# Exploring Extended Warheads of Reversible and Irreversible Cysteine-Targeted Covalent Kinase Inhibitors

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## Abstract

In designing covalent kinase inhibitors (CKIs), the inclusion of electrophiles as attacking warheads demands careful choreography, ensuring not only their presence on the scaffold moiety but also their precise interaction with nucleophiles in the binding sites. Given the limited number of known electrophiles, exploring adjacent chemical space to broaden the palette of available electrophiles capable of covalent inhibition, is desirable. Here, we perform a systematic analysis of the characteristics of warheads and corresponding adjacent fragments for use in CKI design. We first collect all the released cysteine-targeted CKIs from multiple databases and create one CKI dataset containing 16,961 kinase-inhibitor data points from 12,381 unique CKIs covering 146 kinases with accessible cysteines in their binding pockets. Then we analyze this dataset, focusing on the extended warheads (i.e., warheads + adjacent fragments) — including 30 common warheads and 1344 unique adjacent fragments. Thus, we provide structural insights, and delineate chemical properties, and patterns in these extended warheads. Notably, we highlight the privileged patterns observed within reversible CKIs for the popular warheads cyanoacrylamide and aldehyde. This study provides medicinal chemists with novel insights into extended warheads and a comprehensive source of adjacent fragments, thus guiding the design, synthesis, and optimization of CKIs.

## Keywords

Kinome-scale inhibitor database; Cysteinome; Covalent Kinase Inhibitor; Extended Warhead; Adjacent Fragment Library; Drug Discovery

## Abbreviations

AFL: adjacent fragment library

BTK: Bruton's tyrosine kinase

CKI: covalent kinase inhibitor

FDA: Food and Drug Administration

DFG: Asp-Phe-Gly motif

EGFR: epidermal growth factor receptor

FGFR2: fibroblast growth factor receptor 2

FGFR4: fibroblast growth factor receptor 4

JAK3: Janus kinase 3

KLB: klotho beta

NSCLC: non-small cell lung cancer

RCKI: Reversible covalent kinase inhibitor

RSK2: Ribosomal S6 kinase 2

## 1.Introduction

As of November 29, 2023, eighty kinase-targeted small molecule drugs have been approved by the United States Food and Drug Administration (FDA), illustrating the value of kinase drug development for the treatment of a variety of diseases,[1-3] such as non-small cell lung cancer (NSCLC).[4, 5] However, these drugs target just 24 kinases, a small part of the human kinome implying considerable scope remains for developing kinase inhibitors, probes, and drugs.[6-8]

Kinase inhibitors published so far can be divided into Type-I, -II, -III, and Type IV according to their corresponding binding modes.[9, 10] Type-I inhibitors bind into the ATP binding site with the active (DFG-in) kinase conformation like ATP.[2] Typically Type-I inhibitors are composed of an adenine-analog core fragment that forms 2-3 hydrogen-bond interactions with the hinge

region.[11, 12] Type-II inhibitors not only bind into the ATP binding site with the inactive (DFG-out) kinase conformations but also extend into the nearby allosteric pocket, where Type-III inhibitors interact.[13, 14] In contrast, Type-IV allosteric inhibitors bind to pockets away from the ATP binding site, such as those pockets distributed on the C-terminal lobe.[14] To achieve favorable selectivity and potency, other types of inhibitors with promising features have been developed, such as macrocyclic inhibitors and covalent inhibitors.[15-18] Macrocyclic inhibitors refer to, as the name suggests, inhibitors possessing a macrocycle (>12-membered ring).[19] Covalent inhibitors are those compounds that bind into the binding pockets covalently reacting with non-catalytic nucleophilic residues.[20, 21] Due to non-catalytic nucleophilic residues being poorly conserved, covalent interactions offers more opportunities to enhance selectivity across the whole kinome. Thus far, ten covalent kinase drugs have been approved by the FDA, targeting epidermal growth factor receptor (EGFR), Bruton's tyrosine kinase (BTK), fibroblast growth factor receptor 2 (FGFR2), and Janus kinase 3 (JAK3), respectively (**Figure 1**).[5] Since designing covalent inhibitors by manipulating the electrophiles is attractive, tens of common electrophiles used as warheads (e.g., acrylamide) have been applied to the discovery of CKIs, with cysteine, tyrosine, and threonine as nucleophiles.[22-26] However, such a small number of electrophiles is limiting, thus, it is helpful to extend the design of warheads to include adjacent fragments. Here, we define adjacent fragments as those fragments that are immediately adjacent to the warhead and connect the warhead and scaffold moieties. Combining adjacent fragments and warheads offers more combinations to achieve binding.

