Title:

A Perspective on Covalent Inhibitors: Research and Development Trends of Warheads and Targets

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Abstract:

Covalent inhibitors are being used more frequently in drug discovery and is expected to revolutionize how enzyme inhibitors and receptor modulators are created in the future. More than 30 covalent therapies have been approved by the US FDA as a result of this approach, which has produced a wide spectrum of effective treatments. Covalent inhibitors have also made it possible to target previously neglected targets, demonstrating that covalent inhibition has a tremendous opportunity to cure human diseases.

We analyzed the CAS Content Collection to examine the publishing trends of covalent inhibitors with various types of warheads and their target proteins for developing a landscape view of the research effort in this area. We identify potential substructures for specific protein targets by assessing the frequencies of specific warhead structures in covalent inhibitors. In addition, a comprehensive database of warheaded compounds and the corresponding protein targets has been collected, which may be useful for analysis of structure-activity relationships.

Our work provides an overview of covalent inhibitor research and demonstrates how the power of the analysis of large data sets can accelerate research and development.

I. Introduction

Pharmaceutical research for drug discovery has recently begun to investigate the potential of covalent inhibitors after years of mostly focusing on non-covalent reversible inhibitors. The initial reluctance to develop covalent inhibitors as drugs was due to fear of irreversible damage as well as toxicity arising from off target effects [1] In fact, most covalent drugs have resulted from serendipitous discovery rather than by deliberate design. Conventional non-covalent drugs interact reversibly with targets via interactions that are weak and transient (van der Waals interactions and hydrogen bonds). On the other hand, covalent inhibitors form strong covalent bonds to target proteins (Figure 1). Broadly speaking, a covalent inhibitor can be divided into two distinct parts: the "warhead" or "reactive group" which form the covalent bond to their targets and the "guidance system" which determines the selectivity of the inhibitor for its target (Figure 1). Reactive groups are most commonly electrophilic because most of the potential reactive sites on a target protein (either the N-terminal amino groups or amino acid side chains) are nucleophilic in nature. Reactive groups must follow the Goldilocks principle – they must be reactive enough to form a bond to a target upon complex formation but not reactive enough to form bonds to other protein targets or functional groups when unbound.

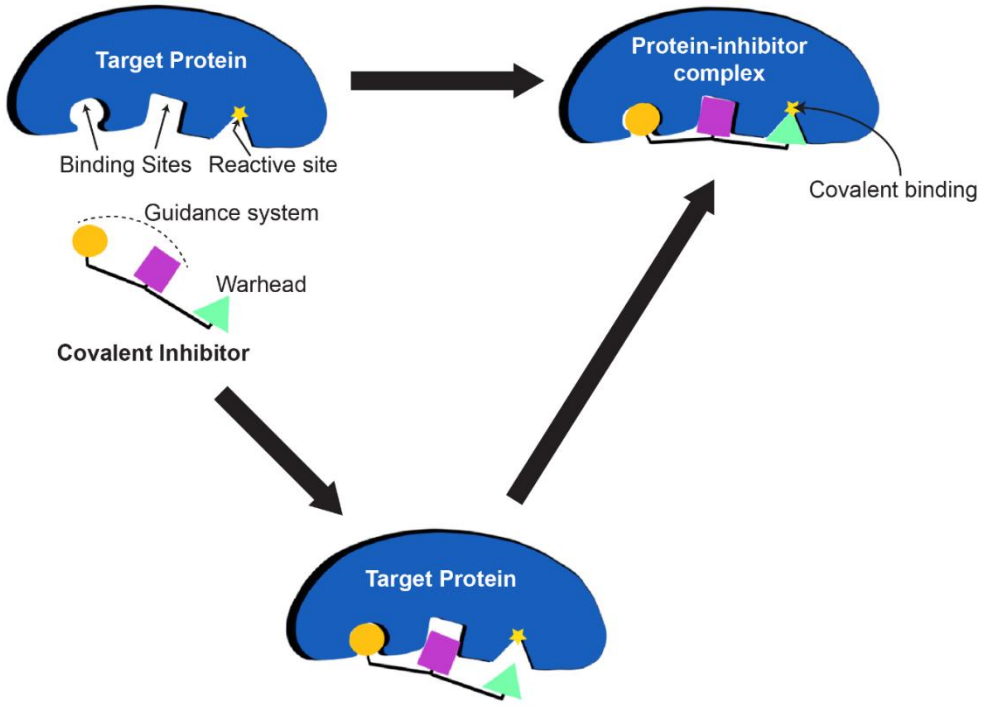

Figure 1. Schematic representation of covalent inhibition of target protein.

In contrast to mechanism-based (or suicide) inhibitors, covalent inhibitors do not require enzyme activation and (in many cases) form bonds to noncatalytic residues of their targets. Covalent inhibitors can be divided into reversible and irreversible covalent inhibitors based on the mechanism and kinetics of inhibition.[2] In reversible covalent inhibitors, protein activity can be recovered via the cleavage of the formed bond; they are continually binding and unbinding with

their target. The rates of dissociation for irreversible covalent inhibitors are lower than the rates of resynthesis of their targets unlike reversible covalent inhibitors which undergo covalent bond cleavage with rates greater than the rates of target resynthesis.[3] Thus, irreversible covalent inhibitors form a permanent covalent bond with their target. The duration of effect of covalent inhibitors depends both on the type of covalent inhibitor and on the rate of resynthesis of their targets.

How do covalent inhibitors work?

Covalent inhibitors with highly reactive groups react nonselectively with their targets; not only may they react at the protein's active site but at other positions within the target protein and with other proteins with similar functional groups. Covalent inhibitors with less reactive groups react with specific functional groups (and hence residues) of the target. The covalent bond prevents or slows down the dissociation of the guidance system from the target protein, providing longer-lasting inhibition than a noncovalent inhibitor. The bonds formed between the covalent inhibitor and its target depend on the amino acid residue and reactive group of the target and inhibitor, respectively. While cysteine residues have been the most common targets for covalent inhibitors, serine, threonine, lysine and tyrosine residues and N-terminal amino groups are also potential reactive sites.[4-6] The choice of reactive group is dictated by the nature/identity of the intended target residues. Acrylamide, cyanoacrylamide, and other α,β-unsaturated carbonyl compounds are common warheads targeting cysteine residues.[7] Aldehydes, boronic acids, and fluorosulfates have been used as amine-reactive warheads (for either lysine residues[7, 8] or with N-terminal amines) in proteins.[5, 6, 9-11] Vinyl sulfones are capable of reacting with both cysteine and lysine residues, depending on the sulfone substituents and the targets.[7]

What advantages and disadvantages do covalent inhibitors have?

