New Electrophiles and Strategies for Mechanism-Based and Targeted Covalent Inhibitor Design

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New Electrophiles and Strategies for Mechanism-Based and Targeted Covalent Inhibitor Design

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ABSTRACT: Covalent inhibitors are experiencing a growing resurgence in drug design and are an increasingly useful tool in molecular biology. The ability to attach inhibitors to their targets by a covalent linkage offers pharmacodynamic and pharmacokinetic advantages, but this can also be a liability if undesired off-target reactions are not mitigated. The discovery of new electrophilic groups that react selectively with specific amino acid residues is therefore highly desirable in the design of targeted covalent inhibitors (TCIs). Additionally, the ability to control reactivity through exploitation of the target enzyme's machinery, as in mechanism-based inhibitors (MBIs), greatly benefits from the discovery of new strategies. This Perspective showcases recent advances in electrophile development and their application in TCIs and MBIs exhibiting high selectivity for their targets.

Most small-molecule inhibitors function by forming a close association with their intended protein target such that its function is impaired. While many molecules associate via intermolecular forces, others can chemically modify the protein's active site by forming covalent bonds. Historically, the pharmaceutical industry largely avoided developing covalent drugs due to concerns of toxicity pertaining to the permanent nature of the bond.[1] Specifically, it was feared that highly reactive small molecules designed for a particular target could react promiscuously off-target with other proteins, nucleic acids, and or other biomolecules.[2] Surprisingly, however, many covalent drugs exhibiting favorable safety profiles have been introduced to market as various treatment options over the past century. Examples of "blockbuster" covalent drugs include aspirin (multipurpose);[3] penicillin,[4] fosfomycin,[5] clavulanic acid,[6] and tazobactam[7] (antibiotics); vigabatrin[8] (antiepileptic); omeprazole and lansoprazole (proton pump inhibitors);[9-10] and selegiline and tranylcypromine (monoamine oxidase inhibitors).[11] Most of these drugs were released to market without a thorough understanding of their mechanism of action and not until subsequent studies were performed was covalent target modification implicated.[12] In 2005, Robertson reported that among all enzyme targets of FDA-approved drugs, more than 25% are inhibited irreversibly by covalent modification.[12-13] Covalent inhibitors therefore represent a major portion of our drug arsenal, with development increasing exponentially over the past decade.[14] The current Perspective presents strategies used in the design of two classes of covalent inhibitors—targeted covalent inhibitors (TCIs) and mechanism-based inhibitors (MBIs)—published since 2013, with a focus on electrophilic warheads.

Non-equilibrium Binding: A Model for Effective Drug Action

Key features for drug efficacy and safety are potency and sufficient drug exposure at the target site to stimulate the desired response with minimal toxicity. A more potent drug requires a lower concentration to achieve efficacy, thereby lowering the risk of side effects.[2,15-17] Accordingly, the present art of drug discovery aspires to design covalent drugs such that inhibition cannot be overcome by competition with very high concentrations of substrates.[1, 14, 18] This is achieved through non-equilibrium binding, in contrast to the equilibrium mechanism of their non-covalent counterparts (Figure 1). Most covalent drugs function by binding to the target through traditional reversible interactions, followed by formation of a covalent inhibitor–protein adduct. If this covalent engagement is irreversible or only slowly reversible within the lifetime of the target protein, it exhibits non-equilibrium kinetics. The potency of reversible inhibitors is defined by the inhibition constant, K_i, which in most cases is governed by the ratio of the rate constants for dissociation and association (k_{off}/k_{on}). Covalent inhibitors are additionally subject to a chemical step (k_{inact}), and consequently, their effectiveness has a time dependence that is best assessed by the second-order rate constant for formation of the covalent complex (k_{inact}/K_I).[19] Here, K_I is the concentration of inhibitor required for half maximal rate of covalent bond formation (i.e., $k_{inact}/2$); for the scheme in Figure 1, K_I is $(k_{off} + k_{inact})/k_{on}$, which is equivalent to K_i for inhibitors that react slowly (i.e., $k_{inact} << k_{off}$).[20] The rate constant for covalent engagement depends on the nucleophilicity of the targeted residue on the protein and the nature of electrophile in the small molecule.

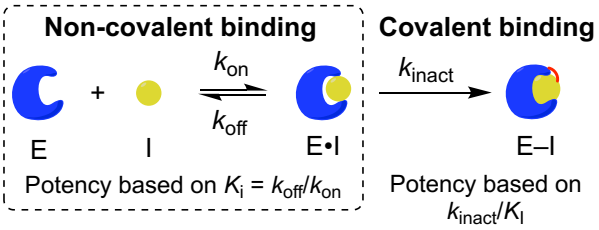

Figure 1. Non-covalent and covalent inhibition. The dashed box represents the reversible, non-covalent binding step common to both types of inhibition.

Non-equilibrium binding kinetics can offer important advantages in drug discovery.[21] An example of a biological challenge where

irreversible inhibition could be particularly beneficial is disruption of protein–protein interactions (PPIs). Because of their large, flat interfaces, PPIs are difficult to target with traditional small molecules that bind reversibly.[22-23] However, the non-equilibrium condition has been utilized by small-molecule electrophilic agents that react covalently with a cysteine residue to disrupt the well-characterized PPI between Kelch-like ECH-associated protein 1 (Keap1) and nuclear factor erythroid 2 related factor 2 (Nrf2),[24] which plays a critical role in the defense mechanisms against oxidative and/or electrophilic stresses that contribute to cancer, neurodegenerative diseases, cardiovascular diseases, and aging.