Fortunately, tens of thousands of CKIs have been published and cumulatively provide a structural basis to explore adjacent fragments.[23, 27] We focus on cysteine-targeted CKIs since they are more widely used and provide a variety of bioactive data.[23] We first integrate multiple

kinase data sources, into a curated CKI dataset, and investigate the CKIs' chemical space, structural characteristics, and target space with emphases on the features of extended warheads, especially for reversible CKIs.

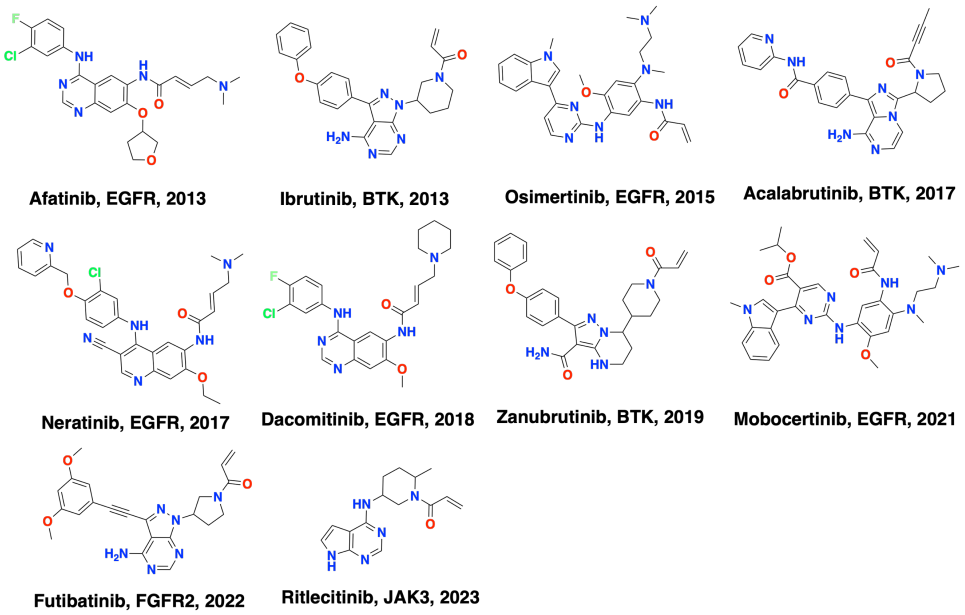


**Figure 1.** Ten FDA-approved CKIs showing their primary target and year of approval.

## 2. Results

### 2.1 CKI Dataset

We integrated all CKIs from the BindingDB and CheMBL databases and the in-house CKI dataset (see Method section). After curation (see Method section), we obtained 16,961 electrophiles-equipped bioactive data points including 12,381 unique CKIs, for 146 out of 208 kinases that possess available cysteines near the ATP binding site.[2] **Figure 2** shows the human kinome phylogenetic tree with the distributions of the 146 kinases, which cover all protein kinase groups (TK, TKL, STE, CK1, AGC, CAMK, CMGC, and Other).[1, 28] 95 out of 146 kinases just have single-digit curated CKIs while the top 9 kinases have more than 500 CKIs (**Figure 3**). Interestingly, the 10 approved CKIs (**Figure 1**) are all distributed among the top 9 kinases (BTK,

EGFR, FGFR2, and JAK3). This indicates that CKI's discovery is challenging, since although thousands of CKIs have been published or patented, only very few ultimately enter the clinic. Conversely, this distribution implies that more promising covalent drugs targeting more kinases are possible with the accumulation of CKIs.