While noncovalent inhibitors associate and dissociate freely from enzymes (depending on binding constant) and thus require sustained concentrations of inhibitor to inactivate an enzyme, (irreversible) covalent inhibitors cannot dissociate ensuring continuous presence of inhibitor. The irreversibility of binding means that binding of the guidance system to the target does not need to be as strong to abrogate activity, and so inhibitors may be smaller and likely less lipophilic and thus (perhaps) more water-soluble.[12] If covalent inhibitors are smaller than traditional noncovalent inhibitors and have longer durations of action, the doses administered can be smaller and less frequent, reducing likely side effects and improving selectivity. In many cases, the separability of reactive groups from guidance systems may make the design of covalent drugs easier. If the strength of initial binding does not need to be as high, an irreversible covalent inhibitor can inhibit enzymes without discrete binding sites such as those involved in protein-protein interactions [13] Finally, because the bonds between irreversible covalent inhibitors and targets do not break, mass spectrometric analysis of binding is facile, making high-throughput screening of inhibition simpler.[3]

However, covalent inhibitors can also have liabilities relative to noncovalent drugs. The irreversibility of binding requires that any binding be selective; off-target binding is irreversible and thus likely to be long-lasting. If sufficiently reactive warheads are used, off-target effects are likely. The irreversibility of binding also allows for immune sensitization to off-target adducts (haptenization), which can lead to long-term harm. As noted in a review by Baillie[3] quoting Barf and Kaptein[14], "the therapeutic applicability or the success of irreversible binding inhibitors is dependent on whether or not the covalent bond can be confined solely to the protein of interest."

Such effects have been observed in less-selective inhibitors such as beloranib and BIA 10-2474.[15, 16] Greater levels of liver toxicities and black-box warnings of severe side effects have been noted for reversible kinase covalent inhibitors than for the corresponding irreversible kinase covalent inhibitors.[17] Reversible covalent inhibitors do not have the possibility of irreversible off-target modification, but (depending on binding constant) the concentrations of inhibitor needed to deactivate protein may be higher, reducing some of the advantages covalent inhibitor.[18] Covalent inhibitors are subject to resistance, however, through mutation of the targeted residues.[19] Since covalent inhibitors tend to target non conserved amino acid residues of target proteins, mutants of the target protein eliding those residues can still function effectively but will be resistant to covalent inhibitors. Finally, accurate kinetic evaluation of covalent inhibitors is more difficult than for reversible inhibitors; while IC_{50} values (the concentrations of inhibitor at which half of the enzyme activity is inhibited) can be useful measures of reversible inhibition, covalent inhibition may require both the binding of inhibitor to the target protein and the rate of covalent modification to be determined.[20] The desired K_{inact}/K_I value can be obtained from the more simply determined fixed time point IC_{50} value for covalent inhibitor analysis if the order of addition of compounds is controlled.[17, 21]

How can we find covalent inhibitors?

The reactivity of warheads (as noted above) determines if covalent bonds are formed selectively. If the warhead is sufficiently selective to react with a desired residue, the selectivity of the guidance system for a particular protein and its strength of binding to the protein determine the rate of protein-inhibitor complex formation. The position of the warhead with respect to the target residue in the protein-inhibitor complex and the reactivity of the warhead determine the rate of inhibition. As noted earlier, irreversible inhibitors can be assayed using mass spectrometric methods. Phenotypic methods look for a desired biological activity directly, while mass spectrometric methods (activity-based protein profiling (ABPP) and isotopic labeling) allow scientists to identify residues that can be modified or are modified by specific inhibitors.[3, 22] Computational methods to model inhibitor binding and protein structure are useful in estimating the positioning of reactive groups in protein-inhibitor complexes. In many cases, however, the underlying protein structural data to determine binding modes and selectivities must be validated by experimental data. If the desired pharmacokinetic behavior for enzyme inhibition is known, kinetic data for warheads can be used to determine appropriate inhibitor if the rate of enzyme resynthesis is known.

Supplementary table S1 shows a collection of intentionally covalent drugs (drugs designed to bind covalently to their targets) approved in the United States since 2000?.

In theory, any enzyme or protein can be inhibited using a covalent inhibitor; nearly all proteins possess an N-terminus and amino acid side chains amenable to reaction with electrophiles. However, previous concerns about off-target toxicity and immune sensitization by covalent drugs mean that they are not used when a noncovalent drug is effective. Many of the enzymes that are important drug development targets are not readily inhibited by noncovalent drugs, making other design strategies necessary. Kinases, for example, have ATP binding sites for which enzyme selectivity is difficult to obtain, and have protein-protein interaction sites with large surface areas and limited interaction densities that make small molecule inhibitor design difficult.[23] Covalent inhibitors have thus been developed for a variety of kinases, including KRAS, Bruton's tyrosine kinase (BTK), epidermal growth factor receptor (EGFR), Janus kinase 3 (JAK3), c-Jun N-terminal kinases (JNK1-3), phosphoinositide 3-kinases (PI3Kα), vascular endothelial growth factor

receptor 2 (VEGFR2), SRC proto-oncogene tyrosine-protein kinase (SRC), never in mitosis, gene A (NIMA) related kinase 2 (NEK2), extracellular signal-regulated kinase (ERK1-2), FMS-like tyrosine kinase 3 (FLT3), AKT (or protein kinase B; PKB), and transforming growth factor-β (TGF-β)-activated kinase (TAK).[24] Targets previously considered undruggable such as KRAS (G12C), Myc and STAT5 (can now be successfully targeted using covalent inhibitors.[25] Nirmatrelvir is a covalent inhibitor of the main SARS-CoV-2 protease $3CL^{PRO}$, while telaprevir, boceprevir, and narlaprevir are covalent inhibitors of the hepatitis C viral protease NS3/4a.[26-28] Voxelotor alters sickle-cell hemoglobin through reaction with the N-terminus of the hemoglobin α-chain.[29] The apoptosis regulator protein MCL1[30], the tyrosine kinases ErbB2 (HER2) and ErbB4[1], and carboxylesterases[31] have all been targets of covalent inhibitors.

