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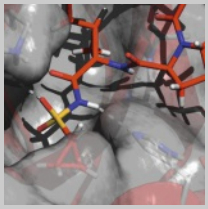

Medicinal Chemistry and Chemical Biology Highlights

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Understanding Intrinsic Warhead Reactivity and Cysteine Druggability in Covalent Drug Discovery

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Drug discovery is a long, expensive and high-risk process with the final goal of identifying new medicines to fight unmet medical needs. Most marketed drugs are low molecular weight (<600 Da) molecules and are produced through chemical synthesis: however, biologicals and new chemical modalities are advancing through clinical trials and these new molecules are capable of targeting diseases in unique fashions.[1] Small molecules usually bind reversibly to their biological target and modulate their activity to achieve a therapeutic effect. Small molecule-enzyme complexes are often short-lived, and the target activity is restored after drug elimination from the body. The introduction of a chemical reactive moiety, so-called warhead, into a drug allows the inhibitor to form a covalent bond with the targeted protein so that its function is permanently blocked and, as a result, protein re-synthesis is necessary before the biological function of the target can be restored. Covalent bonds are remarkably stronger compared to non-covalent interactions, thus irreversible inhibitors have potentially higher potency compared to non-covalent drugs. Moreover, targeting of unique nucleophilic amino acids, within binding sites can result in excellent selectivity of otherwise similar proteins.[2]

Historically, serendipity played a key role in the discovery of covalent inhibitors. For example, aspirin had been used for many years to treat pain, reduce fever and inflammation before the mechanism of action was determined. After the introduction onto the market in 1899, it took more than 70 years to understand that aspirin covalently modifies cyclooxygenases, key mediators of inflammatory pathways.[3] Moreover, electrophile-containing molecules were traditionally disregarded in phenotypic screenings and drug discovery programs due to safety concerns. During the last 15 years, the concept of targeted reactivity was introduced,[4] highlighting that a covalent mechanism of action was not inevitably linked to promiscuity. This led to a renewed interest in the rational design of covalent inhibitors, dubbed targeted covalent inhibitors (TCIs), which combine a reversible binding moiety showing a high affinity towards the targeted protein with a relatively low-reactive warhead.[5] As a result, the optimization of TCIs should be considered as a two-step inhibition process: firstly, the inhibitor forms a reversible complex with the protein (governed by K_i); and secondly, if the warhead is in close proximity to the targeted nucleophilic amino acid, the inhibitor-target covalent bond takes place (k_{inact}, Fig. 1). Thus, the covalent reaction can only occur after a proper molecular recognition (non-covalent binding). The second-order rate constant, k_{inact}/K_i, is used to describe the efficiency of covalent binding and considers the potency of the first reversible interaction and the maximum potential rate of covalent bond formation. The second step is governed by (i) the correct orientation/positioning of the warhead, and (ii) the intrinsic warhead reactivity. Within the context of rational design of drugs, understanding the intrinsic reactivity of the warhead is essential to produce effective and safe molecules.[6] However, it is only recently that this has been accomplished and often, during the past few decades, this was both not appreciated and not completed.[7] For example, replacement on a TCI of an acrylate with an aryl sulfonyl fluoride electrophile was reported to produce a >100-fold enhancement in covalent efficiency, but the different electrophilicity of the warheads was not considered.[8] Even for cysteine-targeting marketed drugs – such as ibrutinib[9] (Fig. 2) and zanubrutinib[10] – structure–activity relationship studies (SAR) involving structurally different warheads did not correlate the *in vitro* activity towards the target with the electrophilicity of the reactive group. With a new appreciation of the necessities for warhead optimization in drug discovery, investigation of warhead reactivity is becoming a gold-standard to rationalize the potency of TCIs. The electrophilicity of the reactive group should be adjusted aiming at selective conjugation reactions with the targeted cysteine, while minimizing the off-target reactions inside the cell.[11] Experimentally, intrinsic warhead reactivity can be assessed with

