversible covalent bond formation. An alternative fragment-based strategy that allows for the selection of compounds with weak inherent affinity was introduced by the group at Sunesis.[48] Termed 'disulfide tethering', it relies upon reversible covalent bond formation. The exchangeable nature of the disulfide bond formed allows for dynamic selection of an optimal ligand, maximising the molecular recognition which is then captured in the thiol-exchange reaction, enabling analysis by MS. This strategy was more recently exploited in the identification of KRAS G12C switch II binding molecules – a particularly powerful demonstration due to the discovery of a ligand-induced cryptic pocket.[49] In this case, optimisation of the reversible motif and replacement of the aforementioned disulfide with a more stable carbon-based electrophile, have ultimately led to the identification of AMG-510 (sotorasib), MTRX849 (adagrasib), JDQ443 and numerous other clinically relevant molecules.[50–53] Importantly, it should be noted at present there is only limited access to an appropriate hit-finding disulfide-exchange library from CRO or commercial vendors.[54] Despite this limitation, this chemistry was used to realise the concept of 'molecular locking', which features a dual-covalent molecule stapling both a cysteine and lysine (Lys) residue simultaneously (*vide infra*). The generated molecules were

effective molecular glues mediating and stabilising the formation an inter-molecular interface between 14-3-3 and ERRγ.[55]

For cysteine-targeted warhead design there are a number of strategies that can be explored, such as the hybridisation level at the reactive atom and the directionality built into the reactive architecture.[56,57] Depending upon the screening strategy chosen, the overall molecular complexity can vary. The fragment-like logic to electrophile libraries has continued to be exploited, but more elaborate enantio- and diasteromeric paired matched sets are now a feature which can facilitate the speed of validation.[58,59] The specificity of warheads[60,61] has also been excellently summarised and there is a continual flow of new options appearing, with some inspired by natural products.[62–64] The demands for new designs will continue as cysteine targets become more ambitious requiring increased creativity from chemists.[65,66] These advances will also undoubtedly continue to pave the road towards targets beyond cysteine.[67]

## Reversible Covalent Ligands

Given the soft, stabilised nature of Cys thiol, it is also possible for it to engage in reversible processes with appropriate covalent handles.[68] Such electrophilic warheads have the possibility to ameliorate off-target effects as well as immunogenic or toxic events associated with more reactive warheads because the ligand is not permanently bound to the target protein. Consequently, the tuning of the reactivity of these warhead types can lead to increased residency and potency along with improvements in the specificity as a result of liganding a proximal specific amino acid.

Protease inhibitors have been a hotbed for compounds acting in a reversible covalent manner, and recent years are no exception. In Pfizer's pursuit of an orally bioavailable main protease ($M^{pro}$) inhibitor for SARS-COV-2 treatment,[69] the inclusion of a nitrile solved issues associated with a hydroxy-ketone that was creating significant pharmacokinetic challenges.[70] The addition of the nitrile reduced the polar surface of the molecule while providing the essential reversible electrophile which forms a thioimidate with the catalytically active Cys145.

Within the PROTAC arena, reversible covalent PoI binders represent another area of promise in the march towards druggability. PROTACs operate catalytically, however this advantage would be lost if the ligand were to covalently engage the target protein irreversibly.[71] Utilising a reversible covalent binder, the necessary ternary complex can be (re)-generated allowing both degradation and drug recycling to be achieved; this was well illustrated recently for BTK-targeting degraders.[72,73]

## Beyond Cysteine

Cysteine's intrinsic reactivity makes it a convenient and attractive site for covalent ligand anchoring, yet it is a relatively rare residue and can be absent in a particular protein of interest.[74] The lower reactivity of other potential nucleophilic residues adds considerable further challenges to their use, not least of which is how to survive the body's cysteine-based clearance methods.

Lysine is far more abundant in the proteome and is being rapidly adopted as a covalent anchor and target for specific bioconjugation strategies. Lysine's nucleophilicity is lower than that of cysteine and its pKa, on average 10.4, renders many Lys residues undruggable at physiological pH. Nonetheless, Lys-specific covalent chemoproteomic workflows have been developed, with Abbasov, Cravatt, and co-workers recently publishing an atlas of proteomic lysine reactivity.[75] They undertook investigation with a wide array of aminophilic probes,[76,77] which have broad and differentiated reactivities. The studies identified non-specific dicarboxaldehydes applicable for use as 'scout' ligands and squaramate and cyano-methyl acyl sulphonamides for selective labeling (Fig. 4). As an example, within the squarates, molecules were identified that were selective for K153 in the Hsc70-interacting protein (ST13).

