Advances in reversible covalent kinase inhibitors

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Abstract
Reversible covalent kinase inhibitors (RCKIs) are a class of novel kinase inhibitors attracting increasing attention because they simultaneously show the selectivity of covalent kinase inhibitors yet avoid permanent protein-modification-induced adverse effects. Over the last decade, RCKIs have been reported to target different kinases, including Atypical group of kinases. Currently, three RCKIs are undergoing clinical trials. Here, advances in RCKIs are reviewed to systematically summarize the characteristics of electrophilic groups, chemical scaffolds, nucleophilic residues, and binding modes. In so doing, we integrate key insights into privileged electrophiles, the distribution of nucleophiles, and hence effective design strategies for the development of RCKIs. Finally, we provide a further perspective on future design strategies for RCKIs, including those that target proteins other than kinases.

KEYWORDS
binding modes, drug discovery, kinase inhibitors, privileged warheads, reversible-covalent kinase inhibitors

1 | INTRODUCTION

Kinases are one of the largest protein families in the human genome, comprising 538 kinase-encoding genes which catalyze the transfer of the γ-phosphate of ATP to phosphorylate specific substrates. In so doing, kinases mediate cell signal transduction which controls a variety of biological functions such as cell proliferation and apoptosis.
Not surprisingly, mutations and dysfunction of kinases are associated with a variety of disease conditions such as cancer, inflammatory disease, cardiovascular disease, neurodegenerative disease, and metabolic disease. As such, kinases have become important therapeutic targets, however, due to the high conservation of the ATP binding site, it is a challenge to design drugs that target kinases with the desired selectivity.

As structural and functional knowledge of kinases has increased, substantial variations such as allosteric sites have been found among different kinases, allowing for greater specificity. Since the first kinase drug, Imatinib, was approved by the U.S. Food and Drug Administration (FDA) in 2001, kinase-targeted drug discovery has been one of the fastest-growing areas of drug development. As of February 12, 2023, 74 small-molecule kinase drugs have been approved by the FDA and over 326 kinase inhibitors are in clinical trials. Correspondingly, there are over 300 kinase targets with released crystal structures providing a substantial structural basis for kinase drug discovery. Undoubtedly, kinase-targeted drug discovery has significantly contributed to clinical targeted therapy, for example, against multiple types of cancer, such as non-small cell lung cancer, melanoma, and leukemia. However, in practice, toxicity and adverse events such as congestive heart failure and cardiogenic shock in some chronic myelogenous leukemia patients require the further development of novel, effective and safe inhibitors.

Of the 74 currently marketed drugs, 65 are noncovalent inhibitors characterized as Type-I, II, or III according to their binding modes. The remaining 9 are irreversible-covalent kinase inhibitors that utilize a noncatalytic cysteine within or near the ATP binding site forming the covalent interactions. Although irreversible covalent kinase inhibitors can have improved selectivity, concerns exist regarding the potential toxicities of such irreversible complexes. A strategy to reduce the potential toxicity is to limit the reactivity of irreversible covalent inhibitors. In this strategy, the inhibitor is considered to be a substrate and catalyzed by its target enzyme. By evolving the chemical structure of the inhibitor, a long-lasting covalent intermediate is formed to achieve the desired inhibitory effect during the catalytic process. Subsequently, with the catalytic action of the enzyme, the covalent bond is broken, the active enzyme is released again and the inhibitor (i.e., substrate) is catalyzed into other products. Currently, the application of this strategy to design low-reactive irreversible covalent inhibitors is just used with enzymes having covalent catalytic mechanisms. However, because kinase inhibitory mechanisms don’t involve covalent interactions with the substrate, the “limited-reactivity” strategy is not suited to developing covalent kinase inhibitors. Most frequently the reversible-covalent approach utilizes an inhibitor equipped with one electrophile. First, the reversible binding part of the inhibitor binds to the corresponding binding site then the electrophile of the inhibitor undergoes a reversible chemical reaction with one reachable nucleophile from an amino acid within the binding site. The reversible covalent binding mode not only ensures a high potency as in covalent interactions, but also allows the adjustment of the residence time by tailoring the electrophilic group and/or noncovalent part of the inhibitor. As such, a reversible-covalent inhibitory mechanism has been applied in developing effective kinase inhibitors, aptly named reversible-covalent kinase inhibitors (RCKIs). To date a variety of RCKIs have been reported; indeed, three have been tested in clinical trials. Here we provide a systematic review of current progress with RCKIs and provide a look ahead. We first focus on all the reported RCKIs and then describe the intrinsic properties of their reversible-covalent reactive warheads, their binding modes, and binding site contextual information. From there we speculate on privileged warheads, nucleophilic groups, and design strategies for future RCKIs.

2 | OVERVIEW OF RCKIS

2.1 | Kinase inhibitors

Various kinase inhibitors, such as Type-I, Type-II, Type-III, and Type-IV, have been developed to achieve the desired selectivity by making full use of the different features of the ATP binding site and beyond. Type-I inhibitors, such as Crizotinib, typically occupy the ATP-binding cavity in the active "DFG-in" kinase conformation.
FIGURE 1 Kinase inhibitor binding modes. (A) Type-I inhibitor Crizotinib in a “DFG-in” conformation (PDB id: 3zbf). (B) Type-II inhibitor Imatinib in the “DFG-out” conformation (PDB id: 1opj). (C) Type-III inhibitor Trametinib (PDB id: 7jur). (D) Type-IV allosteric inhibitor GNF-2 bound to the allosteric pocket of the C-lobe (PDB id: 3k5v). (E) Covalent EGFR kinase inhibitor Osimertinib with a covalent bond interaction with Cys797 (PDB id: 6jxt). [Color figure can be viewed at wileyonlinelibrary.com]
Type-II inhibitors, such as Imatinib (Figure 1B), not only occupy the ATP-binding cavity but also extend into the adjacent allosteric pockets opened up in the inactive “DFG-out” kinase conformation. Type-III kinase inhibitors, such as Trametinib (Figure 1C), are accommodated just in an allosteric pocket adjoining the ATP binding site. In contrast to Type-III kinase inhibitors, Type-IV kinase inhibitors occupy the allosteric pockets away from the ATP-binding pocket, for example, the allosteric pocket located at the C-lobe (Figure 1D). By analyzing all available PDB structures, Yueh et al. identified 10 promising hot spots that are not within the ATP binding site but distributed on the protein surface, as potential binding pockets for designing Type-IV allosteric inhibitors in some kinases.