Another situation that poses a challenge for reversible inhibitors is competition with high concentrations of substrates or endogenous ligands. For example, the ATP-binding site of protein kinases are often surrounded by ATP at concentrations exceeding its K_m by several orders of magnitude. The high concentrations of competitive, reversible drug demanded by this situation could cause undesirable pharmacodynamic and pharmacokinetic effects. The use of an irreversible covalent inhibitor, however, has been shown to mitigate this problem. For example, several FDA approved covalent inhibitors of epidermal growth factor receptor kinase have been shown to circumvent the pronounced ATP-binding affinity of the T790M mutant enzyme in tumor cells in the presence of very high concentrations of ATP.[25-28]

A third advantage of the non-equilibrium mechanism is that it theoretically only requires enough drug such that one molecule engages each target protein (for 1:1 stoichiometry). Thus, for protein targets that are regenerated at a relatively slow rate, the pharmacodynamic half-life of the inhibitor would be sufficiently long compared to its pharmacokinetic half-life.[29-30]

Toxicity and Selectivity

As mentioned earlier, the historical reluctance of the pharmaceutical industry to pursue covalent drug development was largely attributed to fears of toxicity associated with off-target reactivity. Indeed, if the electrophilic "warhead" is too intrinsically reactive, it will exhibit poor selectivity, leading to damage of the surrounding tissue or to formation of a hapten–protein adduct that elicits an immunological response.[31] To mitigate this idiosyncratic toxicity, the broad-target selectivity of the irreversible covalent drug must be tempered by avoiding toxicophores, functional groups that have a tendency to produce a toxic effect.[2, 14, 16]

Covalent inhibitors can be made selective for a target molecule in several ways. Within the body, selectivity can also be achieved by shuttling the distribution and localization of the reactive compounds to a tissue or organ of interest. For example, the anti-obesity drug orlistat, which covalently binds to the active-site serine residue of gastric and pancreatic lipases, exerts its effect exclusively in the gastrointestinal tract due to its poor absorption.[32-33] Activation of prodrugs at the site of action offers another opportunity for avoiding off-target reactivity. For example, within the acidic confines of the stomach, omeprazole is converted to a tetracyclic sulfonamide intermediate, which then binds and covalently modifies the active-site cysteine residue of gastric H^+/K^+-ATPase.[34-35] A third opportunity for selectivity in covalent inhibition is to exploit a unique mechanism utilized by the target enzyme. MBIs, also called suicide inhibitors, are designed such that the enzyme executes part of its normal mechanism, forms a covalent adduct with the inhibitor, and becomes trapped in a stable, inactive state. If the target enzyme's mechanism is shared across a family of enzymes, selectivity in the MBI can be achieved by incorporating binding determinants that ensure a tighter non-covalent association (lower K_I) prior to the chemical step. This concept of packaging an electrophilic group in a molecule with high binding affinity can also be used in a fourth strategy known as targeted covalent inhibition.[2, 14, 27-28, 36] As will be expanded upon in the next section, a TCI is designed from a known tight-binding, reversible inhibitor by adding a weakly reactive electrophile such that it will only form a covalent linkage if it binds appropriately. The main distinction between a TCI and a MBI is that covalent bond formation with a TCI does not utilize the enzyme's mechanism; in fact, TCIs can target proteins that are not enzymes but possess a candidate nucleophile in their binding pocket.

The design of selective covalent inhibitors involves exploitation of structural features of the desired protein target through the use of suitable reactive functional groups. Because most proteins lack electrophilic groups but contain a variety of potential nucleophilic groups (e.g., thiols, alcohols, and amines), inhibitors are constructed to present an electrophilic group to the candidate nucleophile either by proximity upon binding, as for TCIs, or following chemical conversion by the enzyme's machinery, as for MBIs. Accordingly, when designing a covalent inhibitor, it is important to choose a "warhead" that is suitable for the specific amino acid side chain on the target.

In the remaining sections, this article focuses on the design of TCIs and MBIs, with examples from a variety of receptor and enzyme families published since 2013.

Targeted Covalent Inhibitors

TCIs represent a relatively recent and rapidly developing advance in covalent inhibitor design. As described above, these inhibitors function by utilizing non-covalent binding interactions to position the warhead near a candidate nucleophilic residue in the target's active site, enabling a reaction to yield an inactive covalently altered protein.[37] Here, we focus on recent examples that illustrate the design of protein-reactive electrophiles and their corresponding amino-acid selectivity. For a comprehensive account of TCIs, the reader is directed to the definitive review by Gehringer and Laufer.[38]

Because the choice of warhead in TCI design depends on the residues present in the binding pocket, we have arranged the examples alphabetically by the nucleophilic residue.

Aspartate and Glutamate

Growing interest to target a broader range of non-catalytic residues encouraged development of reactive electrophiles that can react with the poorly nucleophilic residues of carboxylate-containing residues aspartate and glutamate. Although many of the electrophiles that react with other amino acid residues, such as sulfonate esters and epoxides (*vide infra*), are capable of forming esters with Asp or Glu, the reactions suffer from poor selectivity. Selective modifications have been achieved with tetrazole reagents[39-40] and diazo compounds,[41] but these are not readily amenable to drug discovery. More recent and selective covalent modification of these carboxylic acid residues in the target's binding site has been shown by Waldmann and co-workers using a modified Woodward's reagent K.[42] In this study, isoxazolium salts (**1**) were shown to selectively target carboxylic acids in the proteome to form very stable covalent enol-ester bonds (Figure 2). Another study led by Shokat functionalized an optimized ligand for the K-Ras switch II pocket with different

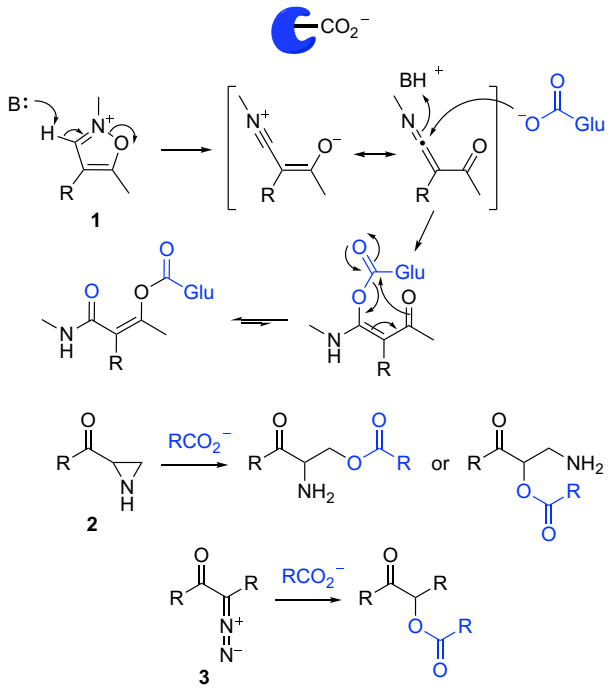

Figure 2. Aspartate- and glutamate-targeted covalent inhibitors.

electrophiles and subjected them to reaction with thiol and carboxylate nucleophiles. Interestingly, aziridines (**2**) and stabilized diazo groups (**3**) were found to react exclusively with the carboxylates over the more nucleophilic thiol (Figure 2).[43-44] Although selective reaction of these moieties with aspartyl and glutamyl residues on proteins remains to be demonstrated, they show promise in labeling these weaker nucleophiles.