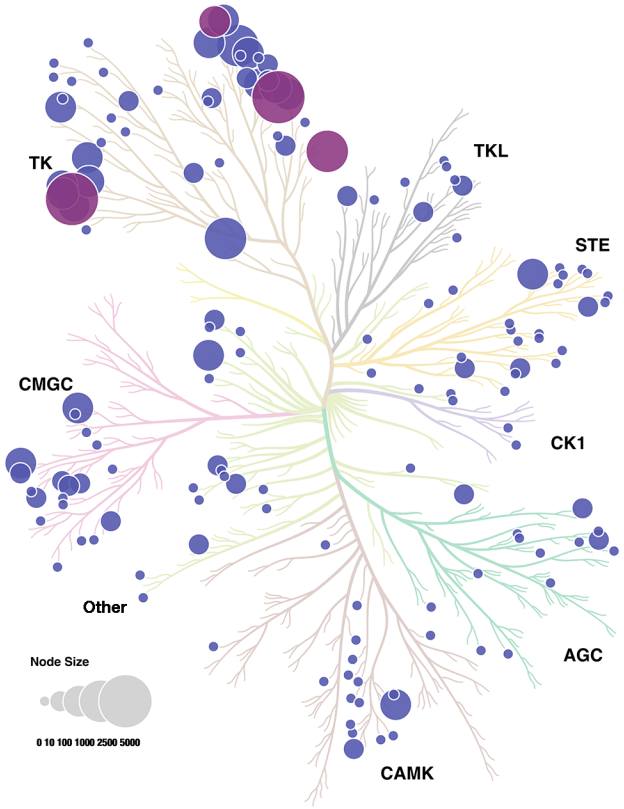


**Figure 2.** Distribution of CKIs on the human kinome phylogenetic tree. Each point represents one kinase with released (blue), or approved CKIs (purple). The point size is proportional to the number of CKIs.

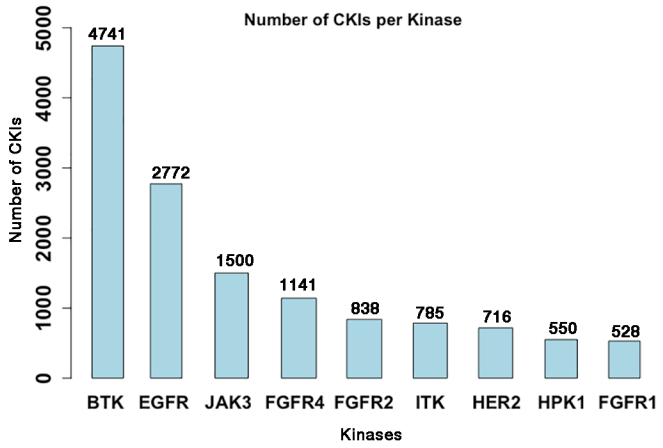


**Figure 3.** The top 9 kinases with >500 CKIs.

## 2.2 CKI Warheads

Dozens of warhead fragments have been successfully applied in the design of CKIs. Here, we surveyed recently published warhead-related reviews and selected 30 commonly used warheads for determining potential CKIs (**Figure 4** and Method section).[2, 18, 22-26, 29] Notably, warheads **1-22** are often used to design irreversible CKIs while **23-30** occur in reversible CKIs. Using the warheads as bait, the corresponding inhibitors equipped with these warheads were fished out, and referred to as potential CKIs in our dataset. **Figure 5** shows the warheads and the distribution of corresponding CKIs. Acrylamide and its derivatives account for the vast majority of CKIs with 10870 (warhead **1**) and 2780 (warhead **2**) CKIs. The second primary component is butynamide and its analog with 976 (warhead **4**) and 45 (warhead **5**) compounds. From the reversible warheads **23-30**, aldehyde and cyanoacrylamide dominate the distribution with 770 (warhead **26**) and 716 (warheads **23-25**) CKIs, respectively.

CKIs with these warheads target different kinases (**Figure 6**). The 13,650 acrylamide-containing CKIs target 132 kinases (**Figure 6a**). The butynamide-containing CKIs target 43 kinases (**Figure**

**6b**). The aldehyde-containing CKIs target 41 kinases and the cyanoacrylamide-containing CKIs target 21 kinases (**Figure 6c-d**). These warheads, which target multiple kinases, show privileged properties,[22] and provide a design strategy for repurposing these warheads in the development of new CKIs.[30] It is worth noting that acrylamide, cyanoacrylamide, and butynamide are similar in structure because they all contain a common amide feature, and have proved tractable in kinase covalent drug design. However, aldehydes have not been commonly used in drug discovery because they can undergo unexpected reactions with off-target enzymes, resulting in toxic side products.[31] For kinase-targeted covalent drug design, an aldehyde that is used as a reversible warhead must be analyzed for its high reactivity, metabolic, and chemical instability.[32] [33, 34]