Type I-IV kinase inhibitors can be classified into covalent kinase inhibitors and noncovalent kinase inhibitors based on the presence/absence of kinase-ligand covalent interactions. For example, Crizotinib, Imatinib, Trametinib, and GNF-2 are noncovalent kinase inhibitors (Figure 1A-D). Whereas, Osimertinib is a covalent EGFR kinase inhibitor due to forming an irreversible covalent bond with Cys797 (Figure 1E).

Typically, covalent kinase inhibitors are designed by combining chemical scaffolds with warheads that participate in covalent reactions. The chemical scaffold is generally a proven noncovalent kinase inhibitor, which binds into the designated binding pocket and then provides the foundation for appending a warhead to bear the covalent interactions with proximal nucleophilic residues such as cysteine, lysine, or tyrosine, near or within the binding sites. Cysteine is targeted much more frequently than other (noncatalytic) amino acids in covalent drug development, due to its high intrinsic nucleophilicity. To date, nine covalent kinase drugs have been approved by the FDA. Nonetheless, owing to potential safety concerns, as a result of protein covalent modification, the pharmaceutical industry remains wary of developing covalent drugs. Accordingly, reversible-covalent inhibition strategies to target protein kinases have been developed. The strategies not only avoid permanent protein modification as found with irreversible covalent inhibitors but can also substantially prolong residence time suggesting superior efficacy. In 2012, Taunton and his colleagues reported the first reversible covalent kinase inhibitor by designing and tweaking the reactivity of the warhead moiety of a given irreversible covalent kinase inhibitor, FMK. Since then, reversible-covalent drug design strategies have been applied to multiple kinase targets and numerous RCKIs are reported. Moreover, a promising RCKI, Rilzabrutinib (formerly known as PRN1008), is in a Phase-III clinical trial for Pemphigus treatment.

2.2 | Reaction mechanisms

Generally, reaction mechanisms of covalent inhibitors can be described in the following process:

\[
R + L \underset{k_f}{\overset{k_i}{\rightleftharpoons}} R\cdot L \rightleftharpoons R-L
\]

where inhibition is a two-step process. First, the inhibitor binds into the receptor binding site forming an initial noncovalent complex. Here, the receptor [R] is in a state of dynamic equilibrium with the inhibitor [L] and the noncovalent complex [R-L], expressed by the noncovalent binding constant (K_i), that is, \( K_i = \frac{k_f}{k_i} \). In the second step the receptor is covalently modified and inactivated by covalent-bond formation of the electrophilic warhead of the inhibitor with the adjacent nucleophilic amino acid of the receptor, leading to the final covalent complex. This step is characterized kinetically using a rate constant of protein inactivation \( k_{\text{inact}} \) (i.e., \( k_2 \) in the equation above). Thus, the overall covalent binding process is expressed as \( k_{\text{inact}}/K_i \), a value used to assess the potential of irreversible inhibitors. Importantly, conventional IC_{50} values are not ideal to measure the potential of irreversible inhibitors.
since they are time-dependent.\textsuperscript{23,50} The $k_{-2}$ is the rate constant for covalent dissociation indicating the reversibility for reversible covalent reactions. Copeland et al.\textsuperscript{51,52} first qualified the residence time ($t$) of the drug molecule within its binding site as the reciprocal of the off rate ($k_{-2}$), namely $t = (k_{-2})^{-1}$, for a binary complex model. Studies have shown RCKIs can be designed by embedding the covalent inhibitor into the kinase binding site and tailoring the chemical nature of their warheads to obtain the desirable pharmacodynamics and efficacy.\textsuperscript{29,53}

### 2.3 | Current status

We manually cataloged all RCKIs published since 2012 using the scholarly literature databases PubMed and Google Scholar. As of May 2021, 64 representative RCKIs, inhibiting 10 different kinase targets have been collected (see Supporting Information S1: Table S1). There are eight types of electrophiles (i–viii) (Figure 2) as warheads reported to exhibit reversible-covalent interactions.\textsuperscript{46} We review the reaction scheme for each warhead with its respective nucleophile on the kinase protein, that is, the reversible covalent binding mechanism (Figure 3). Cyanoacrylamide (warhead i) is the most frequently used covalent-reversible warhead. At the time of writing, 37 representative RCKIs with warhead i (cyanoacrylamide) have been developed (Supporting Information S1: Table S1). Generally, the reversible covalent reaction of cyanoacrylamide is Michael addition-based and follows a direct reverse process (Figure 3).\textsuperscript{54} Compared with the irreversible warhead acrylamide, cyanoacrylamide has one more nitrile group on the $\alpha$-carbon. The nitrile is an electron-withdrawing group, inducing the charge redistribution such that the negative charge density around the olefin decreases.\textsuperscript{28} The decreased negative charge density not only improves the susceptibility of the $\beta$-carbon to nucleophilic attack, but also increases the acidity of the proton on the $\alpha$-carbon of the thiol addition product, which facilitates the deprotonation and elimination of the thiol group through the E1cB mechanism, promoting the reversibility of cyanoacrylamide.\textsuperscript{55} Despite the weak intrinsic reactivity of Chlorofluoroacetamide (CFA, ii), the warhead CFA shows high reactivity by combining with the corresponding scaffold.\textsuperscript{56} Mechanically, CFA as the electrophilic group first undergoes an $S_{N}2$ nucleophilic displacement with the thiol group on the protein (Figure 3). However, the reverse reaction of the CFA-thiol reaction product undergoes hydrolysis under aqueous conditions (Figure 3).\textsuperscript{56} The $S_{N}2$ displacement

![Cysteine-targeted warhead](image1)

![Lysine-targeted warhead](image2)

**FIGURE 2** Warhead moieties in RCKIs. Stars mark the active atoms in reversible covalent reactions. [Color figure can be viewed at wileyonlinelibrary.com]
FIGURE 3  General reaction schemes of the warheads in RCKIs to bind to the corresponding nucleophiles. [Color figure can be viewed at wileyonlinelibrary.com]
step forms a thiol-substituted-fluoroacetamide intermediate, where the increased negative charge from the thiol group is redistributed to the \( \alpha \) carbon. Moreover, the presence of fluorine atoms with strong electronegativities further increases the electrophilicity of the \( \alpha \) carbon atom, leading to the formation of a sulfonium intermediate. Then, water acts as a nucleophile and attacks the \( \alpha \)-carbon atom for the hydrolysis step. In contrast, the chloroacetamide-thiol adduct is stable under the same conditions. Mechanistically, the chloroacetamide-thiol displacement reaction forms a stable C-S bond that is not easily hydrolyzed because there is no electronegative atom linking the carbon atom to increase its electrophilicity, unlike the CFA-thiol adduct.