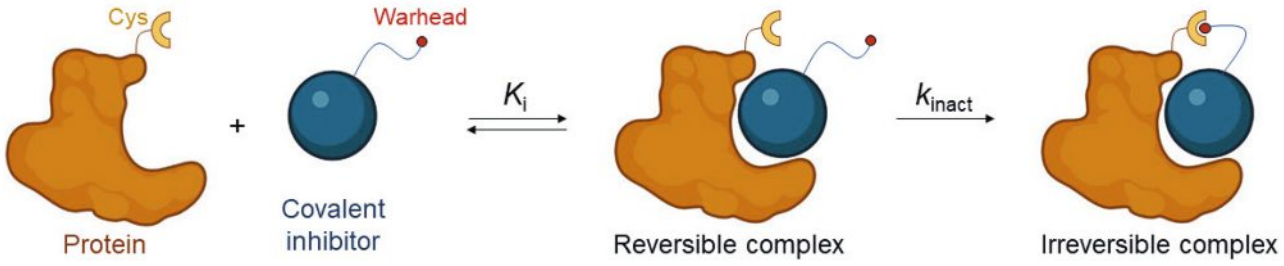

Fig. 1. Two-step binding mechanism of targeted covalent inhibitors (TCI).

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glutathione (GSH) or β-mercaptoethanol (βME), which are used as cysteine surrogates. The half-life of adduct formation ($t_{1/2}$) or the absolute rate constants (k_{chem}) can be measured in covalent drug discovery projects to understand the SAR.[12] Despite experimental measurements being considered the most reliable for measuring the reactivity of cysteine-targeting covalent warheads, the ability to predict warhead reactivity has recently garnered increased interest. *In silico* predictions could also be performed during inhibitor design and before the synthesis of the molecules. Different approaches have been used to predict warhead reactivity, including calculated pK_a and quantum mechanical modelling of GSH adduct formation,[13] NMR chemical shifts and LUMO energies.[14]

Michael acceptors, such as unsubstituted acrylamides, are the most exploited reactive groups to target the thiol side chain of cysteines.[11,15] An acrylamide motif's reactivity can be fine-tuned modifying the substituents in α- or β- positions. While methylation at those positions strongly reduces the reactivity, introducing an electron-withdrawing group (*e.g.* nitrile) in α-position leads to an enhanced reactivity and to a reversible covalent reaction due to the acidity of the α-carbon bearing the nitrile. PRN1008 (rilzabrutinib, Fig. 2),[16] a reversible covalent Bruton's tyrosine kinase (BTK) inhibitor, is currently being investigated in multiple clinical trials including phase 3 trials for pemphigus vulgaris and immune thrombocytopenia.

The presence of a basic amine on the acrylamide β-position has the potential to reactivate the warhead, as intrinsic reactivity is directly correlated with the pK_a of the amine. Among the FDA-approved covalent kinase inhibitors, afatinib[17] (Fig. 2), neratinib[18] and dacomitinib[19] display a tertiary amine group attached to the acrylamide, which is responsible for an intramolecular base catalysis (see legend Fig. 2). Besides the identification of novel substituted warheads, these re-activated acrylamides could also pave the way to innovative chemical probes.[20] It should also be appreciated that the reactivity of a warhead can be affected not only by direct substitutions on the acrylamide moiety, but also by way of different substitutions in proximity to the warheads. For example, a cyanomethyl electron-withdrawing substituent on a warhead-bearing piperazine was able to increase the reactivity of a 2-fluoro acrylamide.[21] The resulting covalent KRAS^{G12C} inhibitor, adagrasib (MRTX849, Fig. 2), is currently under investigation by Mirati in multiple clinical trials (see https://clinicaltrials.gov/).