In the current work we have utilized our access to the CAS Content Collection consisting of over 204 million substances[32] to identify substances showing the presence of warhead groups and thereby capable of acting as covalent drugs/inhibitors. We chose to look at ~33 warhead groups across a wide range of reactivities and analyze our dataset of identified substances/compounds to provide a fuller perspective on the topic. Warheads included in this analysis range from the most used ones (α, β unsaturated carbonyls and their derivatives) to the more obscure ones (such as Selenium-based warheads). In this report we present analysis of published research (journals and patents) pertaining to the chosen warheads. We also looked at FDA approved covalent inhibitors as well as those currently in various stages of clinical trials. Our aim with such a detailed and extensive warhead-specific analysis was to provide insights with respect to substructure preference relative to warhead (as well as protein target where possible).

II. Warheads

For ease of discussion, we divided the analyzed warheads into 5 major groups – Group 1 comprises of the α, β unsaturated carbonyls and its derivatives (-O, -C, -N, -H, -S and -Cl) and have been keep separate from the rest of the warheads due to their tendency to dominate resulting from their large dataset; Group 2 consists of α-ketoamides, aldehydes (CHO), boronic acids and esters, disulfides, Se, seleno sulfides; Group 3 includes epoxides, maleimides, isothiocyanates (NCS), phosphoryl (PO_3), sulfonyl fluorides (SO_2F) moieties; Group 4 comprises of nitriles (R-CN), alkyne carbonyls (YNE-CO), nitro (R-NO_2) and sulfonyls (R-SO_2) where R is either an alkene or alkyne group and Group 5 consists of halogen derivatives FCY, ClCY, BrCY and ICY (where Y = –CN, –CO, –NO_2, –SO).

Based on combined perusal of our CAS Content Collection warhead dataset along with FDA approved covalent inhibitors (Supplementary table S1), we chose to focus on the following warheads: acrylamide (a subset/derivative of α, β unsaturated carbonyls), α-ketoamides and boronic acids, nitriles, epoxides, and aldehydes, due to their long and substantial publication history.

Overall Trend Analysis/Landscape view of chosen warheads

Not surprisingly, the potential of covalent inhibitors as successful drug candidates has attracted many organizations to perform research in this field. Our analysis of the literature since 2000 has shown thousands of organizations have published at least one journal article discussing covalent inhibitors, leading to over 10,000 articles total as of the date this publication. The greatest number of these articles were published by academic institutions with the University of California, Berkeley, the Chinese Academy of Sciences, Scripps Institute and Harvard Medical School all publishing more than 80 articles. The commercial sector has also shown considerable interest

with Pfizer, AstraZeneca and Novartis being the most prolific in terms of publications. Of note, Pfizer stood out with more than 80 publications related to covalent inhibitors, more than twice as many as any other company (Figure 2). The commercial potential of covalent inhibitor therapeutics has led to a correspondingly large number of patents being filed and issued with hundreds of institutions filing at least one patent, leading to over 1,000 patents in total. Predictably, commercial organizations have the most patents with Merck, Janssen, AbbVie and Celgene leading the way. The number of patents published by academic institutions has increased steadily on an annual basis since 2000, with Fudan University in China publishing the most (Figure 2).

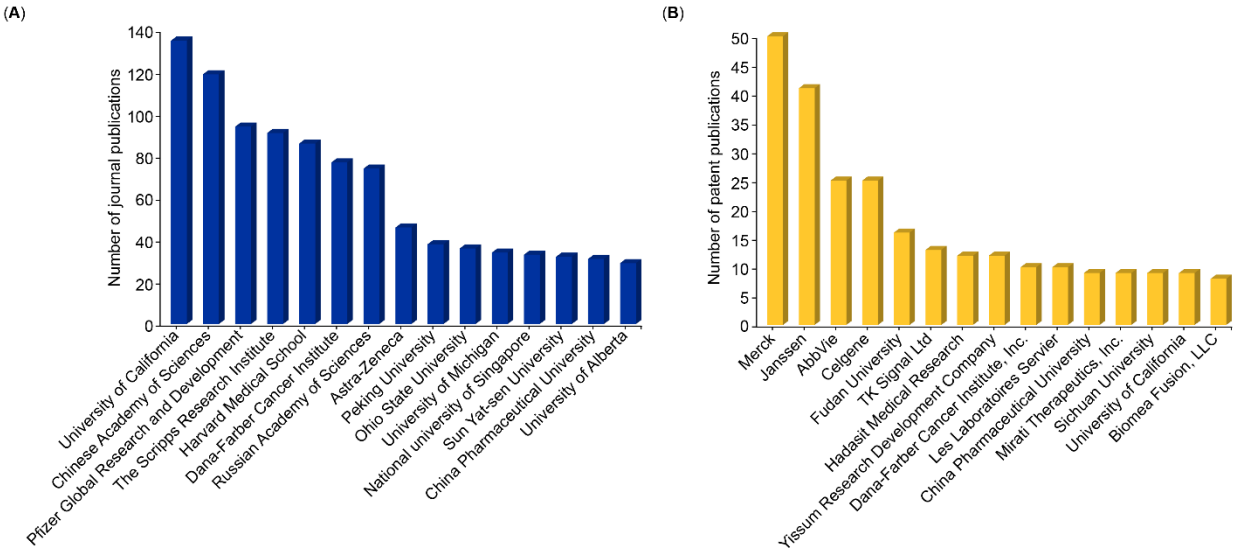

Figure 2. (**A**) Top fifteen patent assignees in the field of covalent inhibitors. (**B**) Top fifteen contributors to journal publications related to covalent inhibitors. For both graphs, data onwards of 2000 was considered.

The relative frequencies of publication and substance indexing indicate that compounds containing α,β-unsaturated carbonyl compounds predominate in covalent inhibitors, occurring in roughly half of the publications discussing covalent inhibitors and making up nearly two-thirds of the compounds indexed from those articles (Figure 3B and 4). The presence of US FDA-approved drugs using this set of warheads (Supplementary table S1) likely reduces the risk of drug development for such compounds relative to those containing a less prevalent warhead; the reactivities of the warheads and their benefits and liabilities are better known (and may also be easier to justify to others). In addition, the acryloyl moiety is readily grafted onto amine or azaheterocyclic groups, making the development of a covalent inhibitor from a noncovalent inhibitor easier. This is reflected by the fast growth in publications for acrylamide (N derivative) warheads as compared to the modest growth of the O and C derivatives (Figure 4). The difference between substance count and publications may be attributed to the presence of a significant number of approved drugs incorporating the warhead; documents comparing a novel warhead or novel substructures are likely to contain compounds with α,β-unsaturated carbonyl compounds (as well as boron compounds and epoxides) as standards.