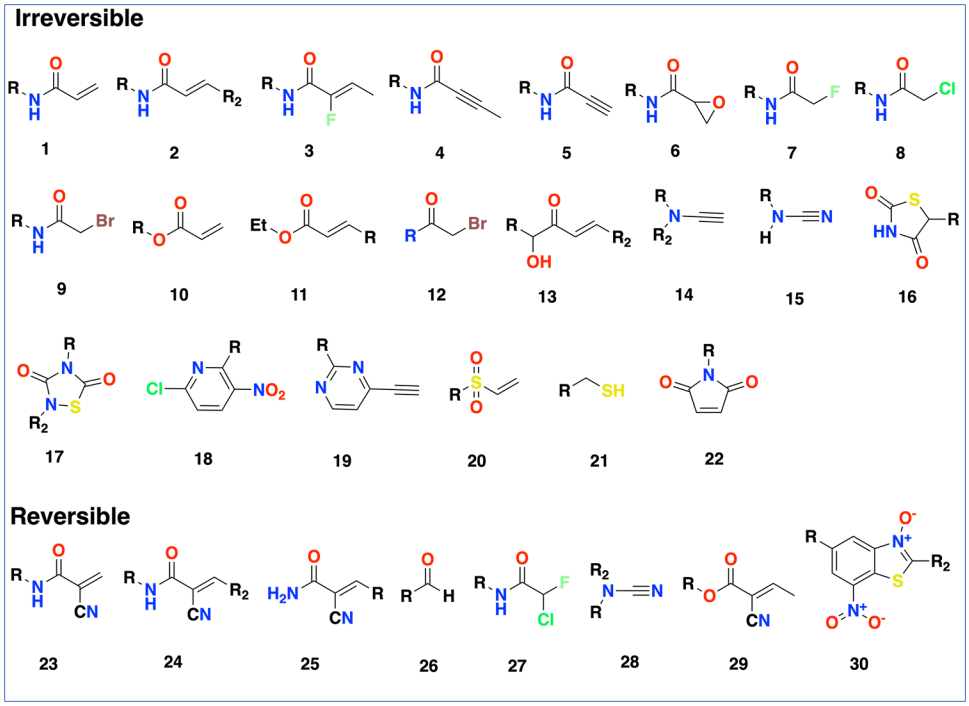


**Figure 4**. CKI warheads, **1-22** are irreversible and **23-30** are reversible.

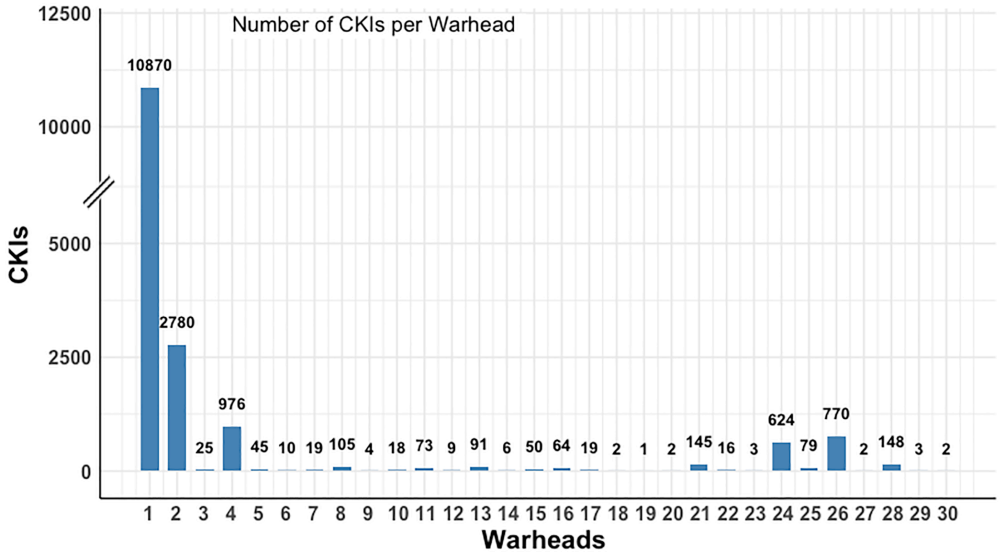


**Figure 5.** CKI warheads and the corresponding number of CKIs.

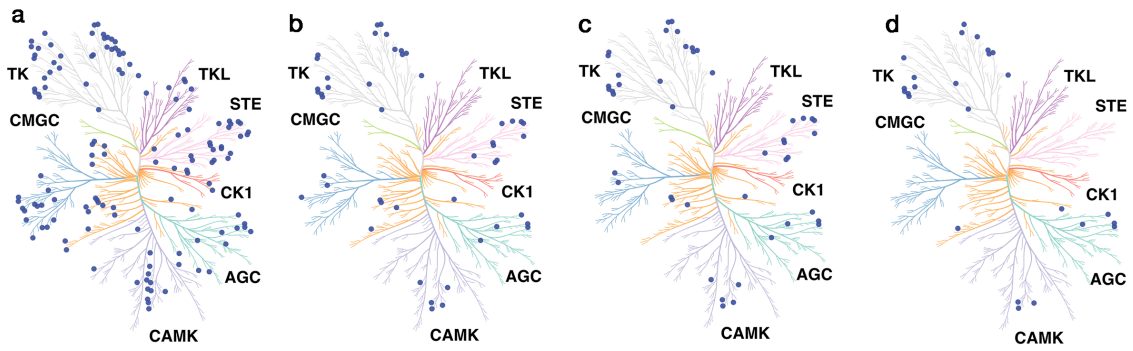


**Figure 6.** Distributions of warheads across the human kinome (dark blue balls). (a) Acrylamide-containing CKIs cover 132 kinases; (b) Butynamide-containing CKIs cover 43 kinases; (c) Aldehyde-containing CKIs cover 41 kinases; and (d) Cyanoacrylamide-containing CKIs cover 21 kinases. Specific kinase names are found in the supporting information (Table **S1**).

## 2.3 Extended Warheads

Improving the selectivity and affinity of compounds by modifying hit-to-lead molecules is a key process in drug design. For CKI design, it is important to have one electrophilic warhead to target the desirable nucleophile. Due to the limited choice of electrophile, modifying adjacent fragments

of the warhead is necessary. Thus, we systematically analyzed the warheads and their adjacent fragments (together called extended warheads) to provide structural insights for designing CKIs. A total of 17,309 adjacent fragments with 1344 unique fragments comprised our adjacent fragment library (AFL, **Table S2**), bridging the scaffold with the corresponding warhead. Using hierarchical clustering analysis, adjacent fragments were clustered into 7 clusters (**C1-C7**, **Figure 7**). Every cluster has different features but 6 of the 7 are cyclo-based fragments, such as aromatic ring-based (**C1**, **C5**, **C6**, and **C7**), heterocycle-based (**C2** and **C5**), or naphthene-based (**C4**). The diversity of adjacent fragments provides a broad conformational space for designing potential CKIs (**Table S2**).