Another type of covalent-reversible warhead is nitrile-related. Generally, nitriles exhibit relative inertness, so the formation of covalent adducts requires strong nucleophiles to attack electrophilic carbon atoms. In practice, the electrophilicity of nitriles can be improved by attaching various electron-withdrawing groups, such as alkylamines in cyanamide (warhead iii), and heteroaryl rings in warhead vii (carbonitrile). In cyanamide (iii), the partial positive charge of the carbon atom can be used as an electrophile through an \( +M \) effect. Moreover, the adjacent nitrogen atom induces the charge redistribution of the nitrile atoms (i.e., -I effect) and thus the positive charge on the carbon atom of the nitrile is amplified, rendering the carbon atom of the nitrile more electrophilic. An aldehyde (iv) is also a very common warhead in proteolytic enzyme inhibitors. However, aldehydes are not often present in drug discovery because the functional group undergoes additional reactions with off-target enzymes, which produces unexpected toxic adducts. Here the aldehyde is used as an RCKI warhead which may reduce toxicity for two reasons. First, RCKIs are generally designed for selectivity. The reversible binding part of the RCKI first binds to the corresponding binding site, and then the electrophile undergoes the covalent chemical reactions. Therefore RCKIs substantially reduce off-targets. Second, by tuning the warhead such that covalent reactivity is reversible to avoid permanent off-target modifications. Nevertheless, the high intrinsic reactivity, poor metabolic and chemical stability, and serious health risks associated with the metabolism of aldehydes make the clinical use of aldehydes problematic. Cyanocrylate (v) is similar to cyanoacrylamide (i) and has the same Michael addition processes as warhead i (cyanoacrylamide). Cyanocrylate (v) was the first warhead used by Taunton and colleagues in 2012 to design RCKIs, leading to various covalent but reversible warheads including cyanoacrylamide (i). Warhead vi was recently developed in designing polo-like kinase 1 (PLK1) inhibitors where the formation of Meisenheimer complexes is considered to be a plausible reaction mechanism (Figure 3). The reversible covalent mechanism was supported by NMR and UV-vis experiments, but no further details were provided, such as binding kinetics assays. Warhead viii was developed based on "iminoboronate chemistry." Here the lysine \( \varepsilon \)-amino group is covalently modified based on the formation of stable iminoboronate with 2-formylbenzeneboronic acid. In designing RCKIs, 2-formylbenzeneboronic acid was morphed into new carbonyl boronic-acid molecules with different scaffolds to achieve prolonged residence time. Next, we review every RCKI based on the described kinase targets and warhead types.

### 2.4 RCKIs

#### 2.4.1 RCKIs of BTK

Currently, there are 20 reported RCKIs with high potency and tuned residence time inhibiting BTK (Figure 4). In 2015, Bradshaw et al. reported three RCKIs based on the Ibrutinib scaffold. The acrylamide warhead of Ibrutinib was replaced by cyanocrylamide (i) capped with methyl (9), isopropyl (10), and tert-butyl (11) (Figure 4A). With the increased steric size of the alkyl capping group, the residence time is prolonged, such that 11—the capping group being tert-butyl—has the longest residence time, showing 55% BTK occupancy 20 h after washout (Figure 4A). The 11-bound BTK crystal structure shows the binding mode of the scaffold to be similar to Ibrutinib (Figure 4B). The covalent bond is formed between the cyanocrylamide warhead and the thiol of Cys481, located on the
front-pocket rim of the ATP binding site. Specifically, the piperidine amide and the tert-butyl capping group both are oriented to shield the proton which is attached to the Ca. This conformation also prevents overlap between the carbonyl π-system and the Ca-H bond, increasing the kinetic and thermodynamic stability. The hydrogen bond between the amide carbonyl of 11 and the backbone NH of Cys481 strengthens the BTK-11 interaction. Two of

![Chemical structures](image)

**FIGURE 4** 2D structures and characteristics of RCKIs inhibiting BTK. (A) Inhibitors 9-11, the corresponding inhibitory potencies and the residence time. (B) the binding mode of inhibitor 11 (PDB id: 4yhf, gray) compared to Ibrutinib (PDB id: 5p9i, light-blue). The covalent bond formation between Cys481 and Cβ. (C–E) Inhibitors 12-17. (F) Inhibitors 18-23, the corresponding inhibitory potencies and the residence time, and (G) the cocrystal structure of the covalent adduct between compound 18 and the mouse BTK kinase domain (PDB id: 6mny). (H–I) PROTACs 24-27 and the corresponding inhibitory potencies and degradation rates. [Color figure can be viewed at wileyonlinelibrary.com]
the capping-group methyls form hydrophobic contacts with Leu483 and Arg525, respectively. Thus, for 11, the hydrogen bond interaction combined with the hydrophobic interaction may further stabilize the covalent complex, leading to a prolonged residence time on the target BTK, compared to RCKIs 9 and 10. Further, Bradshaw et al. identified additional RCKIs (12–15) that improve solubility and oral bioavailability (Figure 4C). Therein, the scaffolds were monofluorinated and more flexible linkers were added to link to the pyrazolopyrimidine scaffolds. Correspondingly, different capping groups were added to the cyanoacrylamide warhead. RCKI 12 showed high potency (IC$_{50}$ = 1.4 ± 0.2 nM) and slow dissociation from BTK (t = 22 ± 3 h). The difference between 12 and 13 is that the methyl-pyrrolidine linkers are a pair of enantiomers where the S-configuration in 13 provides greater potency and slower dissociation (IC$_{50}$ = 0.7 ± 0.1 nM; t = 34 ± 5 h). Based on 13, inhibitors 14 and 15 were synthesized by capping the warhead i (cyanoacrylamide) with polar, branched-alkyl substituents (i.e., morpholine and oxetane in 14 and
that have strong binding potential and longer residence time (IC$_{50}$ = 3.2 ± 0.3 nM; t = 83 ± 14 h for 14 and IC$_{50}$ = 1.9 ± 0.3 nM; t = 167 ± 21 h for 15). Mechanistically, morpholine and oxetane are polar and solvent exposed, forming hydrogen-bond interactions with the solvent and the side chain of Arg525 and therefore improve the stability of the covalent complex in 14 and 15. Further experiments verified that inhibitor 15 has higher selectivity than Ibrutinib based on a 254-nm panel screen. In this evaluation, 1 μM 15 induced >90% inhibition, only BTK and BMX, which are highly homologous and have cysteines at the same positions in the binding sites, were inhibited. A pyrazolopyrimidine fluorescent probe (PP-BODIPY) is an irreversible probe that covalently labels BTK Cys481 with high selectivity and has been shown to penetrate cells.