Cysteine

Owing to its high nucleophilicity, cysteine residues are by far the most commonly targeted by TCIs. Most of the recent FDA approved drugs like afatinib (2013), ibrutinib (2013), osimertinib (2015), and neratinib (2017) react with a unique cysteine residue at the periphery of the active site of the tyrosine kinase family making them the frontline treatment for some cancers. A 450-fold range in reaction rates has been reported for hundreds of acrylates and acrylamides that form an irreversible covalent bond with the thiol nucleophile of the enzyme.[45-55] A number of reviews discuss the strategies and recent examples of warheads to label a non-catalytic cysteine moiety in the target enzyme.[49, 55-56] The most popular electrophiles for reaction with cysteine residues are Michael acceptors, which include acrylates, acrylamides, and their derivatives such as haloacetamides, cyanoacrylates, and vinylsulfonamides (Figure 3A).[57-58] Other historically successful electrophiles include acyl chlorides, sulfonate esters, epoxides, and halomethyl and acyloxymethyl ketones.[28, 56] We direct interested readers to the above review articles for extensive discussions of these inhibitors and turn our focus here to new or underutilized functional groups that have shown selective reactivity with cysteine residues.

Thiuram disulfides, such as the alcohol abuse drug disulfiram, contain a reactive disulfide bond capable of reacting by thiol exchange with cysteine residues to form a mixed disulfide intermediate, which usually reacts with a second cysteine residue to yield a disulfide bridge, rendering the enzyme inactive.[59] Intriguingly, Sun et al. demonstrated that the thiuram disulfide JX06 (**4**) was capable of forming a chemically stable mixed disulfide with a conserved cysteine (Cys240) in pyruvate dehydrogenase kinase PDK1 without subsequent reaction with another thiol (Figure 3B).[60] Other examples of this unusual irreversibility remain to be discovered.

Whereas nitroalkenes are known Michael acceptors for cysteine nucleophiles,[61] their saturated counterparts had not been known to serve as electrophiles until recently. We reported the surprising covalent modification of Cys191 of the tuberculosis target isocitrate lyase (ICL) by 3-nitropropionate (**5**) to form a stable thiohydroximate adduct (**7**, Figure 3C).[62] We have proposed that nitroalkanes can be made electrophilic by conversion to their nitronic acid tautomer (**6**), which in the case of isocitrate lyase is presumably facilitated by Glu285. Although nitro groups pose concerns for pharmaceutical

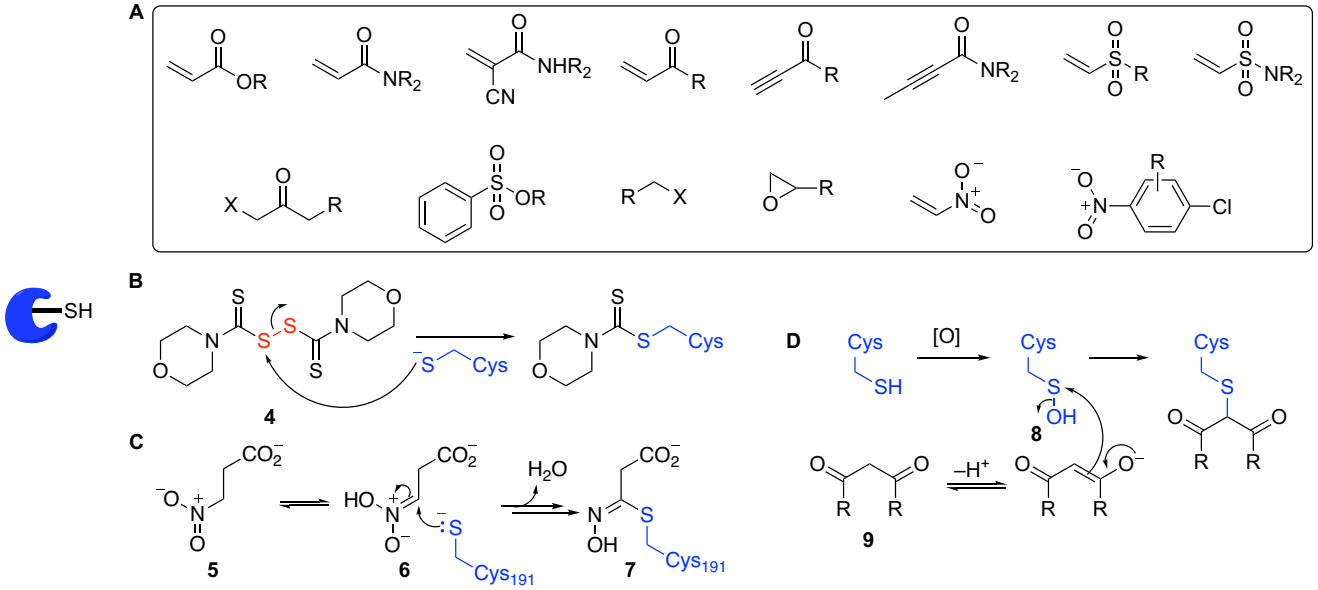

Figure 3. Cysteine-targeted covalent inhibitors. (A) Examples of common cysteine-reactive electrophilic groups. (B) Irreversible reaction of the thiouram disulfide JX06 with pyruvate dehydrogenase kinase 1, yielding a mixed disulfide. (C) Reaction of 3-nitropropionate with isocitrate lyase purportedly via the nitronic acid tautomer. (D) Reaction of a 1,3-dicarbonyl compound with a cysteine sulfenic acid.

applications due to their metabolic instability, they may be useful as a new biological probe for identifying binding sites possessing appropriately positioned cysteine and acidic side chains.