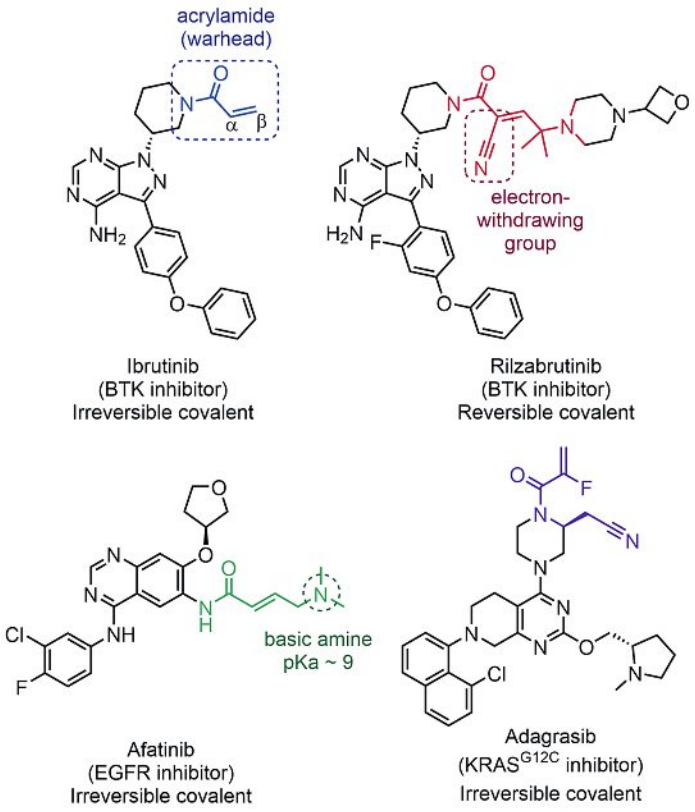

Fig. 2. Selected reversible and irreversible covalent inhibitors in the clinics or in clinical trials. EGFR: epidermal growth factor receptor. BTK: Bruton's tyrosine kinase. pK_a: the negative log of the acid dissociation constant or K_a value. To quantify the basicity of an amine, the pK_a of its conjugate acid is considered. The higher the pK_a of the conjugate acid, the stronger the base. The basic group (see afatinib) has been suggested to be responsible for an intramolecular base catalysis. The tertiary amine serves as a general base to deprotonate the cysteine thiol to the negatively-charged and more nucleophilic thiolate group.

Many parameters affect the electrophilicity of the warhead, but also the reactivity of the thiol group is complex and should be considered for the rational design of covalent chemical probes and drugs. The pK_a of the cysteine residue controls the deprotonation of the thiol to form the reactive anionic thiolate, which promptly reacts with electrophiles. Thus, the rate of covalent modification is strongly influenced by the pK_a of the cysteines, with those having lower pK_a being the most prone to irreversible modification. The cysteines in protein kinases cover a wide range of pK_a (from 7 to 24), resulting in huge differences in their reactivity.[22] Elevated pK_a values lead to cysteines that are not reactive and cannot be targeted with electrophilic drugs. Druggable cysteines in protein kinases have been successfully targeted, leading to nine covalent kinase inhibitors approved by FDA for oncology applications, and to many clinical and preclinical candidates.[15]

Beside protein kinases, medicinal chemists have turned their attention to activated cysteines involved in a catalytic triad or dyad. Cysteine proteases present a Cys-His-Asn triad in the active site, where His acts as a proton acceptor (base, B^- to BH in Fig. 3) increasing cysteine nucleophilicity (pK_a < 6),[23] and Asn plays a role in catalysis by properly orienting His. Cysteine proteases have been successfully targeted for the treatment of SARS-CoV-2. Alongside vaccines, nirmatrelvir (PF-07321332, Fig. 4),[24] a nitrile-substituted covalent inhibitor of the SARS-CoV-2 main protease (M^{pro}), has been approved to fight the COVID-19 pandemic. Beside COVID-19, cysteine proteases represent potential therapeutic targets for the treatment of a variety of diseases, including neurodegenerative disorders. Caspases, calpains, and cathepsins cysteine proteases have been targeted by a variety of covalent inhibitors with the aim of modulating the apoptotic process and developing novel drugs for Alzheimer's, Huntington's and Parkinson's disease. While warheads, such as epoxides, vi-