The PP-BODIPY is a type of molecule designed to selectively bind to a specific target molecule or receptor and emit fluorescent light upon binding. Correspondingly, the target occupancy assay is a method used to determine the extent to which the target molecule or receptor is occupied by the fluorescent probe in a given sample. In practice, the occupancy can be calculated as 100% minus the in-gel fluorescence intensity divided by the control value. As such, PP-BODIPY was used to determine the level of BTK target engagement in rat peripheral blood mononuclear cells (PBMCs) at several times after oral dosing with 40 mg kg$^{-1}$ of 15. In rodent assays, BTK occupancy by 15 revealed that 41% ± 15% of PP-BODIPY-based probe labeling was blocked 24 h after oral dosing. Although the concentration of 15 in plasma fell to 3 ± 3 ng ml$^{-1}$ at 14 h, it showed significant target engagement and slow dissociation from BTK.

Rilzabrutinib (PRN1008, 16 in Figure 4D) from Principia Biopharma is a BTK inhibitor in Phase-III trials to treat immune thrombocytopenia, pemphigus, and other immunologic disorders. With the series discussed in the paragraph above, in contrast to Ibrutinib, its scaffold was monofluorinated and warhead i (cyanoacrylamide) was capped by using a polar, branched-alkyl substituent (Figure 4D). This inhibitor has a high potency (IC$_{50}$ = 1.3 ± 0.5 nM) and long residence time (79% ± 2% of BTK occupancy 18 h after washing in vitro). A further in vitro assay using a 251-nm panel screen showed that 16 has high selectivity. In November 2020 the FDA granted a fast-track designation to Rilzabrutinib for the treatment of patients with immune thrombocytopenia. Another Principia Biopharma-developed reversible covalent BTK inhibitor, PRN473, has the same scaffold structure as 16, just differing in the warhead (Figure 4D). Here, the warhead is capped with a tert-butyl group, forming the reversible covalent interaction with Cys481, analogous to the BTK inhibitors discussed above. Currently, PRN473 has completed Phase-I clinical trials for the treatment of neutrophil-mediated tissue damage.

Besides warhead i (cyanoacrylamide), Shindo et al. introduced a chlorofluoroacetamide (CFA) (warhead ii) to develop a BTK-targeted RCKI (17 in Figure 4E). RCKI 17 was designed based on Ibrutinib and has the same scaffold. The difference is that RCKI 17 has a CFA warhead bearing a cis-4-substituted cyclohexane linker, unlike Ibrutinib, which has an acrylamide warhead with a 3-substituted piperidine linker. RCKI 17 exhibits strong inhibitory activity on in-cell BTK autophosphorylation (IC$_{50}$ = 44 nM). Moreover, in a Ramos cell assay, RCKI 17 maintained an 82% BTK occupancy 12 h after cell washout, which suggests RCKI 17 has a long residence time.

Another kind of warhead used to design BTK-targeted RCKIs is warhead iii (cyanamide, Figure 4F–G). Schnute et al. observed that the aminopyrimidine of Ibrutinib forms two conserved hydrogen bonds with the BTK hinge residues Glu475 and Met477 (Figure 4G). Consequently, the authors replaced aminopyrimidine with aminopyrazole carboxamide, without affecting the two conserved hydrogen bonds, resulting in an Ibrutinib-like pseudo-bicyclic arrangement. This modification was used to design a series of novel RCKIs with warhead iii (cyanamide) which achieve reversibility (Figure 4F). The addition reaction was carried out between the cyanamide carbon of warhead iii and the thiol of Cys481 (Figure 4F, 18–23). Further modification of the scaffolds, such as pyridinyl substitution (21–23), did not impact the potency (IC$_{50}$) or residence time (t) (Figure 4F).

As stated, proteolysis targeting chimeras (PROTACs) are receiving more attention as a treatment modality. Typically, PROTACs are composed of three parts: a protein target binder, a linker, and an E3 ubiquitin ligase ligand. Upon binding, PROTAC forms a ternary complex with the target protein and E3 ubiquitin ligase, leading to ubiquitination and proteasomal degradation of the target protein. Introducing covalent interactions between the PROTAC and the protein target is one way to strengthen the binding affinity. Recently, several successful
irreversible covalent PROTAC degraders have been designed. However, irreversible binding may negate the catalytic properties of PROTAC, reducing PROTAC’s potency. Gabizon and Guo et al. recently reported multiple reversible covalent BTK PROTACs (24–27) with high target occupancy and effectiveness as a degrader (Figure 4H). All four PROTAC degraders are Ibrutinib scaffold-based and the electrophile is cyanoacrylamide (i). The different linkers capping cyanoacrylamide (i) yield the different IC50s and DC50s (i.e., compound concentration inducing 50% protein degradation, Figure 4I). PROTAC dissociation times of 10–20 h are similar to the BTK RCKIs with the warhead cyanoacrylamide, which may lead to reduced catalytic efficiency compared to rapid degradation. Importantly, reversible covalent PROTACs maintain the strong covalent interaction with the kinase BTK like RCKI, and significantly improve the selectivity. Therefore, reversible covalent PROTAC degraders are promising, especially for the degradation of a target which has no high-affinity reversible ligands available, and targets where the selectivity of their reversible covalent inhibitors needs to be improved.