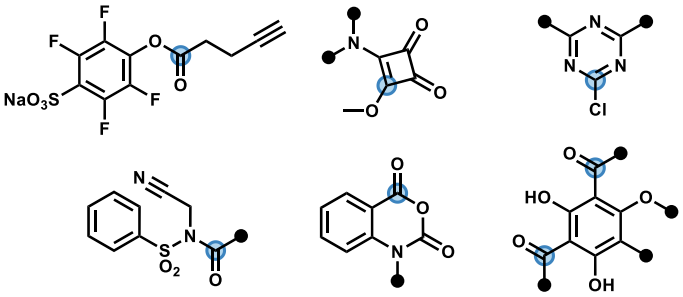


Fig. 4. A representative selection from the diverse aminophilic probes used to target lysine and develop a proteome-wide atlas, with highlighted reaction centre.

Although active site serine targeting has been viable for decades, its generally low reactivity (pKa 13–14) renders these residues challenging to target when they are not in catalytic diads and triads. Following the determination that covalent approaches could effectively inhibit KRAS G12C,[49] a similar solution to address the inhibition of the cancer-relevant mutant KRAS G12S has been reported. Many regio- and stereo-isomers of a series of electrophilic lactones were synthesised and the critical positioning, despite highly similar intrinsic reactivities, of only one of the lactones led to the selective and preferential labelling of the non-catalytic mutant serine.[78]

Aspartic acid (Asp) lies still further down in the nucleophilicity hierarchy, though granted its much more labile acidic proton may present some advantages (pKa 3.9). A patent application targeting KRAS G12D (highly prevalent in pancreatic, colorectal and lung cancers)[79] highlighted that carbodiimides, aziridines and oxazolines are effective electrophilic warheads capable of modifying the carboxylate functional group.[80]

Even side chains with no ionisable nucleophilic residue are now being exploited for covalent drug discovery.[81] Founded on their breakthrough report in 2017,[82] Chang and Toste, in collaboration with Novartis, recently applied their ABPP-based Redox-Activated Chemical Tagging (ReACT) strategy to the discovery of allosteric methionine (Met) binding sites in cyclin-dependent kinase 4 (CDK4).[83] The use of a designed oxaziridine-derived fragment library identified an allosteric Met site, M169, which when oxidised prevented the phosphorylation of the activating T172 and blocked cell cycle progression (Fig. 5). This insight into the redox regulation of CDK4 offers a new potential therapeutic intervention and showcases the power of the chemoproteomics methodology. Such redox-active approaches to covalency will likely be pursued heavily in future years as the community takes aim at more recalcitrant residues.

To conclude, covalency can aid hit finding, target finding, or be used as an optimisation strategy as compounds move through optimisation and toward the clinic. Hopefully, through the highlighted examples it can be appreciated that covalency in drug discovery is not a catholicon for all, but when used with thought and with careful consideration of the biological target it offers a new modality to drug difficult targets that would otherwise not be approachable. New advances have rightfully placed this chemistry at the front of drug discovery, reframing our perspective into the universe of the 'undruggable'.

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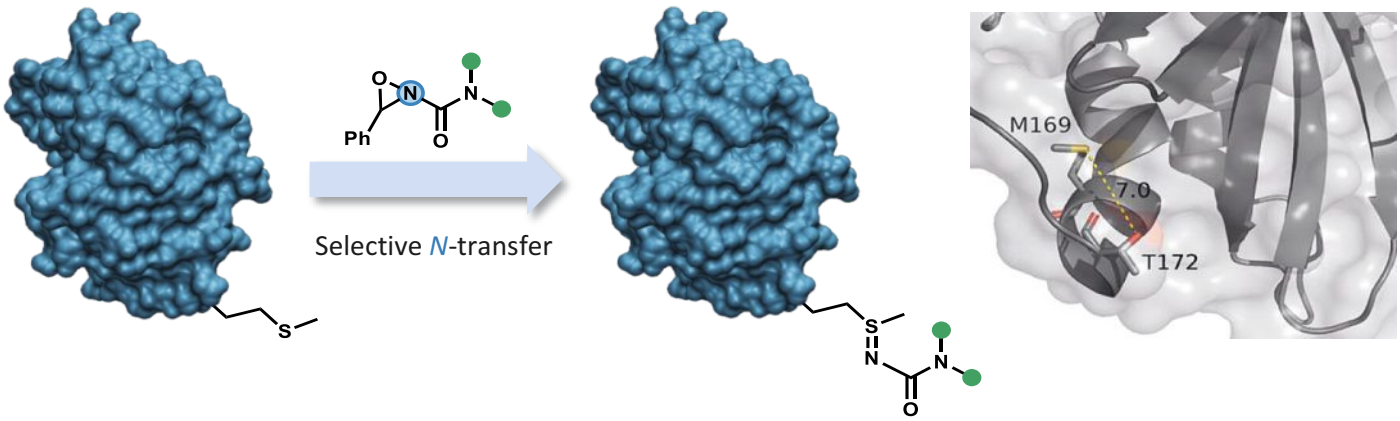


Fig. 5. Oxaziridine (ReACT)-based library to interrogate methionine function in CDK4 by use of selective N-transfer from oxiziridine and ABPP workflow.


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