It is worth noting that some cysteine residues are subject to oxidation, existing as sulfenic acids (**8**, Figure 3D). These residues are not nucleophilic and therefore cannot be modified with the above electrophilic reagents. However, as demonstrated by Caroll and coworkers, these oxidized residues enable an umpolung approach to covalent modification by means of *nucleophilic* warheads, such as 1,3-dicarbonyl compounds (Figure 3D).[63-64] Due to the high concentration of reactive oxygen species associated with cancer cells, cysteine sulfenic acids present an attractive target for covalent pharmaceuticals.

Histidine

Histidine is an underinvestigated non-catalytic residue in the context of TCI design. Currently, the only known examples were discovered serendipitously from natural products or high-throughput screening. Taking inspiration from the natural product fumagillin, Morgen et al. developed a series of spiroepoxytriazoles (**10**) that modify His231 of methionine aminopeptidase 2 (Figure 4A).[65] A research team at AbbVie found α-cyanoenones (**11**) covalently attached to His315 in the active site of isocitrate dehydrogenase 1 (Figure 4B).[66] While a few other examples of aza-Michael addition to α,β-unstaturated carbonyl compounds are known,[38] competition by more reactive cysteine residues has likely limited the discovery of histidine-selective covalent inhibitors.

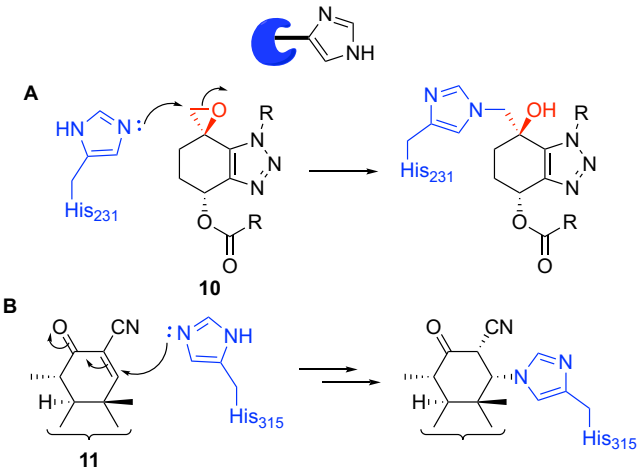

Figure 4. Histidine-targeted covalent inhibitors. (a) Selective epoxide opening of **10** by His231 of methionine aminopeptidase 2. (b) Aza-Michael addition by His315 of isocitrate dehydrogenase 1.

Lysine

The ample number of non-functional lysine residues on the outer surface of enzymes and N-terminal amino groups of the protein has posed a challenge for targeting active-site lysine residues. Additionally, posttranslational modifications (e.g., acylation) and its propensity to exist as a cation hamper its ability to react selectively. Nevertheless, recent investigations have led to the development of electrophilic warheads capable of targeting non-catalytic lysine residues present near the active site (Figure 5A).

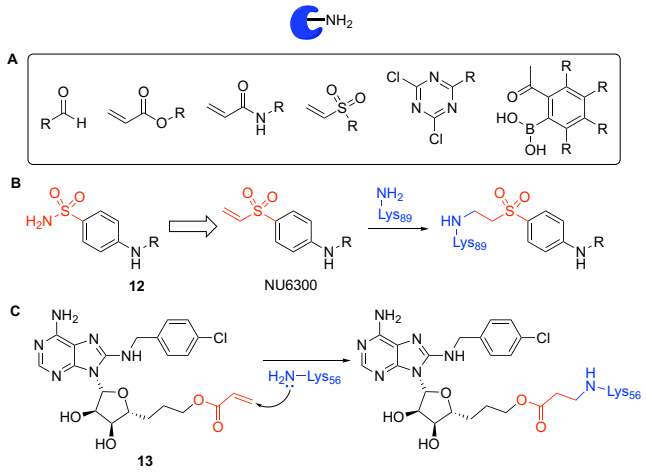

Figure 5. Lysine-targeted covalent inhibitors. (A) Examples of common electrophilic groups that can react with lysine. (B) Introduction of a vinyl sulfone in place of the sulfonamide in **12** resulted in covalent reaction of NU6300 with Lys89 of CDK2. (C) Acrylate **13** reacts selectively with Lys56 of HSP72, at the exclusion of candidate cysteine residues.

Anscombe et al. replaced the sulfonamide group in a previously known reversible inhibitor (**12**) of the cancer target cyclin-dependent kinase 2 (CDK2) with a vinyl sulfone warhead, recognizing the proximity of this moiety to a pair of non-catalytic active site lysine residues (Figure 5B).[67] The resulting compound, NU6300, served as an irreversible covalent inhibitor by reacting with Lys89 and was shown to be active in cells.[67]

Although Michael acceptors commonly react with cysteine residues, they have been found to react serendipitously in some cases with lysine residues. For example, while targeting Cys17 of heat shock 70 kDa protein 1 (HSP72), which is implicated in several cancers, the group led by Cheeseman discovered that adenosine-based acrylate ester **13** instead selectively reacted with Lys56 (Figure 5C).[68] These investigators subsequently reviewed historical and recent developments of electrophilic warheads that covalently engage lysine residues, and interested readers are referred to this text and to the references therein for additional examples.[69]

Serine, Threonine, and Tyrosine

The alcohol-containing side chains of serine, threonine, and tyrosine in diverse enzyme targets have long been recognized as sites of modification by covalent inhibitors. The catalytic triads of proteases and hydrolases feature an activated alcohol, making them especially susceptible to reaction with a wide range of electrophiles. In the spirit of this Perspective, however, attention will be focused on serine, threonine, and tyrosine residues that do not serve as nucleophiles in their catalytic function and are therefore more challenging to target. Due to their modest nucleophilicity, alcohols typically require more reactive electrophiles, and competition with lysine and cysteine residues can occur; examples include sulfonyl fluorides, fluorosulfates, fluorophosphonates, diphenyl phosphonates, diphenyl phosphoramidates, epoxides, and coumarins (Figure 6A).[70-71]