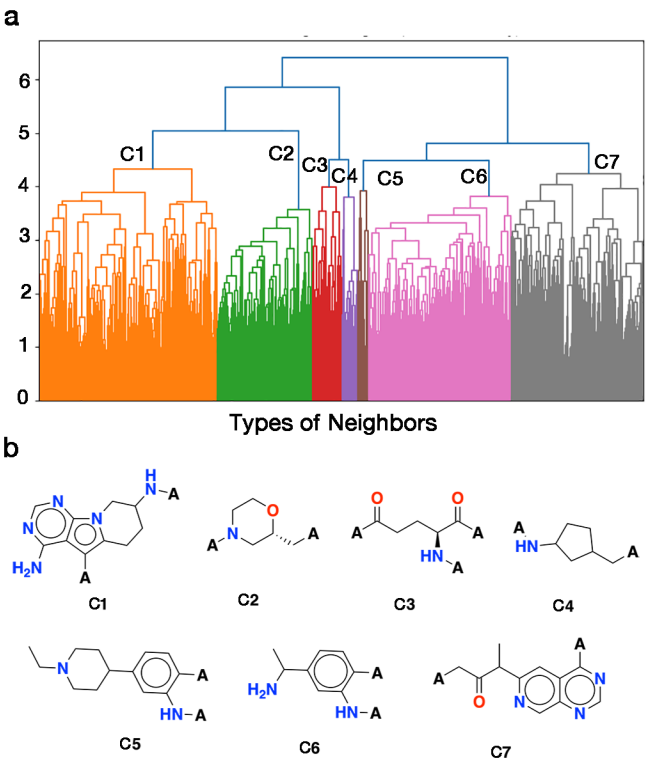


Figure 7. Hierarchical clustering. (a) 7 clusters of adjacent fragments are highlighted in different colors. (b) An example from every cluster illustrates the different types of adjacent fragments.

We counted the frequency of occurrence of the 1344 different adjacent fragments in the CKI dataset. Among them, nearly 900 molecular fragments appear exclusively in 1 to 2 CKIs. In contrast, there are 6 frequently used adjacent fragments: Benzenamine (**T1**), Piperidine (**T2**), 6-Azaspiro[3.4]octane (**T3**), Quinazoline (**T4**), Pyrrolidine (**T5**), Piperazine (**T6**) (**Figure 8a**) found in > 490 CKIs. They all occur in multiple CKIs targeting different kinases (**Figure 8b**) or are equipped with different warheads (**Figure 8c**), showing strong plasticity (adaptable to different CKI warheads or scaffolds). For example, **T2**, the adjacent fragment to 10 kinds of warheads, occurs in 24 kinases. The aromatic ring-based **T4**, as the adjacent fragment of 3 kinds of warheads, occurs in 61 kinases, showing strong resilience to different CKIs targeting different kinases. Of course, a variety of adjacent fragments yields different chemical properties, such as different structural sizes, or being based on aromatic rings, offering a rich arsenal with versatile features for CKI design (Table **S2**).

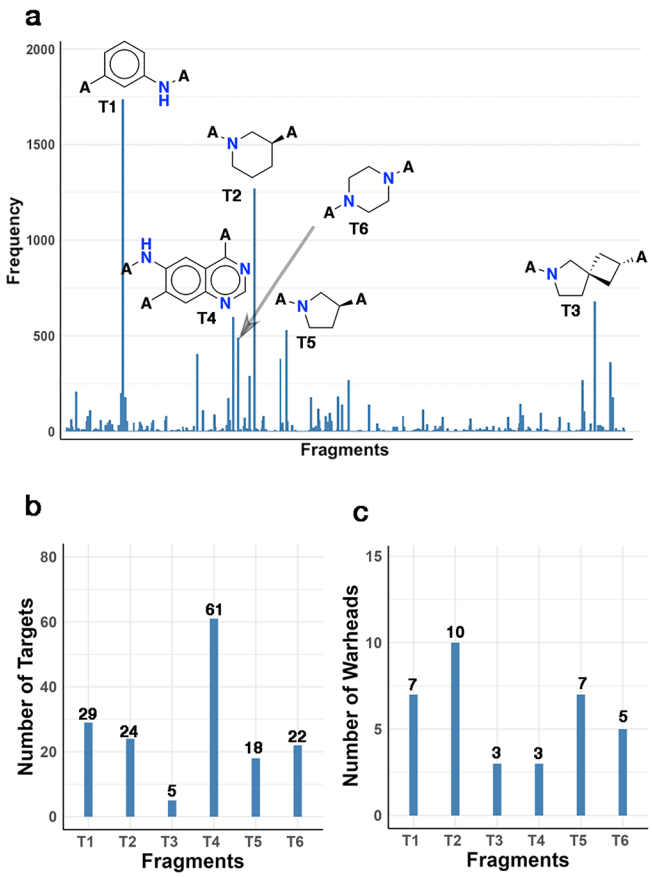


**Figure 8.** (a) Top 6 frequently used fragments out of 1344 unique adjacent fragments. (b) The number of kinases covered by the top 6 frequently used adjacent fragments. (c) The number of warheads equipped with the top 6 frequently used adjacent fragments.