2.4.2 | RCKIs of EGFR

Rauh’s lab reported a series of EGFR-targeted RCKIs to address EGFR drug resistance (Figure 5). These RCKIs (28–32) were designed based on the co-crystal structure of EGFR and pyrazolopyrimidine-based

![Diagram of EGFR RCKIs](image)

**Table: IC50 Values for EGFR RCKIs**

| EGFR | IC50 (nM) |
|------|-----------|
| WT   |           |
| L858R| 538 ± 3889|
| L858/R790M| 229 ± 62  |
| L858/R790M| 4541 ± 543|
| WT   | >10 μM    |
| L858R| 385 ± 207 |
| L858/R790M| 2543 ± 420|
| WT   | 102 ± 75  |
| L858R| 21 ± 20   |
| L858/R790M| 80 ± 51   |

**Table: IC50 Values for EGFR RCKIs**

| EGFR | IC50 (nM) |
|------|-----------|
| WT   |           |
| L858R| 96 ± 26   |
| L858/R790M| 10 ± 8    |
| L858/R790M| 20 ± 13   |
| WT   | 338 ± 69  |
| L858R| 85 ± 34   |
| L858/R790M| 213 ± 49  |

**Figure 5** (A) Binding mode of the pyrazolopyrimidine-framework covalent inhibitor (PDB id: 5j9y); (B, C) RCKIs of EGFR and the corresponding binding affinities. [Color figure can be viewed at wileyonlinelibrary.com]
inhibitors published by the same lab. (Figure 5A). The acrylamide warhead was replaced by cyanoacrylamide (warhead i), which forms a covalent bond with Cys797 located at the front pocket of the rim of the binding site. The different R substituent groups affect the potency of the corresponding inhibitors (Figure 5C). Inhibitor 31 is a strong inhibitor and is over fivefold more selective for the L858R/T790M mutant (IC\textsubscript{50} = 20 ± 13 nM) over wildtype EGFR (IC\textsubscript{50} = 96 ± 26 nM). Thus, inhibitor 31 can be used as a promising starting point for developing more selective mutant EGFR inhibitors. The authors used a mass-spectroscopy method to characterize the reversible features of RCKIs. This method includes a three-step protocol to characterize RCKIs. First, the apo kinase results in a single peak with a defined m/z value. Second, treatment with an RCKI leads to a characteristic shift of the peak by the molar mass of the RCKI (Peak + ΔMRCKI). Third, after incubation with another covalent inhibitor, another shift of the peak by the molar mass of the covalent inhibitor will occur, with the Peak + ΔMRCKI disappearing if the RCKI tested is reversible in the timescale of the assay.

2.4.3 | RCKIs of JAK3

The Janus kinase (JAK) family, comprising JAK1, JAK2, JAK3, and TYK2, are attractive targets in the development of anti-inflammatory drugs. Achieving selectivity among JAK family members is an essential yet challenging step in drug discovery resulting from the high degree of structural similarity. The kinase cysteinome reveals JAK3 as having a noncatalytic cysteine residue, Cys909, at the rim of the ATP binding site which does not exist in other JAKs, namely JAK1, JAK2, and TYK2. Over the entire human kinome, MAP2K7, TEC, TXK, ITK, BTK, BLK, HER2, EGFR, and HER4 kinases also contain a cysteine amino acid in the same position as Cys909 of JAK3. Thus, targeting this cysteine is a very promising strategy for achieving isoform-selectivity for JAK3. Forster et al. reported two JAK3-specific RCKIs (33 and 34) that bind to the ATP-binding pocket and induce a small binding cleft in the area of the front pocket (Figure 6A-B). Inhibitors 33 and 34 with IC\textsubscript{50} values in the picomolar range (127 pM and 154 pM, respectively) show high selectivity (Figure 6A). A binding kinetics assay shows that inhibitor 33 has a prolonged residence time of 50 min for JAK3. The authors determined the crystal structure of JAK3 kinase domain in complex with 34, showing the coexistent binding modes of 34 covalently and the non-covalently bound to JAK3 (Figure 6B). Both inhibitors, 33 and 34, use warhead i (cyanoacrylamide) as an electrophile to form reversible covalent interactions with Cys909. The interactions between the nitrile function groups of inhibitors 33 and 34 and nearby residues Arg911, Asp912, and Arg953 induce the shallow pocket (Figure 6B), contributing to the selectivity of 33 and 34. In 2014, London et al. reported a potential RCKI (35 in Figure 6C) using warhead i (cyanoacrylamide) which covalently targets Cys909 (IC\textsubscript{50} = 49 nM) and was shown to be reversible based on a dilution experiment. Inhibitor 35 has multiple off-targets, such as BLK (IC\textsubscript{50} = 22 nM) and HER4 (IC\textsubscript{50} = 44 nM), and has to be further improved.

Casimiro-Garcia et al. identified a set of JAK3 RCKIs. As exemplified by RCKI 36 (Figure 6D), it uses warhead iii (cyanamide) as the electrophile, covalently targets Cys909 (IC\textsubscript{50} = 456 nM, >22-fold selectivity vs JAK1), and is reversible (residence time = 154 min). There are two hydrogen-bond interactions between the pyrrolopyrimidine of inhibitor 36 and the residues Glu903 and Leu905 at the hinge region of JAK3 (Figure 6E). The covalent bond occurs between the nitrile moiety and Cys909 to give an isothiourea adduct (Figure 6E), similar to the binding mode reported for the aforementioned BTK RCKIs (see inhibitor 18).

