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Targeting biomolecules with reversible covalent chemistry

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Interaction of biomolecules typically proceeds in a highly selective and reversible manner, for which covalent bond formation has been largely avoided due to the potential difficulty of dissociation. However, employing reversible covalent warheads in drug design has given rise to covalent enzyme inhibitors that serve as powerful therapeutics, as well as molecular probes with exquisite target selectivity. This review article summarizes the recent advances in the development of reversible covalent chemistry for biological and medicinal applications. Specifically, we document the chemical strategies that allow for reversible modification of the three major classes of nucleophiles in biology: thiols, alcohols and amines. Emphasis is given to the chemical mechanisms that underlie the development of these reversible covalent reactions and their utilization in biology.

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Introduction

In a living cell, various molecules interact with one another in a dynamic and highly selective manner. The exquisite selectivity of molecular interactions underlies essentially every aspect of biology. Similarly, a guiding philosophy in drug discovery has been to develop selective inhibitors of culprit proteins. In order to achieve such targeted molecular recognition, a cognate ligand typically interacts with its target protein via an array of noncovalent interactions including hydrophobic packing, electrostatic interactions, hydrogen bonding, and others. These noncovalent forces collectively stabilize the desired complex over competing structures (Figure 1). The reliance on noncovalent interactions also renders the binding process reversible when needed. Nature rarely uses covalent chemistry to drive molecular interactions in normal physiology; this is perhaps not surprising as dissociation of a covalent complex may be challenging. One exception is the disulfide bond formation, which presents a prominent feature and a stabilizing mechanism for folded proteins and protein complexes. A disulfide bond can form *reversibly* with redox regulation. It can exchange with free thiols of cysteine side chains to allow thermodynamic control of protein-protein interactions. The reversibility of the disulfide chemistry has been exploited by nature in the redox regulation of transcription factors [1], as well as in protein glutathionylation [2]. Additional reversible covalent chemistries like the disulfide bond formation would greatly empower chemists toward the development of molecular probes of important biomolecules.

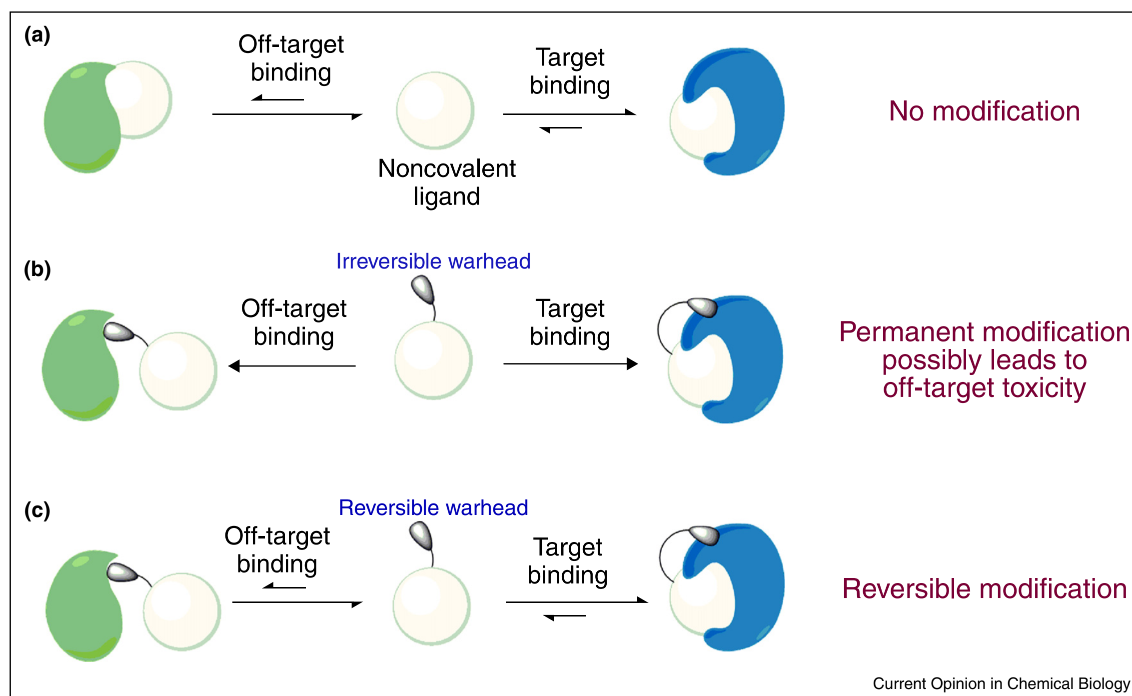
Different from the molecular interactions in normal physiology, which largely rely on noncovalent interactions, considerable success has been achieved in drug discovery by developing covalent inhibitors of target enzymes. In fact, about one-third of all validated enzyme targets have one or more covalent inhibitors approved for therapeutic use [3•]. In principle, reversibility is not required, and perhaps should be purposely avoided toward potent inhibition of pathogenic proteins; this makes covalent drugs appealing. However, concerns arise from the 'off-target' effect (Figure 1), in which irreversible modification of non-target proteins leads to toxicity [4]. This problem can be potentially mitigated by exploiting *reversible* covalent inhibitors that do not result in permanently labeled proteins.