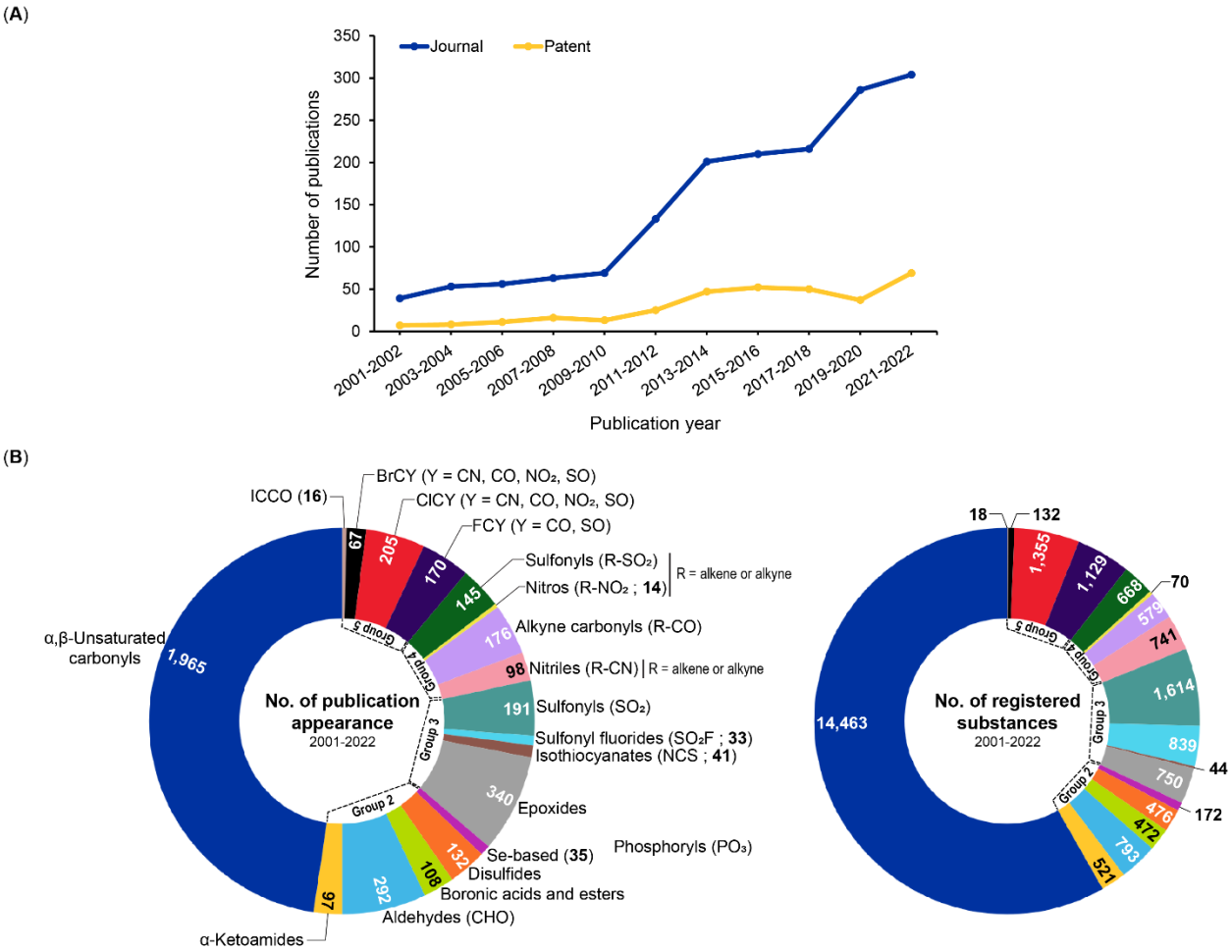

Figure 3. (**A**) Growth of documents (journal and patents) in the CAS Content Collection for our chosen groups of warheads. (**B**) Distribution of our chosen group of warheads across publications (journals and patents) and registered substances in the CAS Content Collection database over a period of little over two decades (2001-2022). α, β unsaturated carbonyls dominate both publication appearances as well as registered substances.

Publications discussing epoxides and aldehydes belonging to groups 3 and 2, respectively, make up the next largest fractions of warheads (after α,β unsaturated carbonyls), though their fractional contribution to the total number of warhead-containing substances is smaller than their contribution to publication count (Figure 3B). Both epoxides and aldehydes are also present in approved covalent inhibitors (Supplementary table S1), making them less risky than less prevalent warheads; however, both epoxides and aldehydes may react with a variety of residues, including N-terminal amines and lysine, serine, threonine, and cysteine residues.

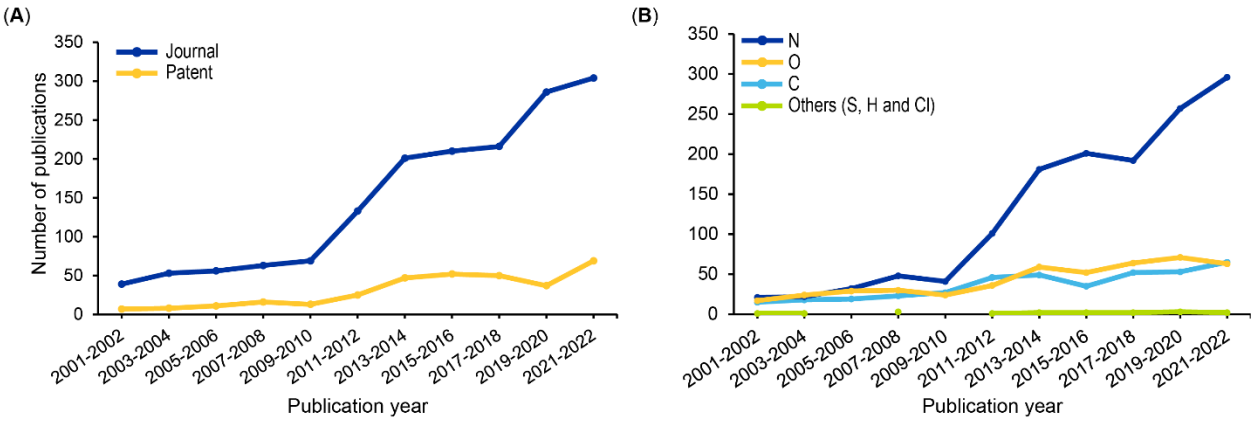

Figure 4. (**A**) Yearly growth of documents (journal and patents) in the CAS Content Collection for Group 1 warhead (α, β unsaturated carbonyls) over 2001-2022. (**B**) Breakdown of growth of documents for α, β unsaturated carbonyl derivatives. The N derivative of α, β unsaturated carbonyls (i.e., acrylamide warhead) showed the greatest growth in the last decade with O and C derivatives showing a more conservative growth.