Of these, sulfonyl fluorides have gained the most attention in the design of TCIs.[72] Being biocompatible and relatively stable in aqueous solution, (2-aminoethyl)benzenesulfonyl fluoride (AEBSF) and phenylmethylsulfonyl fluoride (PMSF) are two common sulfonyl

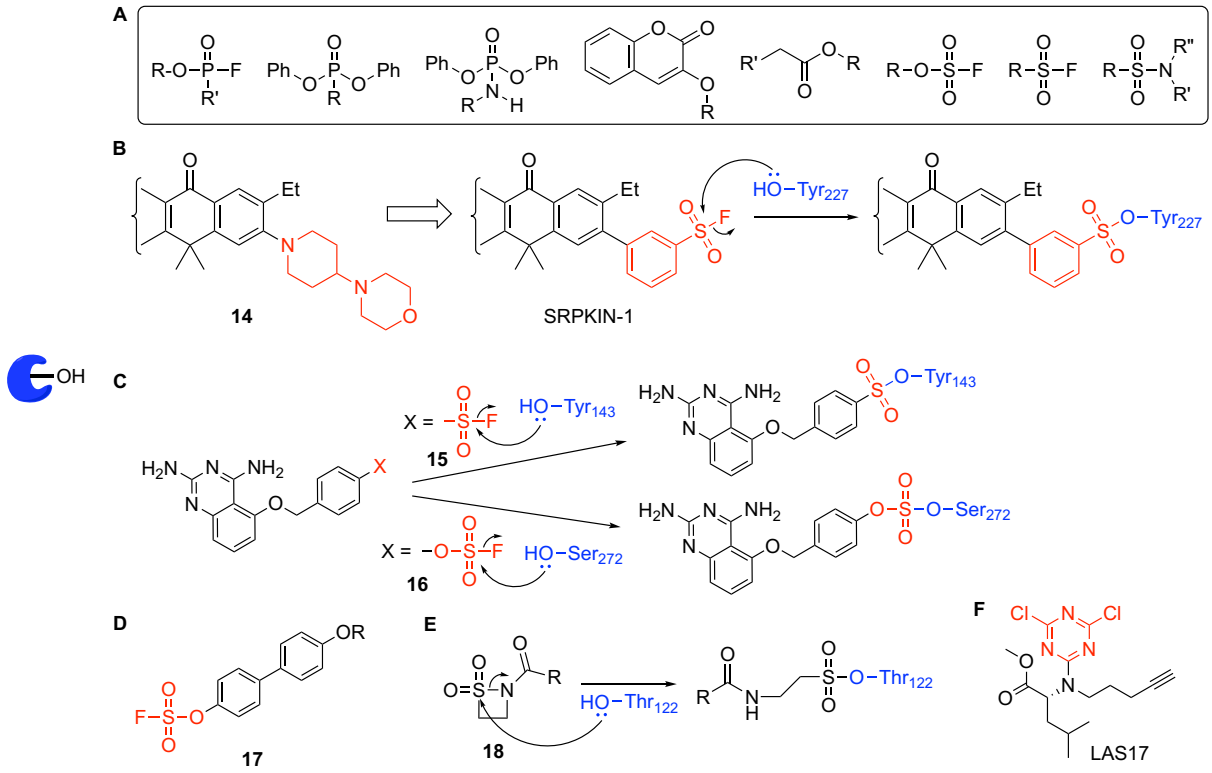

Figure 6. Covalent inhibitors that target serine, threonine, or tyrosine residues. (A) Common alcohol-reactive electrophilic groups. (B) Reversible SRPK 1 inhibitor **14** served as the scaffold for the sulfonyl fluoride SRPKIN-1, which reacts selectively with Tyr227. (C) The selectivity of sulfonyl fluoride **15** for Tyr143 of DcpS changed to Ser272 upon modification to the fluorosulfate **16**. (D) Fluorosulfates used as chemical probes of CRABP2 in HeLa cells. (E) β-Sulfams are capable of reacting with alcohols of non-catalytic residues. (F) Dichlorotriazine inhibitor that reacts by nucleophilic aromatic substitution by Tyr108 of GSTP1.

fluoride reagents that have been used for decades to inactivate serine proteases in the preparation of cell lysates to prevent the degradation of purified proteins.[73] Sulfonyl fluorides have also shown promise in selective modification of otherwise non-reactive alcohols. An active-site tyrosine residue in serine/arginine protein kinase (SRPK) 1, which regulates splicing of pro-angiogenic vascular endothelial growth factor A, was recently exploited through the application of a sulfonyl fluoride. Dysfunctional splicing initiates development of multiple diseases; hence, the inhibition of this enzyme can help in restoring the balance of pro/antiangiogenic isoforms to normal physiological levels.[74] Recognizing the proximity of the morpholine group of a nanomolar reversible inhibitor (**14**) to Tyr227, Hatcher et al. substituted a benzenesulfonyl fluoride to yield serine arginine protein kinase inhibitor (SRPKIN-1), the first kinase inhibitor to target tyrosine (Figure 6B).[75] Sulfonyl fluorides have also been used to target specific tyrosine residues in the active site of the mRNA-decapping scavenger enzyme DcpS, a pyrophosphatase used in the maturation of mammalian mRNA and microRNA. The study by Jones and co-workers remarkably demonstrated the selective reaction of **15** with either of two tyrosine residues despite the proximity of a lysine residue (Figure 6B); in fact, the authors suggested that the reactivity of the tyrosine residues is dependent on the neighboring basic residue.[76]The related fluorosulfates function similarly but exhibit superior chemical and metabolic stability over sulfonyl fluorides. Once again studying the inhibition of DcpS, Jones and colleagues replaced the sulfonyl fluoride of the above inhibitor with a fluorosulfate (**16**), yet to their surprise, a non-catalytic serine residue was modified rather than one of the two candidate tyrosine residues in the active site (Figure 6C).[77] In another example, Chen et al. developed aryl fluorosulfate probes (**17**) designed to react selectively with lipid-binding proteins, which were subsequently derivatized with a biotin tag via a click reaction, purified, and characterized by mass spectrometry.[78] These aryl fluorosulfates demonstrated much lower background reaction with the proteome than sulfonyl fluorides. Analysis of the covalent adduct formed between these probes and cellular retinoic acid binding protein 2 (CRABP2) within HeLa cells revealed the site of modification to be a conserved tyrosine residue (Tyr134, Figure 6D). Treatment of breast cancer cells with one of these probes inhibited retinoic acid signaling.