2.4.4 | RCKIs of FGFR1

The FGFR kinases, FGFR-1, -2, -3, and -4, are promising therapeutic targets for multiple types of cancer. Bradshaw et al. reported a series of FGFR1-targeted RCKIs 37-47 (Figure 7A) based on warhead i (cyanoacrylamide) and a pyrimidopyridine scaffold previously used to design irreversible FGFR inhibitors.
Here, Cys486, located within the P loop, was used as the nucleophile to form the reversible covalent interaction with the cyanoacrylamide derivative-based warheads in RCKIs 37–47. RCKIs 37–47 are strong inhibitors (IC_{50} \leq 6 \text{nM based on enzyme-activity assays}). The different capping groups (R1, see inhibitors 37–47) on the cyanoacrylamide warhead allowed for the tuning of residence times with the change of 24-h occupancy rate (%) ranging from 0 ± 10% to 99 ± 8%. As such, the range of residence times was from 11 ± 11 h to >150 h, highlighting again that simple modifications to the capping group change residence time, which is important for designing drugs with the desired durability. Conceptually, the capping groups, attached to the β-carbon of reversible covalent warheads, form noncovalent interactions with the protein targets near the new covalent bond, resulting in conformational stabilization and masking of the proton on the α-carbon, therefore stabilizing the complex, leading to prolonged residence times.29 Thus, modifying the capping group has become an effective strategy in designing inhibitors with a variety of residence times.53,72
FGFR4 is a driver of some solid tumors, for example, rhabdomyosarcoma (RMS) and hepatocellular carcinoma (HCC), and hence has attracted efforts to seek highly selective inhibitors. In FGFR4, a cysteine (Cys552) is located at the GK + 2 position of the hinge region. In the entire kinome there are only five kinases with cysteines at the hinge region.

![Diagram of FGFR4](image)

**FIGURE 7** (A) 2D chemical structures of RCKIs of FGFR1. Warhead i is shown in the dashed rectangle. (B) Binding mode of Roblitinib based on the co-crystal complex structure (PDB id: 6yi8). The yellow dash lines show the H-bond interaction between Roblitinib and the amino acids R483, V500, and A553. (C) The FGFR4-targeted RCKIs with the potency and the corresponding residence time. [Color figure can be viewed at wileyonlinelibrary.com]
Based on previous chemical experiments showing that thiols can react with cyanoacrylates with rapid reversibility at physiological pH,

54 Serafimova et al. designed RCKIs based on an irreversible covalent RSK2 inhibitor, FMK (a fluoromethylketone-based inhibitor).

28,86 The irreversible warhead (fluoromethylketone) of FMK was replaced by cyanoacrylate (warhead v) and its derivative to obtain reversible covalent inhibitors 57 and 59, and replaced by cyanoacrylamide (warhead i) to obtain reversible covalent inhibitor 58 (Figure 8A). The co-crystal structure with inhibitor 59 shows that the thiol group of Cys436 on the β2 sheet forms the covalent C-S bond with warhead v (cyanoacrylate). Further experiments verified the dissociation of the covalent bonds, as evidenced by unfolding or proteolysis,

28 showing the reversible nature of 57-59. This pioneering work,28 demonstrates the introduction of the reversible warhead cyanoacrylate for designing the first RCKI, and further development of the warhead cyanoacrylamide. Moreover, the authors

28 concluded that the reversible thiol-addition and elimination chemistry seemed
to be a general characteristic of cyanoacrylamide, which is very important for a reversible covalent targeting strategy applied to all kinases.

In a separate study, Taunton et al. used an electrophilic fragment-based design strategy to develop RCKIs 60 and 61 (Figure 8B). Inhibitor 60 targets wild-type and T493M RSK2 with a strong potency ($IC_{50} = 15 \pm 2$ nM and $3 \pm 1$ nM, separately) and was designed using a trimethoxyphenyl-substituted indazole as the scaffold and warhead (cyanoacrylamide) as the electrophilic group. The scaffold of inhibitor 61 is the same as 60, but warhead (cyanoacrylamide) was capped using a 1,1-dimethyl-2-hydroxyethyl group, which keeps the strong inhibitory ability against wild-type and T493M RSK2 ($IC_{50} = 13 \pm 2$ nM and $< 2.5$ nM, separately). The co-crystal structure of 61 and T493M RSK2 shows a covalent bond formed between the electrophilic $\beta$-carbon of the cyanoacrylamide (warhead i) and the thiol of Cys436 (Figure 8B). Based on an unfolding experiment of the 61/RSK complex using guanidinium-HCl, the reversibility of inhibitor 61 was verified. The authors also tested whether inhibitor 61 could inhibit the MSK1 C-terminal kinase domain through a reversible covalent interaction with Cys440 ($IC_{50} \approx 100$ nM).

Taunton's group and Shoichet's group reported a virtual covalent docking method (http://covalent.docking.org) used to discover reversible covalent chemical probes by screening large virtual libraries of electrophilic small molecules. As expected, a series of cyanoacrylamide inhibitors were predicted to target RSK2 and MSK1, including inhibitor 62 (Figure 8C), which was an RSK2-targeted probe with a strong potency ($IC_{50} = 40$ nM). The web server and screening scheme provide a valuable resource for the rapid discovery of potential reversible covalent chemical probes.

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**FIGURE 8** (A) 2D chemical structures of RCKIs 57–59 and the binding modes of 59 shown in the co-crystal inhibitor-RSK complexes (PDB id: 4d9u). (B) 2D chemical structures of RCKIs 60–61 and the binding modes of 61 (PDB id: 4jg8). (C) 2D chemical structures of RCKI 62. [Color figure can be viewed at wileyonlinelibrary.com]
2.4.7 | RCKIs of PLK1

Overexpression of Polo-like Kinase 1 (PLK1) is a common feature of cancers, such as gastric and breast cancer.\(^\text{88}\) Pearson et al.\(^\text{62}\) reported a series of PLK1-targeted RCKIs including inhibitor 63 (Figure 9). Inhibitor 63 forms a covalent bond with Cys67, which is located on the β2 sheet close to the P-loop region. A subsequent experiment verified that inhibitor 63 could effectively inhibit the overexpression of PLK1 with high selectivity (IC\(_{50}\) = 2.47 ± 1.23 uM). Inhibitor 63 contains the benzothiazole N-oxide scaffold (warhead vi) and forms a reversible covalent bond as a Meisenheimer complex (MC), which is stable and fully reversible under normal conditions.\(^\text{89}\) The suggested covalent mode of action was supported by UV-Vis spectrophotometry and NMR experiments. The electron rearrangement when forming the adduct between the arene carrying three electron-withdrawing groups and a nucleophile is shown in Figure 9. Changing the electron-withdrawing groups modulates electrophilicity and with it the compound's potency such that inhibitor 64 has higher binding potential (IC\(_{50}\) = 0.39 ± 0.07 uM) due to the difference in the R1 substituted group (SCF\(_3\)), which has a stronger potential to interact with the ATP binding site.\(^\text{16}\)