While the development of covalent inhibitors reactive with amino acids such as lysine is important to find more broadly active covalent inhibitors, it also may make the reactivity of epoxide- or aldehyde-containing covalent inhibitors more difficult to predict. Among the other members of the Group 2 warheads, disulfides, boronic acids and esters and α-ketoamides exhibit comparable modest but steady growth in publications over the past two decades (Figure 5A). Disulfides may react reversibly with enzymes, which may provide both opportunity and uncertainty to development in covalent inhibitors.[7] Boron compounds and α-ketoamides have been used in FDA approved covalent inhibitors (Supplementary table S1) as is evidenced by an increased number of publications (Figure 5A). Articles pertaining to use of selenium warheads have shown a small but sharp growth in the last 3 years (Figure 5A). This can be partly attributed to the discovery of Ebselen's inhibition of lens-epithelium-derived growth-factor[33] and a resurgence of interest in selenium containing small molecules.[34, 35] Isothiocyanates belonging to group 3 warheads, show very limited growth in publications (Figure 5B) while sulfonyl fluorides appear to show a faster rate of growth since 2017 (Figure 5B). This could be attributed to their reactivities with both lysine and cysteine residues providing sulfonyl containing covalent inhibitors with a broader scope of targets, though fewer methods likely exist for their synthesis than for chloroacetamides or acrylamides

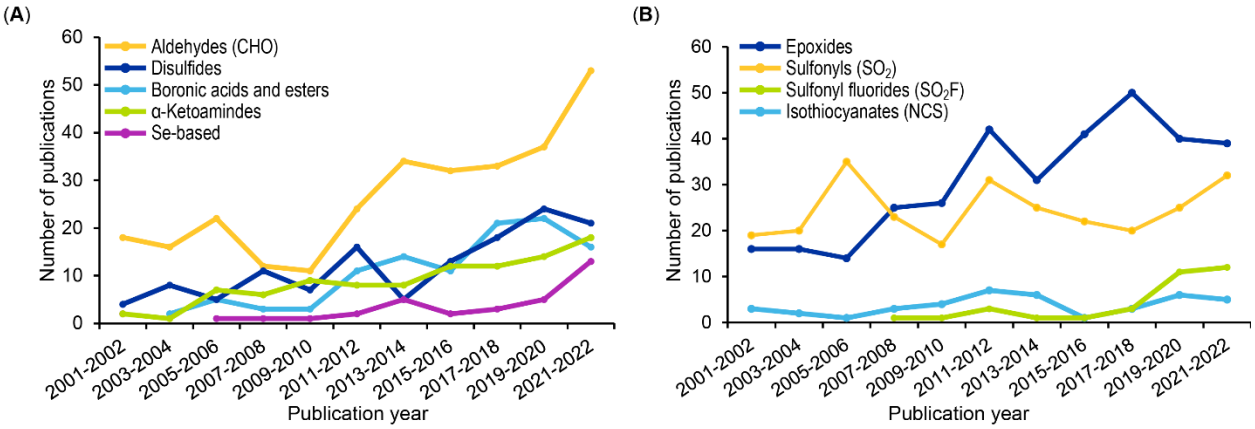

Figure 5. Growth of publications in the CAS Content Collection for (**A**) Group 2 warheads consisting of α-ketoamides, aldehydes (CHO), boronic acids and esters, disulfides and Se-based and (**B**) Group 3 warheads consisting of epoxides, isothiocyanates (NCS), sulfonyls (SO_2) and sulfonyl fluorides (SO_2F) containing substances over 2001-2022.

Alkyne-containing carbonyl and sulfonyl compounds and vinyl sulfonyl compounds make up significant fractions of covalent inhibitor publications and smaller contributions to the total substance count (Figure 3B). Like boron compounds and α-ketoamides, alkyne carbonyl compounds have also appeared in approved covalent inhibitor drugs (Supplementary table S1), making them potential starting points for further development. In group 4 warheads, alkyne-containing carbonyl compounds (R-CO) have shown maximal growth in publications, followed by alkyne or alkene-containing sulfonyls (R-SO_2) and nitriles (R-CN) with the nitro compounds showing the minimal/least growth (R-NO_2) (Figure 6A). The need for multi-step synthesis of the warheads may make them more difficult to incorporate into existing noncovalent inhibitors. The increased reactivity of some warheads (alkyne carbonyl and sulfonyl compounds) or the variable selectivities of others (boron-containing inhibitors, vinyl sulfonyl compounds) may also hinder development explaining differences in their relative rates of growth.

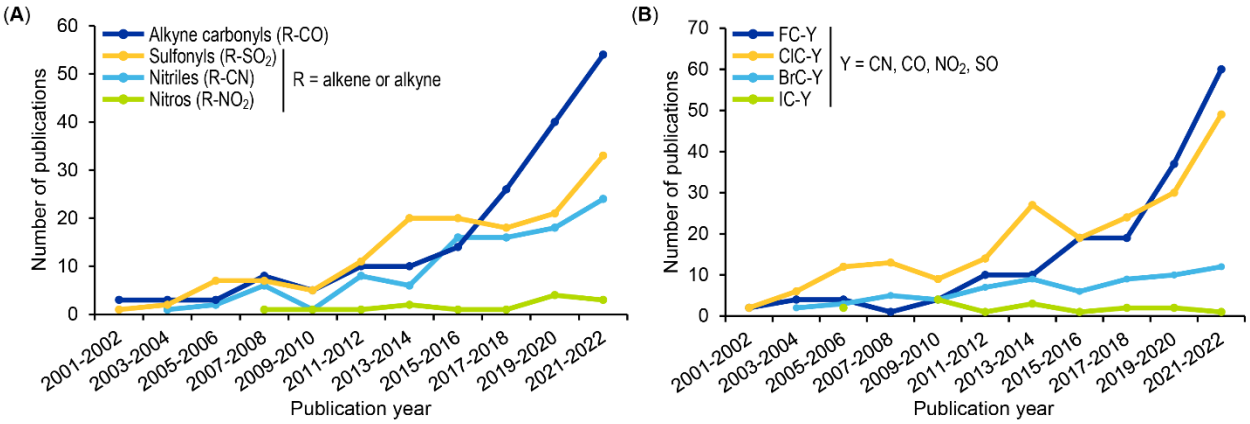

Figure 6. Growth of publications in the CAS Content Collection for (**A**) Group 4 warheads consisting of nitriles (R-CN), nitros (R-NO_2) and sulfonyls (R-SO_2) where R is either an alkene or alkyne group and alkyne carbonyls (R-CO) and (**B**) Group 5 warheads consisting of halogen containing derivatives FCY, ClCY, BrCY and ICY where Y = CN, CO, NO_2, and SO over 2001-2022.