A new class of β-lactam analogues known as β-sultams (**18**) were recently shown to react with a conserved threonine residue outside the active site of azoreductases in bacterial cells from various species (Figure 6E).[79] This unprecedented selectivity was surprising, as the β-sultams were expected to react like their lactam counterparts with the active-site serine residues of penicillin binding proteins, elastase, and β-lactamase.

Dichlorotriazines represent another promising candidate for labeling tyrosine residues. Whereas a simple dichlorotriazine probe was shown by Weerapana and co-workers to preferentially react with lysine residues via nucleophilic aromatic substitution,[80] the leucine derivative LAS17 was surprisingly found to bind selectively to Tyr108 in glutathione S-transferase Pi (GSTP1), despite the preponderance of generally more reactive candidate nucleophiles (Figure 6F).[81]

Mechanism Based Inhibitors (MBIs)

MBIs or suicide inhibitors are usually dormant molecules that need to be activated by the target enzyme's catalytic machinery for inhibitory activity.[20, 82] MBIs are usually substrate analogues that upon binding are transformed by the enzyme into a reactive species that subsequently modifies the enzyme covalently, rendering it inactive. As the inhibitor is a substrate analogue, it typically binds to the target with high selectivity and reacts specifically with residues within the active site.[83] Because of their high selectivity and specificity, MBIs have been used as therapeutics for decades.[84] Due to re-emerging interest in MBIs as an approach for designing selective and specific inhibitors, we herein describe a few representative examples of new MBI strategies.

Aryl aldehyde: Formation of a stable thioester adduct

Aldehyde dehydrogenase (ALDH) is a NAD(P)$^+$-dependent enzyme that catalyzes the oxidation of aldehydes to carboxylic acids. ALDH7A1, an isoform of ALDH that is overexpressed in several types of cancer stem cells, provides resistance to anticancer drugs. Despite being a known inhibitor of ALDH for decades, 4-diethylaminobenzaldehyde (DEAB) was only recently established to be a MBI by Tanner and co-workers.[85] DEAB was proposed to undergo nucleophilic attack by the catalytic cysteine residue as seen with the substrate. This leads to the formation of a hemithioacetal, followed by a hydride transfer to give rise to an acyl-enzyme intermediate (Figure 7). In the case of a functional substrate, this acyl-enzyme intermediate undergoes hydrolysis, releasing the carboxylic acid product and free enzyme. However, it has been hypothesized that electron-donating ability of the 4-amino group in DEAB serves to deactivate the thioester group toward electrophilic reaction with water, resulting in accumulation of a stable covalent adduct.

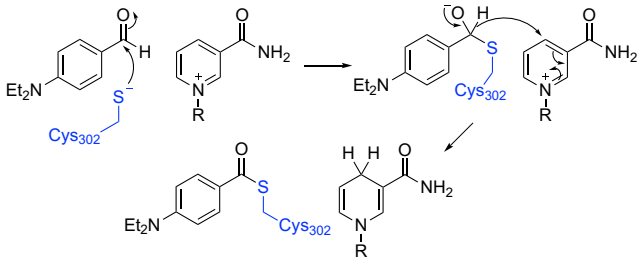

Figure 7. Mechanism-based inactivation of ALDH by DEAB.

Difluorosaccharide: Trapping a glycosyl-enzyme intermediate

Sialidase (also known as neuraminidase) is a viral enzyme that plays a major role in the life cycle of the influenza virus. This enzyme catalyzes the hydrolysis of sialosides and is a drug target to prevent the spread of influenza infection. X-ray crystallographic, kinetic isotope effect, mutational, and molecular modeling studies have helped to elucidate the mechanism of this enzyme. Hydrolysis of sialosides occurs via formation of an acetal intermediate formed between the substrate's anomeric carbon and the phenolic oxygen of the conserved tyrosine residue present in the enzyme active.[86] Evidence suggests that formation and breakdown of this covalent adduct proceed through oxocarbenium ion intermediates stabilized by the negatively charged active-site pocket.[87] The covalent mechanism prompted the group led by Stephen Withers to design MBIs that could form a covalent bond with Tyr406 of the viral enzyme. The group designed a new class of specific, mechanism-based drugs

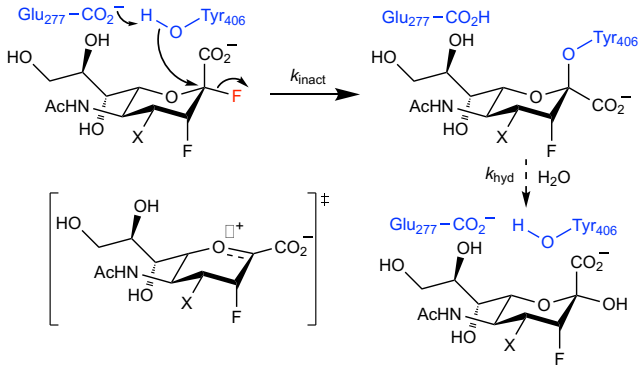

Figure 8. Mechanism-based inactivation of sialidase (neuraminidase) by difluorosialosides. The transition state is shown for formation and hydrolysis of the covalent intermediate. X = OH, NH_2, NHC(=NH)NH_2.

known as difluorosialic acids (DFSAs), which have been successful in inhibiting drug-resistant strains in vitro.[88-89] The fluorine atoms in DFSAs serve two important roles in ensuring covalent adduct formation with minimal subsequent hydrolysis (Figure 8): (1) the fluorine at C-2 provides a good leaving group to maintain a high k_{inact}, and (2) the electronegativity of the fluorine at C-3 inductively destabilizes the oxocarbenium ion transition states for formation and hydrolysis of the covalent adduct, slowing k_{hyd} (this also slows k_{inact} but this is more than compensated by the fluorine at C-2). DFSAs have shown effectiveness against the neuraminidase from zanamivir- and oseltamivir-resistant influenza virus strains.[89]