2.4.8 | RCKIs of eEF-2K

Elongation factor 2 kinase (eEF-2K) belongs to the atypical group of kinases and plays an important role in maintaining cellular homeostasis and tumor-cell survival and proliferation,\(^\text{90}\) making eEF-2K a potential target for cancer treatment, notably breast cancer. Devkota et al. reported an RCKI inhibitor 65, 2,6-diamino-4-(2-fluorophenyl)-4H-thiopyran-3,5-dicarbonitrile, discovered through structure-based virtual screening (Figure 10A).\(^\text{90}\) Kinetic experiments and molecular docking suggest that RCKI 65 acts via a reversible covalent mechanism of inhibition where the carbonitrile group (warhead vii) forms a reversible thioimidate adduct with the eEF-2K’s Cys146 located on the β2 sheet of the P-loop region of the ATP binding site (Figure 10B).

2.4.9 | RCKIs of ABL

The BCR-ABL fusion gene is associated with chronic myelogenous leukemia (CML). Even though multiple ABL-targeted drugs have been developed, frequent acquired drug resistance occurs. Lack of available cysteines at the binding site precludes the design of cysteine-targeted covalent ABL kinase inhibitors. Recently, Quach et al.\(^\text{42}\)
designed a class of reversible covalent small-molecule inhibitors targeting the catalytic lysine residue of the β3 sheet in the N-lobe of ABL (part of the ATP binding pocket) using an iminoborionate strategy. The two inhibitors 66–67 were designed based on a noncovalent pan-ABL inhibitor PPY-A91 (Figure 11A) equipped with the reversible covalent warhead viii (aldehyde boronic acid, Figure 2). The co-crystal structure of the inhibitor 66-bound ABL complex illustrates that the aldehyde (warhead viii) forms an imine by reacting with the amine of Lys271 in the ATP-binding pocket (Figure 11B). The boron atom of the boric acid functional group and the imine nitrogen of Lys271 form the coordinate bond interaction, suggesting that boric acid plays a key role in the stabilization of the imine during the condensation process. The covalent interaction yields high potency: inhibitor 66 with IC50 (WT) = 1.7 ± 0.2 nM, IC50(T315I) = 0.1 ± 0.1 nM, and IC50 (E255K) = 0.5 ± 0.03 nM; and inhibitor 67 with IC50(WT) = 5.0 ± 0.4 nM. The reversibility of inhibitors 66–67 was confirmed using a NaBH3CN labeling experiment and an alkyne-containing analog (68). NaBH3CN is a reducing agent that traps the imine by reduction to a stable amine.64 In the presence of NaBH3CN, after incubation (i.e., NaBH3CN reacts with the imine to form an intermediate iminium, and then the intermediate is reduced, resulting in the formation of the amine), an increase in the fluorescence intensity of 68-labeled proteomic samples was observed. In contrast, washing the 68-labeled proteome with cold acetone and methanol in the absence of NaBH3CN significantly reduced or eliminated the
fluorescence signals. Thus, the reversibility of the iminoboronate bond was confirmed. The authors also showed further evidence for the reversibility of the iminoboronate bond using $^1$H NMR studies of the imine product formed between 2-formylphenyl boronic acid and Ac-Lys-NHMe.42

3 | RCKIS DESIGN STRATEGIES

3.1 | Major strategies to develop RCKIs

Based on prior work, three major strategies for developing RCKIs emerge. The first is to tune the electrophilic groups of existing irreversible-covalent kinase inhibitors towards reversibility. Existing covalent kinase inhibitors have a well-defined scaffold and the nucleophilic group has been validated to be within striking distance. For example, Ibrutinib is a prototypical BTK covalent kinase inhibitor. Based on Ibrutinib, many BTK-targeted RCKIs, such as RCKIs 9–11, have been reported by creating reversible warheads.

The second strategy is to engineer a reversible covalent electrophilic group based on an existing noncovalent kinase inhibitor. For example, JAK3-targeted RCKIs 33 and 34 were developed by using a noncovalent JAK3 compound (IC$_{50}$ = 63 nM, and Supporting Information S1: Figure S1) as the starting point. Specifically, a linker moiety bearing an electrophile was added to the compound to suitably interact with the Cys909 of JAK3.78 Thus, high potency and selectivity of RCKIs 33 and 34 were obtained and proven in a cellular model.78 Given the large number of known noncovalent kinase inhibitors, and the increasing repository of kinase structures,16 this noncovalent inhibitor-based strategy is a valuable approach.

The third strategy is a high-throughput screen. In this RCKI screening protocol, a covalent docking method is used to anchor the covalently interacting atoms. High-throughput screening extends the chemical conformation space of RCKIs and provides opportunities to design chemically novel RCKIs. For example, London et al. applied a virtual screening scheme for discovering novel RCKIs and AmpC β-lactamase-targeted reversible-covalent inhibitors.31 However, virtual screening method while easy to use, may produce false hits and artificial binding poses, thus further experimental validation is warranted.92

3.2 | Privileged reversible-covalent warheads

The electrophile is the centerpiece of designing RCKIs. The nature of the electrophilic groups determines, among other factors, the reversibility and the residence time. Currently, there are 8 types of warheads reported (Figure 2). The corresponding derivatives, such as cyanoacrylamide, capped with different substituents contribute to the selectivity and residence time. For example, inhibitors 37–47 achieve different residence times by modifying warhead i (cyanoacrylamide) using different substituent groups (Figure 7). Therefore, choosing a potential electrophile and further tuning its reversible-covalent electrophilic properties are essential steps. One way to choose an electrophile is from available warhead libraries.93 Given the success of irreversible covalent kinase drugs, multiple electrophilic warhead databases for irreversible covalent kinase drug design are available.30,41,93 With this warhead toolbox researchers can tweak the characteristics of the chosen irreversible covalent electrophile to establish the reversible covalent interactions. Krishnan et al.55 used a computational method to analyze the intrinsic reaction trends of different electrophilic groups during the thiol-Michael addition reaction. This intrinsic trend is beneficial in guiding the design of the desired reversible covalent interaction. The other way to choose an electrophile is based on the intrinsic reversibility of chemical reactions between the electrophile and nucleophile. For example, experiments in 1968 found that the chemical reaction between thiol and cyanoacrylamide was reversible.54 Currently, cyanoacrylamide has been used to successfully design RCKIs to target BTK, EGFR, JAKs, and others. As such,
cyanoacrylamide is a privileged reversible-covalent warhead based on the intrinsic reactive properties and proven successful applications.