Fluoro and chloro-containing compounds which include carbonyls, nitriles, nitro compounds and sulfones make up a notable fraction of publications but an even larger proportion of covalent inhibitors (Figure 3B). The ready availability of chloroacetylating agents and the modularity of chloroacetamides likely allows for the rapid synthesis of chloro-containing covalent inhibitors by adaptation of noncovalent inhibitors accounting for the rapid growth in publications in the last decade (Figure 6B). The greater stability of chloro-containing covalent inhibitors to storage than bromo- and iodoacetyl compounds accounts for the preference for chloro-containing carbonyl compounds over other halocarbonyl compounds which show a slower growth (Figure 6B). While the potential reactivity of chloroacetamides with both amines and thiols requires disambiguation, the lack of steric hindrance and flexibility in reactive conformation may make them attractive for covalent inhibitor development.

In depth look at selected warheads:

α,β-unsaturated carbonyl compounds have the structures C::CCOX (X is an alkoxy group, an amino group, a proton, or an alkyl or aryl group; α,β-unsaturated cyanides may be included where the carbonyl is replaced with a C:::N group). In most cases, the alkene is terminated with a methylene group, CH_2; in some cases, one of the protons may be substituted with an alkyl group

such as dimethylamino (Me_2NCH_2) or piperidinylmethyl ($C_5H_{10}NCH_2$). Cyclic α,β-unsaturated carbonyl compounds are uncommon in covalent inhibitors, as are cycloalkenyl carbonyl compounds.

α,β-unsaturated carbonyl compounds have polarized bonds because carbon and oxygen differ in electronegativity, with the carbon acquiring a partial positive charge and the oxygen acquiring a partial negative charge. Through resonance, the end of the alkene furthest from the carbonyl group also acquires a partial positive charge. Nucleophiles with significant amounts of electron density add to the alkene forming an enolate and a new single bond. The enolate is protonated at carbon to form a new C-H bond and to reform the carbonyl group. Two single bonds are stronger than the original olefin, providing the energy to drive the reaction forward. This reaction is called a Michael addition reaction.

Additions to other double bonds or equivalents such as α-keto amides and nitriles are commonly reversible because the electrons from the double or triple bond are placed in the products on a heteroatom (oxygen or nitrogen), eventually forming an amine or alcohol. Alcohol and amine protons are moderately acidic, and the barrier to their removal is low. To reverse the Michael addition, a proton must be removed from the carbon atom next to the carbonyl group, and this proton is significantly less acidic (in most cases) than an amine or alcohol. Cleavage of the C-H bond is in most cases slower than deprotonation of an amine or alcohol, slowing down the retro-Michael reaction and rendering the addition irreversible under physiological conditions. However, if the C-H bond is made more acidic by, such as, the substitution of a proton with a second carbonyl group or nitrile, the deprotonation may be made rapid enough to make the retro-Michael reaction reversible. Bond formation at the terminal alkene carbon is an important step in Michael addition; steric hindrance at that carbon lowers the rate of Michael addition and may prevent it.

α,β-unsaturated carbonyl moieties react with nucleophiles, and the most nucleophilic groups of a protein are amines (lysine side chains or the N-terminal amino group) and thiols (from the side chains of cysteine residues). The acidity of thiols and the higher nucleophilicity of sulfur make Michael reactions with thiols more favorable than for amines and determine the bias of α,β-unsaturated carbonyl compounds for cysteine residues over lysine residues. The electron deficiency of the carbonyl compound (or more generally, the alkene or alkyne substituent) helps to determine its reactivity as a covalent inhibitor. Nitro compounds tend to be more highly electron-deficient than ketones or aldehydes, which are in turn more electron-deficient than unsaturated sulfones or amides. Unsaturated carboxylic acids are generally resistant to Michael addition because deprotonation of acids is facile and the anions formed from their deprotonation sate the electron-deficiencies of the remaining carbonyl group. High reactivity with nucleophiles is likely to render the warhead less selective, both with the type of nucleophile and with the enzyme target, while low reactivity may mean that the warhead cannot react with the target, and so α,β-unsaturated carbonyl compounds with intermediate reactivities are great options for warheads. A majority of covalent inhibitors using α,β-unsaturated carbonyl moieties as warheads rely on α,β-unsaturated amides. Amides are some of the least electron-deficient carbonyl compounds, tempering the olefin's reactivity, but are sufficiently reactive to undergo Michael addition with biological nucleophiles. In addition, amides are easily prepared with a variety of nitrogen substituents and amide couplings are some of the most common reactions in medicinal chemistry.[36, 37] The distance of nitrogen substituents from the reactive sites of unsaturated amides allows unfettered substitution; unsaturated amides can be assembled from unsaturated carboxylic

acids or their derivatives and readily prepared amines, allowing covalent inhibitors to be prepared with separate warhead and binding modules and with a wide variety of structures.

A variety of covalent inhibitors use α,β-unsaturated carbonyl moieties as their warheads; second only to β-lactams. The kinase inhibitors afatininb, ibrutinib, osimertinib, olmutinib, adagrasib, sotorasib, dacomitinib, acalabrutinib, zanabrutinib, neratinib, and futibatinib (all US FDA-approved medications; Supplementary table S1) rely on unsaturated amides as warheads, as well as the failed drug candidate rociletinib (Supplementary table S2). Most α,β-unsaturated carbonyl containing covalent inhibitors rely on either terminally unsubstituted or substituted with a single alkyl group because steric hindrance deters Michael addition. Abiraterone and omaveloxolone are exceptions to this observation; both compounds contain more highly substituted α,β-unsaturated ketone moieties (Supplementary table S1). The increased steric hindrance of the olefin moiety in α,β-unsaturated ketones is likely countered by the greater electron-deficiency as compared to α,β-unsaturated amides. Dimethyl fumarate (Tecfidera), used in the treatment of multiple sclerosis, is an electron-deficient unsaturated ester (Supplementary table S1) whose actual mechanism of action is unknown[24] though covalent modification via Michael addition is one plausible mechanism.