Aryloxycarbonyl hydroxamate

Bacteria that produce β-lactamases are emerging as a major clinical threat due to their resistance towards classic antibiotics like penicillins, cephalosporins, cephamycins, and carbapenems. One approach to overcoming this challenge is to selectively inhibit β-lactamases so that the β-lactam antibiotic can reach and inhibit its target.[90] Class C β-lactamases hydrolyze β-lactams by covalent catalysis using general base-activated serine nucleophile. To combat this class of β-lactamases, Pratt and co-workers designed a MBI that inactivates *Actinomadura* RR DD-peptidase by diverting the covalent adduct to a conformation that is resistant to hydrolysis.[91] The *O*-aryloxycarbonyl hydroxamate **19** was found to rapidly form acyl-enzyme adduct **20** by displacement of the phenoxide leaving group (Figure 9). Molecular modeling indicated that unlike the acyl-enzyme intermediates formed with other inhibitors, **20** subsequently undergoes a conformational change that disengages the inhibitor from the active-site contacts required for hydrolysis in order to adopt favorable polar interactions with residues in the neighboring

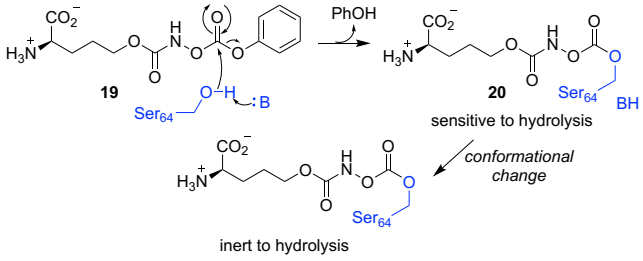

Figure 9. Mechanism-based inactivation of *Actinomadura* R39 DD-peptidase by an aryloxycarbonyl hydroxamate. A conformational change displaces the covalently attached inhibitor from the active site, protecting it from hydrolysis.

Ω loop. This inhibitor reflects a unique mechanism of action where the inactivation of the enzyme is sheltered from active-site residues by a non-covalent conformational change.

Bicyclobutonium cation: An oxocarbenium ion mimic

Glycosidases are ubiquitous enzymes in nature that catalyze the hydrolysis of carbohydrates from various biomolecules. As carbohydrates play an essential role in all organisms, the ability to modify the activity of these enzymes has fascinated researchers for decades.[92-93] Glycosidases can be categorized as retaining or inverting based on the relative stereochemistry of the anomeric center of their substrate and product.[94] A common mechanism utilized by retaining glycosidases involves a double-displacement reaction assisted by two active-site carboxyl groups. The first step of the reaction utilizes one of these as a general acid to assist departure of the aglycone, simultaneously causing nucleophilic attack by the other carboxylate upon the anomeric center, forming a glycosyl-enzyme intermediate. Following dissociation of the aglycone, the carboxylate residue serves as a general base to assist water in acting as a nucleophile upon the glycosyl-enzyme intermediate. The transition state in both steps possesses oxocarbenium ion-like character (similar to that shown in Figure 8) due to the development of a substantial positive charge on the carbohydrate.[94-96] In an attempt to discover new structural motifs to inhibit glycosidases, Bennet and co-workers designed an unusual bicyclo[4.1.0]heptyl analogue (**21**) of galactose for retaining α-galactosidases (Figure 10).[96] Exploiting the fact that cyclopropylmethyl derivatives undergo S_N1 reactions at enhanced rates relative to the corresponding acyclic homologues, Bennet proposed that **21** undergoes reaction within the active site to form a bicyclobutonium ion intermediate (**22**). This intermediate is then intercepted by an aspartate residue to form a stable covalent adduct that renders the enzyme inactive. This class of covalent inhibitor is the first example that involves delocalized stabilization of positive charge through a transient non-classical carbocation rather than by resonance from an adjacent heteroatom.[93, 96-97]

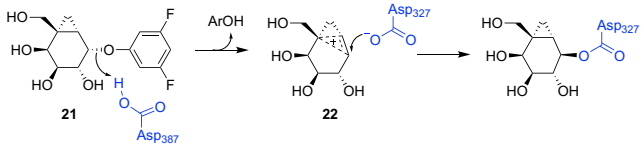

Figure 10. Mechanism-based inactivation of galactosidase via a transient non-classical carbocation. The cyclopropyl group stabilizes the adjacent developing positive charge as non-classical bicyclobutonium carbocation **22**, which reacts with Asp327 to generate a stable covalent adduct.

Allylic alcohol: A masked Michael acceptor

The observation that the catalytic cysteine residue of ICL can react with electrophiles such as 3-bromopyruvate and 3-nitropropionate (see TCI section) prompted Meek to design a MBI carrying a masked electrophile that would become reactive after conversion by the enzyme.[98] ICL catalyzes the reversible retro-aldol cleavage of isocitrate into succinate and glyoxylate. 2-*C*-Vinyl-D-isocitrate (**23**) was envisioned to react as an isocitrate analogue to form succinate and 2-vinylglyoxylate (**24**), which possesses an α,β-unsaturated carbonyl. Kinetic and structural data indicated that after retro-aldol cleavage of **23** and dissociation of the succinate product, **24** served as a Michael acceptor for reaction with Cys191 (Figure 11). The development of similar isocitrate analogues that carry a group that becomes electrophilic after being processed by ICL provides promise for combating *Mycobacterium tuberculosis*, which continues to pose a major threat globally.

