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Beyond cysteine: recent developments in the area of targeted covalent inhibition

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Over the past decade targeted covalent inhibitors have undergone a renaissance due to the clinical validation and regulatory approval of several small molecule therapeutics that are designed to irreversibly modify their target protein. Invariably, these compounds rely on the serendipitous placement of a cysteine residue proximal to the small molecule binding site; while this strategy has afforded numerous successes, it necessarily limits the number of proteins that can be targeted by this approach. This drawback has led several research groups to develop novel methodologies that target non-cysteine residues for covalent modification. Herein, we survey the current literature of warheads that covalently modify non-cysteine amino acids in proteins.

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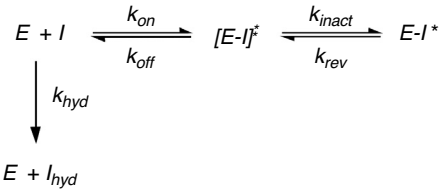
Introduction

Despite the prevalence of covalent inhibitors amongst approved drugs, medicinal chemists have historically avoided the intentional development of agents that irreversibly modify their target protein [1•]. As a result, clinically validated drugs have often been demonstrated to possess a covalent mechanism of action (MOA) long after their approval [2]. Certainly, this distrust of covalent inhibitors is not unfounded. The necessary introduction of a reactive electrophilic species raises the possibility of indiscriminate modification and idiosyncratic toxicity as has been demonstrated with some commonly used therapeutics such as acetaminophen [3]. These effects, however, are often the result of highly reactive metabolites, and armed with a greater understanding of the reactivity requirements for covalent inhibitors there has been a resurgence of interest in the field [4]. Indeed, covalent inhibitors offer several advantages when compared to traditional reversible ligands, including prolonged residence time, increased potency, and, when the target residue is poorly conserved across the proteome, enhanced selectivity. Importantly, these benefits have been demonstrated to be clinically relevant, and rationally designed targeted covalent inhibitors (TCIs) are beginning to enter the market [5]. Invariably, these compounds rely on the accessibility of a cysteine residue in or near a small molecule binding site. It should be noted, however, that the mere presence of a reactive residue near a ligand binding site is not sufficient to justify a covalent strategy; the local chemical environment of the target residue must be carefully considered to determine the suitability of this approach [6••]. While the highly nucleophilic nature of cysteine makes it ideal for the development of TCIs, the fact that it is amongst one of the least common amino acid residues found in proteins reduces the possible applications; this is further exacerbated by the fact that many cysteines are present within disulfide bridges and are thus inaccessible for covalent modification [7]. For this reason, several research groups have recently explored the development of novel warheads that are capable of targeting other nucleophilic amino acid side chains for the generation of irreversible inhibitors. While this concept is not new [8•], these approaches can now be evaluated in the context of marketed covalent drugs. In particular, when considering a typical two-stage covalent binding event (Figure 1), acrylamides (the standard warhead used for the modification of cysteines) typically benefit from relatively fast rates of cysteine modification (k_{inact}) and very low (or zero) rates of warhead hydrolysis/decomposition (k_{hyd}). As a result, installing a suitably positioned acrylamide into ligands possessing suboptimal k_{on} or k_{off} rates may still result in functional target inhibition. In contrast, non-cysteine residues typically afford lower k_{inact} rates, requiring more reactive warheads. This greater reactivity, however, typically comes at the expense of increased k_{hyd} rates, thus requiring a careful tuning of both reactivity and non-covalent binding interactions (thereby optimizing k_{on} and/or k_{off}) in order to obtain the desired target inhibition profile.

Nevertheless, assuming that these conditions can be met, it is becoming increasingly apparent that this class of compounds possesses great potential provided some points of concern can be ameliorated (Figure 1).