## 2.4. Extended Warheads for Reversible CKIs

A concern with covalent inhibitors is unexpected toxicity due to off-target covalent modifications. Reversible CKIs (RCKIs) provide long-residence-time covalent binding, but avoid permanent protein modification. Thus far, RCKIs have been established by tuning the warhead-nucleophile reactivity for ten kinases, such as EGFR, BTK, JAK3, and Ribosomal S6 kinase 2 (RSK2).[35-39] Correspondingly, multiple warheads have been used successfully in RCKI design, for example, warheads **23-30** (**Figure 4**). Given our CKI data, the largest number of RCKIs are

770 aldehyde-equipped CKIs (**Figure 5**, warhead **26**). Out of 770 RCKIs, there are 121 different adjacent fragments (**Figure 10a**). The top 3 are a tetrahydronaphthyridine fragment (**A1**), a methoxyphenol fragment (**A2**), and a betanaphthol fragment (**A3**), respectively. All have aromatic rings. The extended warheads target 1 to 2 kinases thereby showing strong specificity (**Figure 10b**). Currently, one of the RCKIs, Roblitinib (FGF401), which targets fibroblast growth factor receptor 4 (FGFR4) to treat hepatocellular carcinoma and other solid tumors with positive FGFR4 and klotho beta (KLB) expression, is in phase-II clinical trial.[34]

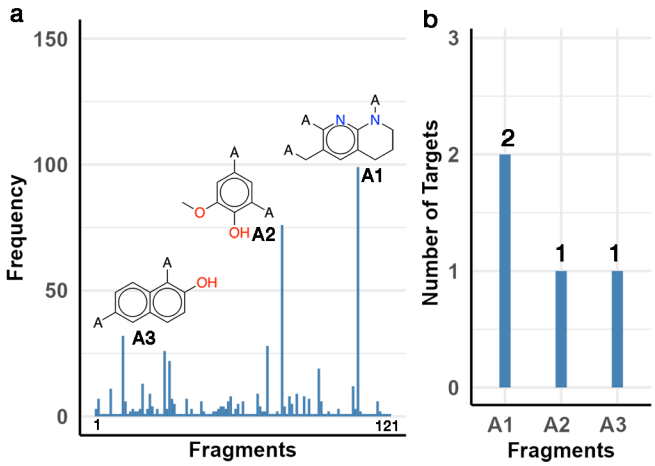


**Figure 10**. Adjacent fragments in aldehyde-equipped reversible CKIs.

Cyanoacrylamide-equipped CKIs are the second largest group with 716 RCKIs. 98 different adjacent fragments were extracted and the top 3 are piperidine (**Cy1**), 2-methylpyrrolidine (**Cy2**), and 3-aminopiperidine (**Cy3**) (**Figure 11a**). Compared to the aldehyde-based RCKIs, cyanoacrylamide-equipped RCKIs can adapt to targeting multiple kinases, such as **Cy1** + cyanoacrylamide-based RCKIs targeting 10 kinases. The top 1-3 adjacent fragments are all heterocycle-based (**Figure 11b**), different from the aromatic ring-base adjacent fragment of the aldehyde warhead (**Figure 10b**). It is worth noting that cyanoacrylamide is also a derivative of

acrylamide. Currently, two cyanoacrylamide-equipped RCKIs, PRN473 and Rilzabrutinib, are in Phase-I and Phase-III clinical trials, respectively, for treating pemphigus vulgaris and immune thrombocytopenia.[40, 41]

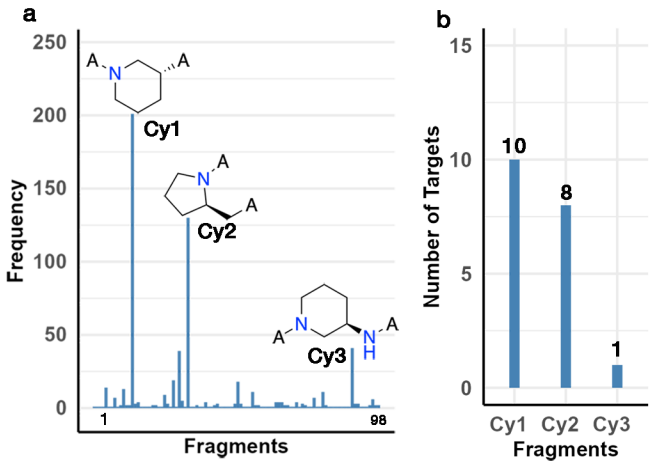


**Figure 11**. Adjacent fragments in cyanoacrylamide-equipped reversible CKIs.

## 3. Conclusion

Covalent drug design mainly focuses on designing an electrophile as a warhead to strike the accessible residues based on existing noncovalent inhibitors as scaffolds.[18] Due to the limited types of warheads, additional adjacent fragments need to be considered to strike the desired residues. Here, we systematically explored adjacent fragments and the resultant warhead patterns, providing more opportunities to achieve covalent inhibition.