Halopyridine: Trapping by nucleophilic aromatic substitution

The DNA methyltransferase (DNMT) family uses *S*-adeonsyl-L-methionine (SAM) to methylate DNA, causing epigenetic changes that can moderate the chromatin to regulate gene expression. In cancer, these epigenetic changes can cause hypermethylation of some

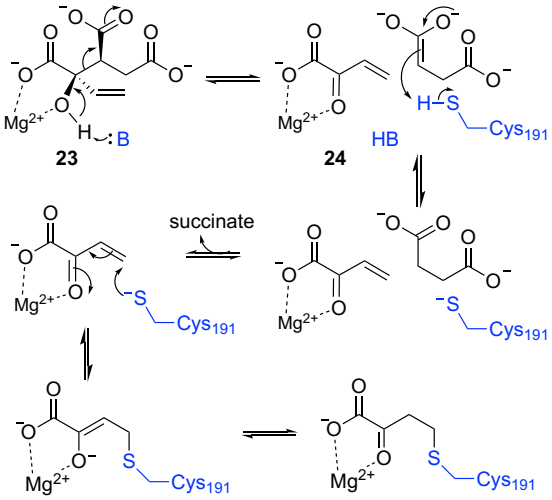

Figure 11. Mechanism-based inactivation of isocitrate lyase by 2-*C*-vinyl-D-isocitrate. After retro-aldol cleavage of **23** by the enzyme and dissociation of succinate, Cys191 reacts with **24** by Michael addition to yield an inactive *S*-homopyruvoyl derivative.

genes, repressing tumor suppressor genes, thereby promoting oncogenic pathways. Hence, DNMT has been recognized as a potential target for cancer therapy.[99] One of such DNMT recognizes CpG sequences in DNA and catalyzes the methylation of cytosine at the C5 position to yield 5-methylCpG. Recently, Sato et al. designed oligonucleotides containing 2-amino-halopyridine-*C*-nucleosides that act as MBIs for DNMTs.[100] Methylation of cytosine by cytosine 5-methyltransferase first proceeds by nucleophilic attack by the active-site cysteine residue upon C6 of the pyrimidine, a step that is promoted by concurrent protonation of N3 (Figure 12A). The thioether intermediate becomes methylated by SAM at C5 and then the cysteine group is removed by an elimination reaction. However, by including a halogen leaving group at the carbon that is attacked by cysteine, nucleophilic aromatic substitution occurs instead, in which the C–X bond is cleaved in preference to the C–S bond to the enzyme (Figure 12B). The ability of these inhibitors to reduce cell proliferation by inhibition of human DNMT1 was demonstrated in HeLa cells, giving promise that this strategy may result in a drug lead for cancer chemotherapy.[100]

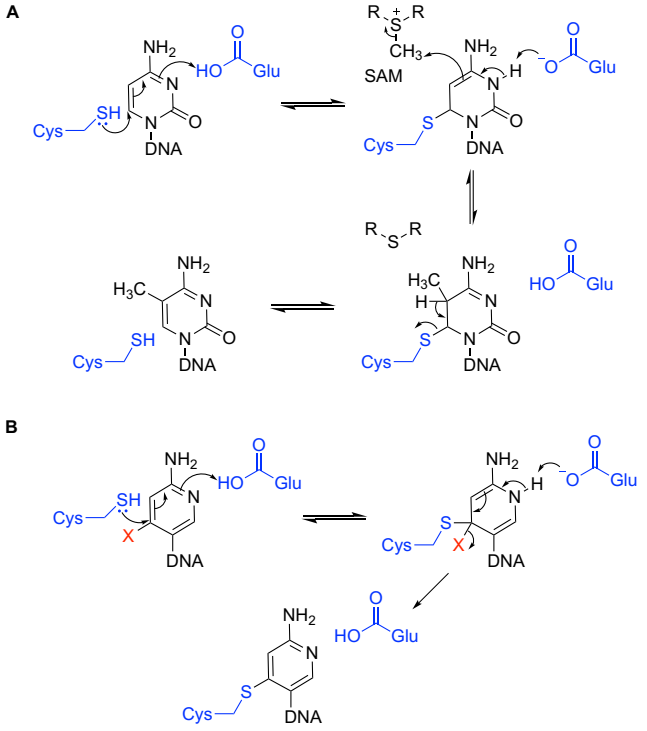

Figure 12. Mechanism-based inactivation of cytosine 5-methyltransferase. (A) The mechanism of cytosine methylation at C5. (B) 2-Amino-4-halopyridines undergo an analogous nucleophilic attack by cysteine, but the thioether intermediate undergoes elimination of the halide instead of cysteine. SAM = *S*-adenosyl-L-methionine; X = F or Cl.

Conclusion

Highlighted by the recent FDA approval of afatinib (2013), ibrutinib (2013), osimertinib (2015), and neratinib (2017), the rational design of covalent drugs is a validated approach to drug design that has undergone a resurgence over the past decade.[28, 101-104] TCIs and MBIs present two strategies for modifying drug targets with selectivity, an important factor for ensuring drug safety particularly in light of the long lifetimes characteristic of the covalent linkage. This Perspective has showcased several recent examples of novel electrophilic groups that have been successfully incorporated into inhibitor scaffolds to react with specific amino acid groups in the target's binding pocket. Additionally, previously unrealized strategies for exploiting the target enzyme's mechanism have been presented. As interest in covalent inhibitor design continues to blossom, the toolkit of electrophiles available is expected to expand, followed closely by the pipeline of covalent drug candidates based on them.

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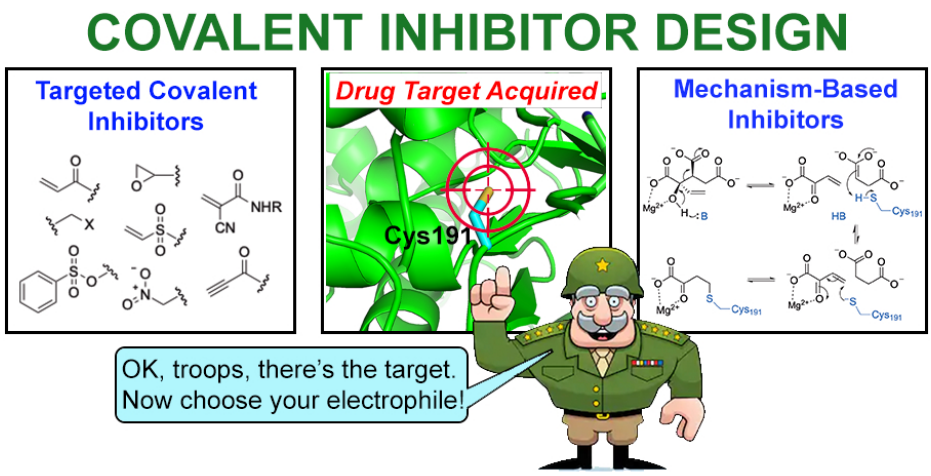

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