Figure 1



Challenges	Benefits
Off-target reactivity; idiosyncratic toxicity	Opportunity for selective target modification
Difficulty in assessing target potency	Typically higher potency achievable
PK/PD relationship can be difficult to manage	Potential to access "undruggable" targets
Instability of warheads	
Prolonged target engagement	
Potential for complete target inhibition (until resynthesis)	

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(a) Model for two-stage covalent modification of a protein target (E) by a covalent substrate (S). $[E–S]^{‡}$ represents the initial non-covalent complex formed between E and S; $E–S^{*}$ represents the covalent adduct. P represents an inactive byproduct arising from irreversible hydrolysis of S. **(b)** Challenges and benefits of potential covalent therapeutic molecules.

Covalent modification of lysine (Lys)

The pK_a of the ε-amino group of lysine has been determined to be ~10.0–10.5 [9]. Consequently, lysine residues on exposed protein surfaces exist almost entirely in the protonated form and are essentially non-nucleophilic under physiological conditions. In contrast, the pK_a of internal lysines can be highly perturbed through interactions with surrounding residues, and have been reported as low as 5.3 [10]. This, along with its prevalence, has made lysine an attractive target for non-cysteine based TCIs. Moreover, the amino group is a hard nucleophile, and thus shows a preference for reactions with hard electrophiles. Indeed, Gilbert and co-workers have demonstrated that substituted vinyl sulfones react faster with N^{α}-acetyl-L-lysine than with glutathione [11].

Several natural products are known to inhibit their target via an irreversible linkage with lysine. Possibly the most well-known amongst these is wortmannin, **1** [12], which has been demonstrated to modify the catalytic lysine of the phosphoinositide 3-kinases (PI3K) family of kinases (Figure 1). Recently, Campos and coworkers have taken this approach further and designed a set of selective PI3Kδ covalent inhibitors that also target the catalytic lysine, (**2**, Figure 2) [13••]. In this work, electronically diverse phenolic esters were examined for their ability to react selectively with PI3K family members. Compound **2** was identified as being the most selective for PI3Kδ, with the site of modification being unambiguously determined through crystallography (PDB: 6EYZ). Interestingly, K_{inact} of the library of phenol esters did not correlate with reactivity, and thus the authors propose a more complex mechanism than the standard model for covalent inhibition described above (for more details, see supplementary discussion [13••]). In contrast, Anscombe and coworkers, targeted a non-conserved solvent channel lysine in cyclin-dependent kinase 2 (CDK2), a kinase involved in the cell cycle [14]. Incorporation of a vinyl sulfone motif into a previously identified hit [15], allowed for covalent modification of Lys^{89} (**3**), as demonstrated by co-crystallography (PDB: 5CYI) and single point mutation (CDK2-K89A) studies (Figure 2). While the compound did display improved cellular potency when compared to the non-covalent analogs, the rate of bond formation was found to be slow, presumably due to the solvent exposed nature of the lysine (vide supra) and hence low nucleophilicity at physiological pH.

In work by Kelly and coworkers, a series of covalent stabilizers of the amyloidogenic protein transthyretin (TTR) were reported that modify the non-catalytic residue Lys^{15}, as demonstrated by crystallography (PDB: 1DVS, 5KMS, 4YDM) and point mutation studies (TTR-K15A). Phenol esters (**4**) [16], sulfonyl fluorides (**5**) [17], or fluorosulfates (**6**) [18], were utilized to generate the covalent linkage (Figure 2).

An unexpected instance of covalent modification of a lysine residue was reported by the Cheeseman group in their search of heat shock 70 kDa protein 1 (HSP72) inhibitors [19]. Although initially attempting to target Cys^{17}, crystallography suggested that **7** modified HSP72 through Lys^{56}, which was later corroborated through a point mutation study (HSP72-K56A) (Figure 2). A second serendipitous discovery of lysine modification came from Jinno *et al.*, who found that the previously described toll-like receptor 4 signal transduction inhibitor TAK-242, **8**, modified a lysine residue of human serum