Systematic analysis of our current CKI dataset found 16,961 covalent inhibitors covering 146 kinases, accounting for 70% of the 209 kinases that have at least one cysteine in the binding site. Further, we conducted systematic analysis of extended warheads, whereupon 1344 unique adjacent fragments associated with the corresponding warheads were extracted. The complete adjacent fragment library with associated properties can be accessed (Table **S2**) where **T1-T6** shows the

highest adaptability. Adjacent fragments increase the conformational space of the warhead providing more options for refining the hit-to-lead molecules anchoring the desired nucleophile or even dual-nucleophiles.[42, 43]

Along with the development of CKIs, more attention has been placed on RCKIs for exploiting sustained potency while avoiding unintended permanent protein modification.[44] However, the RCKI warheads are difficult to design. To help we collated current RCKI warheads and extracted the corresponding adjacent fragments with aldehyde and cyanoacrylamide showing broad utility. Importantly, their adjacent fragments present distinctive characteristics: aromatic ring-like adjacent fragments for aldehyde warheads; and heterocyclic-like ones for cyanoacrylamide warheads.

The diversity of adjacent fragments illustrates the potential in designing precise covalent inhibitors. Exploration of adjacent fragments and the corresponding warheads offers insights into the interaction modes of fragments, important for drug design using fragment blocks.[45] The exploration of adjacent fragments described here should provide useful information as an arsenal for developing new CKIs with appropriate warheads.

## 4. Methods

### 4.1 Curating the CKI Dataset

First, a CKI dataset was curated and filtered for the purpose of extending warheads from different databases (accessed on 17 October, 2023) including BindingDB,[46] CheMBL,[47] and an in-house CKI library including 200 manually curated CKIs (see reference *Pharmaceuticals (Basel)* **2022**, 15 (11) ).[22] A total of 521,736 kinase-compound pairs were obtained using thresholds of inhibitory activity values less than 10,000 nM (Ki, Kd, or IC50) and the highest confidence score

of 9. Second, we double-checked that there are 208 kinases with available cysteines near the binding sites among the whole human kinome (see Table **S3**, which was adapted from the supplementary information S3 in Gray's papers).[2, 18] Third, we filtered all the kinase-compound data points that target the 208 kinases, resulting in 162,165 unique kinase-compound pairs. Finally, based on 30 warheads (**Figure 4**) that have been applied in practice, we extracted all the CKIs that have the warhead fragment. Duplicate kinase-CKI pairs were deleted regardless of the essay's values from different sources. All CKIs were represented in canonical SMILES format.

### 4.2 Extracting adjacent fragments

Adjacent fragments were extracted using the Chem.Recap module of the RDKit chem lib.[48, 49] First, every compound was fragmented using the Recap method, which is a hierarchy of nodes. Then, we teased out those nodes with two leaves at the same branch, where if one is a warhead, the other is an adjacent fragment. Further, adjacent fragments at the distal end of CKIs are excluded, while those bridging warheads and scaffolds are retained. We traversed all CKIs to provide an adjacent fragment library (a framework module in Python is enclosed in Table **S4**). The warhead input format is represented as a SMART pattern using the module MolFragmentToSmarts in RDKit.[48]

Adjacent fragments were clustered using hierarchical clustering with an average linkage method based on pairwise cosine similarities of Morgan fingerprints (radius=2, nBits=2048) using RDKit.[48] The properties of adjacent fragments were extracted using the Descriptors module in RDKit.[48] All fragments were drawn using ChemDraw v20.0.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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**Supporting Information**

Table **S1:** Specific kinases covered by acrylamide-, butynamide-, aldehyde-, and cyanoacrylamide-equipped CKIs, respectively. Table **S2**: the complete adjacent fragment library. Table **S3**: 208 kinases with available cysteines in the binding sites. Table **S4**: The Python framework for extracting adjacent fragments of CKIs.

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