This review highlights recent advances in reversible covalent chemistry that has been developed to target biomolecules. We focus on the chemistries developed to target abundant nucleophiles in biology including thiols, alcohols, and amines. Particularly, emphasis will be put on the mechanistic underpinnings that have enabled the development and applications of these reversible reactions.

Reversible covalent chemistry targeting thiols

As one of the least abundant amino acids, cysteines play vitally important roles in catalysis, signaling, and redox regulation of gene expression [5]. Not surprisingly, cysteines have attracted much interest in the pursuit of covalent probes and inhibitors of proteins. Taking advantage of the disulfide bond formation, Wells and coworkers have devised a fragment screening strategy, in which protein-specific ligands are selected through disulfide crosslinking of the ligand to a cysteine residue of the target protein [6••]. Much work in the field of covalent

Figure 1



Comparison of **(a)** noncovalent, **(b)** irreversible covalent and **(c)** reversible covalent inhibitors.

drug development has focused on targeting cysteines with Michael acceptors. In particular, acrylamide-based inhibitors has found great success, yielding the marketed anticancer drugs ibrutibib and afatinib, which covalently inhibit BTK kinase and EGFR respectively [3•]. Directed by the noncovalent interactions between an inhibitor and its cognate protein, the acrylamide warhead irreversibly crosslinks the inhibitor and the target enzyme via Michael addition of a cysteine residue. To better avoid the off-target effects of covalent inhibitors, Taunton and coworkers have recently demonstrated the use of α-cyanoacrylamides (or acrylates) as reversible modifiers of cysteines [7••,8,9,10•]. Inspired by an earlier report [11], in which conjugation of simple thiols to 2-cyanoacrylate was found to give an unstable product, the Taunton group showed that the reaction between cysteine and compound **1** (Figure 2a) is rapidly reversible, yielding a K_a value of $\sim 10^2\ M^{-1}$. The facile thiol addition to the α-cyanoacrylate is owing to the two electron withdrawing groups at α-carbon. On the other hand, the increased α-proton acidity of the cyanoacrylate adduct drives the reverse reaction through a β-elimination mechanism. The rapid reversibility and the relatively low K_a value preclude random modification of cysteines in the proteome. Grafting such cyano-substituted acrylate or acrylamide motifs onto a known kinase-binding scaffold yielded potent and highly selective inhibitors of the RSK2 kinase [7••]. Crystallography studies clearly revealed the covalent linkage between the inhibitor and the enzyme (Figure 2b), in addition to the noncovalent interactions that stabilize the complex. Interestingly, the covalent linkage rapidly breaks off when the target protein unfolds or is proteolytically degraded, leaving no modified peptide fragments. Despite the potential reversibility, the inhibitory effect of the cyanoacrylamide-based inhibitors was found to be as long-lasting as an irreversible inhibitor. This is presumably due to the cooperative action between the covalent bond formation and various noncovalent interactions that give rise to slow-dissociating drug–enzyme complexes. Unfolding or degradation of the protein abolishes the noncovalent interactions and exposes the cysteine adduct, which rapidly dissociates as expected for quick reversibility and the low millimolar affinity of the reaction (Figure 2a).

The Taunton group further shows the residence time of a reversible covalent drug can be systematically tuned via structural modification of the α-cyanoacrylamide warhead [10•]. Specifically, a series of BTK kinase inhibitors displaying end-capped α-cyanoacrylamide warheads were synthesized and found to display biochemical residence times ranging from minutes to seven days (Figure 2c). The reversible conjugation with cysteines is not limited to α-cyanoacrylamides; α-substituted acrylonitriles with various electron-withdrawing groups were found to react with cysteines in a reversible manner as well [9]. The

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