α-ketoamides have vicinal (adjoining) carbonyl groups, one of which is attached to a nitrogen atom and the carbon chain, and the second of which is attached to the amide carbonyl and an alkyl or aryl group. The ketone carbonyl is rendered even more electron-deficient by the presence of the amide and thus more reactive with nucleophiles than other carbonyl groups. This electron deficiency and lack of relative steric hindrance allows for addition by nucleophilic nitrogen and sulfur atoms with protonation of the carbonyl oxygen to yield hemiaminals or thiohemiketals. If protons are available, hemiaminals can eliminate water to form imines which can undergo addition of a second nucleophile if present. Dinucleophiles with sufficiently short linking groups such as an N-terminal cysteine, serine, or threonine moiety can undergo multiple addition reactions to form thiazolidine or oxazolidine rings. The presence of lone pairs on nucleophiles and the facile deprotonation of heteroatom-hydrogen bonds renders addition to ketoamides potentially reversible; cyclization makes the reaction less likely to be reversed.

α-ketoamides can react with amines (both lysine and N-terminal amines), alcohols (serine and threonine residues), and thiols (cysteine residues). In actual use, the α-ketoamides boceprevir and telaprevir (Supplementary table S1) inhibit hepatitis C proteases NS3/4a by reaction with their catalytic serine residues.[9] They were approved as anti-hepatitis C agents by the US FDA in 2011, but telaprevir was withdrawn in 2014 for adverse effects, while boceprevir was withdrawn in 2015 after being superseded by newer anti-hepatits C agents. Another example of reversible covalent inhibitor containing α-ketoamide moietyis narlaprevir, also an anti-hepatitis C agent targeting the enzyme NS3/4A serine protease.[28, 38]

Boronic acids and boronates contain a boron atom attached to carbon; boronic acids have a boron atom substituted with two hydroxy groups, while boronates are esters of boronic acids with one or both boronic acid protons substituted with alkyl or aryl groups. Boron atoms are trivalent, and unusual for main-group atoms, the boron atoms lack an octet of electrons. Boron compounds thus tend to form complexes with compounds such as amines or alcohols which have lone pairs of electrons available for donation. The complexes are formally charged because the lone pairs from the nucleophilic atom (oxygen or nitrogen) are given to boron but are less polar than depicted by the formal Lewis structures. The presence of pendant nucleophilic groups on boronates such as aldehydes can allow for tandem condensation and complexation reactions of

boronates with functional groups such as amines to form adducts that do not revert easily to starting materials and thus effectively irreversible.[7, 39]

Boronic acids and boronates commonly react with nucleophiles such as alcohols (serine and threonine residues) and amines (lysine and N-terminal amino acids) but do not form stable complexes with thiols.[9, 40] Boron compounds may also form complexes with imidazole rings of histidines.[41] Examples of FDA approved boronic acid/boronate covalent inhibitors are the proteosome inhibitors bortezomib and ixazomib (Supplementary table S1), which form boronate complexes with a threonine residue of the proteosome.[42, 43] The antibiotic tavaborole (Supplementary table S1) binds to the hydroxy groups of leucyl-tRNA to prevent protein synthesis, forming a chelated complex which is not easily displaced.[9, 44] Vaborbactam (Supplementary table S1) inhibits beta-lactamase by forming a complex with an active-site serine, preventing bacteria from inactivating β-lactam antibiotics.[45]

Aldehydes are carbonyl compounds in which one of the substituents of the carbonyl carbon is a hydrogen atom (RHC::O). Aldehydes have only one alkyl or aryl group and no electron-donating groups, so aldehydes are among the most electron-deficient of carbonyl compounds, and the presence of only one substituent makes the carbonyl carbon unhindered. Addition of a nucleophile (an amine, alcohol, or thiol) to the carbonyl carbon is thus facile and involves transfer of a proton yielding an alcohol-containing compound (a hemiaminal, hemiacetal, or hemithioacetal). Primary amines can undergo addition to aldehydes followed by elimination of water to yield an imine, which revert to amines and aldehydes much more slowly than direct addition intermediates. Aldehydes or nucleophiles with pendant nucleophiles (such as N-terminal serine, threonine, or cysteine residues) can also undergo multiple addition and elimination reactions to form oxazolidine or thiazolidine heterocycles which undergo cleavage much more slowly.

Aldehydes can react with amine and alcohol nucleophiles and thus with lysine, serine, and threonine residues, with N-terminal amines, and with N-terminal serine, threonine, and cysteine residues. A variety of aldehydes have been used as covalent inhibitors. For example, the US FDA-approved pharmaceutical voxelator (a treatment for sickle-cell disease) (Supplementary table S1) reacts at the N-terminus of the β-chain of hemoglobin to form an imine; the covalently modified hemoglobin forms complexes with enhanced oxygen affinity. Roblitinib (Supplementary table S1) was developed as an FGFR4 inhibitor using an aldehyde as a warhead.[46] A variety of antiviral compounds have been developed as inhibitors for the main protease M^{pro} of SARS-CoV-2 for treatment or prevention of COVID-19 infection using an aldehyde moiety (or protected aldehyde moieties such as α-hydroxysulfinates) and bind to a cysteine residue in the active site.[47, 48]

Nitriles are compounds possessing a carbon-nitrogen triple bond (R-CN). The triple bond renders the nitrile group linear and relatively unhindered, making it more easily available for reactions with nucleophiles. The terminal lone pair on the nitrogen atom makes nitriles potentially reactive with electrophiles, but the product is likely to have enhanced reactivity towards nucleophiles. The carbon atom of nitriles is electrophilic, both because of the hybridization of the carbon atom (the increased s character in bonds to the carbon make the carbon atom effectively more electronegative) and because carbon is less electronegative than nitrogen. Nitriles act as less hindered and more electron-deficient analogs of carbonyl compounds. Nucleophiles add to the carbon atom to form substituted imines (after protonation); the presence of a lone pair on the

nitrogen atom renders addition reactions of heteroatomic (nitrogen, oxygen, and sulfur) nucleophiles reversible.