3.3 | Nucleophilic residues in the binding site

Just as important, are nucleophilic groups within warhead striking distance.\(^{41,94}\) So far, multiple nucleophilic amino acids, including cysteine and lysine, from 10 distinct kinases have been targeted as RCKIs (Figure 12A). The 10 kinases belong to 4 kinase groups (Figure 12A): (1) the TK group including the kinases ABL, and BTK. EGFR, JAK3, FGFR1, and FGFR4; (2) the AGC group including the kinases MSK1 and RSK2; (3) the kinase PLK1, and (4) the atypical group including the kinase eEF-2K. More importantly, the nucleophilic groups are located at different positions within the binding sites (Figure 12B), including the cysteine (Cys552) at the Hinge region targeted by the RCKIs of FGFR4; the cysteine (Cys486) at the P-loop targeted by the RCKIs of FGFR1; the cysteines (Cys481 in BTK, Cys797 in EGFR, and Cys909 in JAK3, respectively) at the Front pocket at the rim of binding site targeted by the RCKIs of BTK, EGFR, and JAK3; the cysteines (Cys436 in RSK1, Cys440 in MSK1, Cys67 in PLK1, and Cys146 in eEF-2K, respectively) at the β2 sheet targeted by the RCKIs of RSK1, MSK1, PLK1, and eEF-2K; and the catalytic lysine (Lys271) at the β3 sheet targeted by the RCKIs of ABL1. Targeting lysine amino group with covalently interacting electrophilic ligands has been a challenging task due to the high pKa (~10) leading to complete protonation under physiological condition (pH=7.4). However, buried lysines in a hydrophobic environment frequently have an altered (lower) pKa which makes them more likely to be available. For example, RCKIs 66 and 67 were designed and validated as targeting the conserved catalytic lysine of ABL1 (Lys271). These results suggest that designing selective RCKIs to target the conserved lysine in ATP binding sites may be practicable.\(^{42,95}\) Based on the previous study of the cysteinome,\(^{9,39,41}\) there are over 200 kinases with accessible cysteines within striking

![Figure 12](image-url)
distance, providing an abundant structural resource to design RCKIs. Our previous study concluded that the microenvironment of the cysteine inside the binding sites affects the possibility of covalent reactions. Microenvironment also affects the reversibility of the covalent reaction. For example, Shindo reported a series of RCKIs using a chlorofluoroacetamide (CFA, Warhead ii) as the electrophilic group and experimentally validated that the CFA-based thiol adduct was easily hydrolyzed under neutral aqueous conditions. Based on the molecular architecture of Afatinib as the scaffold, showed high reactivity to the Cys797 of EGFR. The linkers connecting the warhead CFA and the scaffold affected the reaction rate. Moreover, the CFA-based EGFR probes were stable in the solvent-sequestered EGFR ATP binding pocket (t1/2 > 72 h). In contrast, the CFA-based probes showed reversible properties when targeting Cys481 of BTK, such that maintained an 82% BTK occupancy rate 12 h after cell washout, which indicated that Cys481 in BTK is more solvent-accessible than the Cys797 in EGFR. This suggests that the influence of the microenvironment near the nucleophilic residue should be taken into account during designing RCKI warheads, especially for CFA, a solvent environment-sensitive warhead, to target more noncatalytic cysteines located at different positions of the kinase domain.

4 | CONCLUSION AND OUTLOOK

Here we systematically describe progress with RCKIs, summarizing the different types of reversible covalent warheads and the corresponding striking nucleophilic residues among 10 different kinases, highlighting design strategies for RCKIs, the privileged reversible covalent warhead cyanoacrylamide, and effects of microenvironments near the nucleophilic residues especially for the solvent-sensitive warhead CFA. We summarize three RCKI design pipelines, which have benefited from the current successes with reversible covalent kinase inhibitors and diverse kinase inhibitors. Notably, a large number of active kinase inhibitors, such as the ~54,000 active compounds in ChEMBL Kinase SARfari, provide abundant structure–activity relationships and design opportunities. Also, elaborating upon the reversibility of electrophilic groups is an essential step in obtaining reversible covalent characteristics. Multiple research groups have systematically studied the intrinsic reactivity of diverse warheads, which helps determine the right electrophilic groups as the starting point for designing RCKIs. Fortunately, warhead i (cyanoacrylamide) showed privileged reversible covalent properties and has been the most frequently used electrophilic group and has been designed to target different kinases including BTK, EGFR, JAK3, RSK2, and MSK1. Correspondingly, abundant cysteine residues, located in different positions of the binding sites in about 200 kinases, and catalytic lysine residues across the whole kinome, can act as nucleophiles, thereby providing tremendous opportunities for developing RCKIs. The reversibility of electrophilic groups should be specifically estimated when the nucleophilic groups are located in different microenvironments, such as solvent-accessible or solvent-sequestered sites.

Measured against the whole human kinome, the field of RCKI development is far from mature. With the anticipated approval of reversible covalent drugs, their advantages, especially the tunable residence time, will attract more attention in reversible covalent drug discovery to treat chronic diseases. For instance, Rilzabrutinib, a Phase-III drug, illustrates the potential by utilizing its rapid reversibility avoiding unwanted adverse effects on the immune system. RCKIs may have another advantage with targets having a high turnover, as shown in the development of FGF401, where the rapid turnover of FGFR4 led the Novartis researchers to pursue a reversible-covalent approach instead of an irreversible one. In conclusion, we can expect more kinases to be targeted by reversible covalent inhibitors.

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DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article.
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**SUPPORTING INFORMATION**

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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