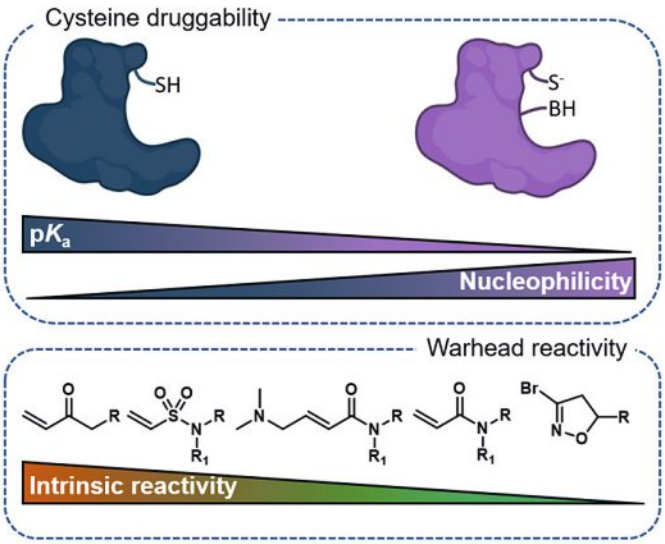

Fig. 3. Cysteine druggability and warhead intrinsic reactivity: two sides of the same coin.

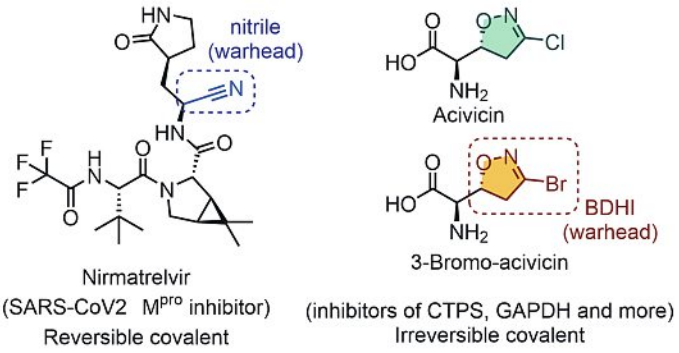

Fig. 4. Selected reversible and irreversible covalent inhibitors targeting an activated cysteine in a catalytic triad or dyad. SARS-Cov-2 M^{pro}: SARS-CoV-2 main protease. CTPS: cytidine triphosphate synthetase. GAPDH: glyceraldehyde-3-phosphate dehydrogenase. BDHI: 3-bromo-4,5-dihydroisoxazole core.

nylsulfones, halomethyl ketones, resulted in inhibitors lacking selectivity, Michael acceptors included in an appropriate recognition group (aza-peptide) led to selective caspases inhibitors.[25] A cysteine-histidine-aspartate catalytic triad is also present in glutamine amidotransferases, such as Cytidine Triphosphate Synthetase (CTPS) which has been highlighted as a target for the treatment of Human African trypanosomiasis. Acivicin (Fig. 4) and its synthetic derivatives 3-bromoacivicin (3-BA) was found to inhibit CTPS.[26] The mechanism of action involves the nucleophilic attack by the activated cysteine to the electrophilic C3 of the 3-chloro or 3-bromo-4,5-dihydroisoxazole core (BDHI, Fig. 3). The BDHI scaffold is able to inhibit also glyceraldehyde-3-phosphate dehydrogenase (GAPDH)[27] and aldehyde dehydrogenase (ADH)[28] through an addition-elimination mechanism on a nucleophilic cysteine. The catalytic Cys152 of hGAPDH is involved in a catalytic dyad with a histidine residue (His179) which led to an enhanced nucleophilicity (pK_a ~6). Very recently, the intrinsic reactivity of the BDHI moiety was disclosed, revealing that this warhead does not react with GSH[29] or βME[30] at physiological pH (7.4). In contrast, at pH 9 the BDHI warhead showed a k_{chem} comparable to that of the drug-like acrylamide-based covalent inhibitors.[30] As for acrylamides, substituents on the BDHI ring could fine-tune the reactivity of the resulting molecules. In general, the BDHI core can selectively react with activated cysteines (low pK_a), minimizing the off-target interaction with cellular thiols.

In conclusion, understanding pK_a values of targetable cysteines, as well as intrinsic warhead reactivity, is essential in covalent drug discovery to pinpoint the correct electrophile and achieve a selective irreversible modification of the desired target.

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