Covalent inhibitors that use nitrile warheads are not uncommon. Of marketed drugs, the anti-COVID-19 agent nirmatrelvir (Supplementary table S1) inhibits the SARS-CoV-2 main protease M^{pro} by the reversible reaction of an active site cysteine residue with its nitrile moiety.[49] Odanacatib (Supplementary Table S1) was an anti-osteoporosis drug developed by Merck but terminated for increased risk of stroke[50]; it acts by reversible covalent binding to an active site cysteine residue of cathepsin K.[51] Related analogs were prepared as inhibitors of the cysteine protease cruzain.[52] A piperidinecarbonitrile PF-303 was prepared as a reversible covalent inhibitor of BTK (Supplementary Table S1).[53, 54] While nitriles are in theory capable of reacting with nucleophilic residues (such as lysine, threonine, and serine residues), nitriles appear to be most reactive with cysteine residues.

Epoxides are substances containing three-membered rings in which one of the ring atoms is oxygen. Three-membered rings pull the carbon-carbon and carbon-oxygen bond angles away from their preferred angles, generating a large amount of strain which can be relieved most easily by cleavage of one of the carbon-oxygen bonds of the ring. The relative electronegativities of carbon and oxygen make the carbon atoms susceptible to nucleophilic attack. Reaction of a nucleophile with one of the carbon atoms of an epoxide (in most cases, the least sterically hindered carbon atom) forms a new bond to the carbon atom and an alcohol (after protonation of the oxygen atom). The strain of the three-membered ring is large enough to render the reverse reaction (reclosure of the epoxide and expulsion of the nucleophile) difficult under physiological conditions, rendering epoxide opening (and enzyme inhibition) irreversible.

There are fewer covalent inhibitors using epoxide warheads as compared to other types of warheads, likely because of the carcinogenicity of polycyclic aromatic hydrocarbon-derived epoxides and because of the presence of epoxide hydrolases that may inactivate epoxide-containing covalent inhibitors[55], but covalent inhibitors containing epoxides are known and used. The US FDA-approved proteosome inhibitor carfilzomib (derived from the natural product epoxomicin; Supplementary table S1)[9] contains an epoxyketone moiety which undergoes both epoxide ring opening and cyclocondensation with the N-terminal threonine residue of the 20S proteosome to form a morpholine ring[55]; the combination of reactions renders inhibition irreversible. The antibiotic fosfomycin inhibits UDP N-acetylglucosamine enolpyruvyl transferase[56] by reacting with Cys115 and preventing transfer of phosphoenolpyruvate.

Lipinski's rule of five and covalent inhibitors

Lipinski in his landmark paper[57] defined the Rule of 5 as "...poor absorption or permeation is more likely when there are more than 5 H-bond donors, 10 H-bond acceptors, the molecular weight (MWT) is greater than 500 and the calculated log P (CLogP) is greater than 5 (or MlogP > 4.15)." (MLogP = Moriguchi Log P calculation).[58] A compound complies with Lipinski's rule if it violates no more than one of the conditions. While once considered central to drug design for drugs intended to be orally administered, the number of drugs including those with FDA approval that violate/are exceptions to the rule of 5 has been growing.[59-61] For our set of warhead containing compounds (~25 K), we calculated the number of H-bond donors, H-bond acceptors, the molecular weight and CLogP. We chose to classify our set of covalent inhibitors by how many aspects of the Rule of 5 each compound obeyed. Since most of the compounds studied are likely to be delivered orally, the use of the Rule of 5 is likely pertinent. As shown in Figure 7, an almost negligible fraction of substances (0.05%) followed none of Lipinski's rules while roughly 80%

compounds violated none or one of the Rule of 5 conditions. About 15% and 4% of the remainder followed two or one of the rules, respectively. Thirty of 74 US FDA-approved kinase inhibitors violate Lipinski's rule of five.[59] Of the covalent inhibitors we found, alkyne and chloro carbonyl compounds disproportionately obeyed all of the conditions of the Rule of 5, while α,β-unsaturated amides and epoxides made up a disproportionate fraction of compounds that violated one or two of the conditions of the Rule of 5. As noted by Jagannathan[62] , roughly 60% of approved antitumor agents obeyed the molecular weight cutoff for the Rule of 5; since α,β-unsaturated carbonyl compounds such as acrylamides can be derived by addition of an acrylamide or other unsaturated acyl moiety to a known noncovalent inhibitor, the molecular weights of covalent inhibitors are likely higher than the corresponding noncovalent inhibitors and more likely to exceed the molecular weight threshold of the Rule of 5. Disulfides and selenium compounds tended to flout 3 out of 4 rules; the reason for this is unclear.

Biological targets

We extracted information about biological targets for identified substances showing presence of warheads from multiple databases (CAS, CAS_PROJECTED, EVOLVUS_ADME, GOSTAR, GOSTAR_PROJECTED). Out of all the identified substances, a very small fraction of compounds had biological data associated with them. Figure 8A shows the top 20 most targeted proteins and include protein tyrosine kinases such as BTK, Tec and protein kinases such as Akt, MAPK1, JAK3 among others. Protein tyrosine kinases can be sub-divided into two families: receptor and non-receptor protein kinases with the latter being targeted by covalent inhibitors almost 7.5 times more than the former (Figure 8B). Covalent inhibitors containing the following warheads appear to target protein tyrosine kinases: α,β-unsaturated carbonyls, sulfonyls (SO2), nitriles (R-CN), halogen derivatives (Cl-CY, Br-CY and F-CY where Y = CO, $SO_{(n)}$ or NO_2), alkyne carbonyls (R-CO), aldehydes (CHO) and epoxides. Perhaps unsurprisingly, α,β-unsaturated carbonyl containing substances have the largest number of targets associated with them (Supplementary Figure 1 and Figure 2). This is followed by nitriles (R-CN), fluoro derivatives (FCY) and aldehydes (CHO). Among the warheads, only for nitriles (R-CN), the number of indexed targets associated with patents are far greater than those for journals (Supplementary Figure 1). On the other hand, aldehydes (CHO), boronic acid and esters, epoxides, bromo derivatives (BrCY), α-ketoamides, Se-based and disulfide containing substances have targets associated only with journals (Supplementary Figure 1). Detailed breakdown of protein targets for each warhead is shown in Supplementary Figure 2.

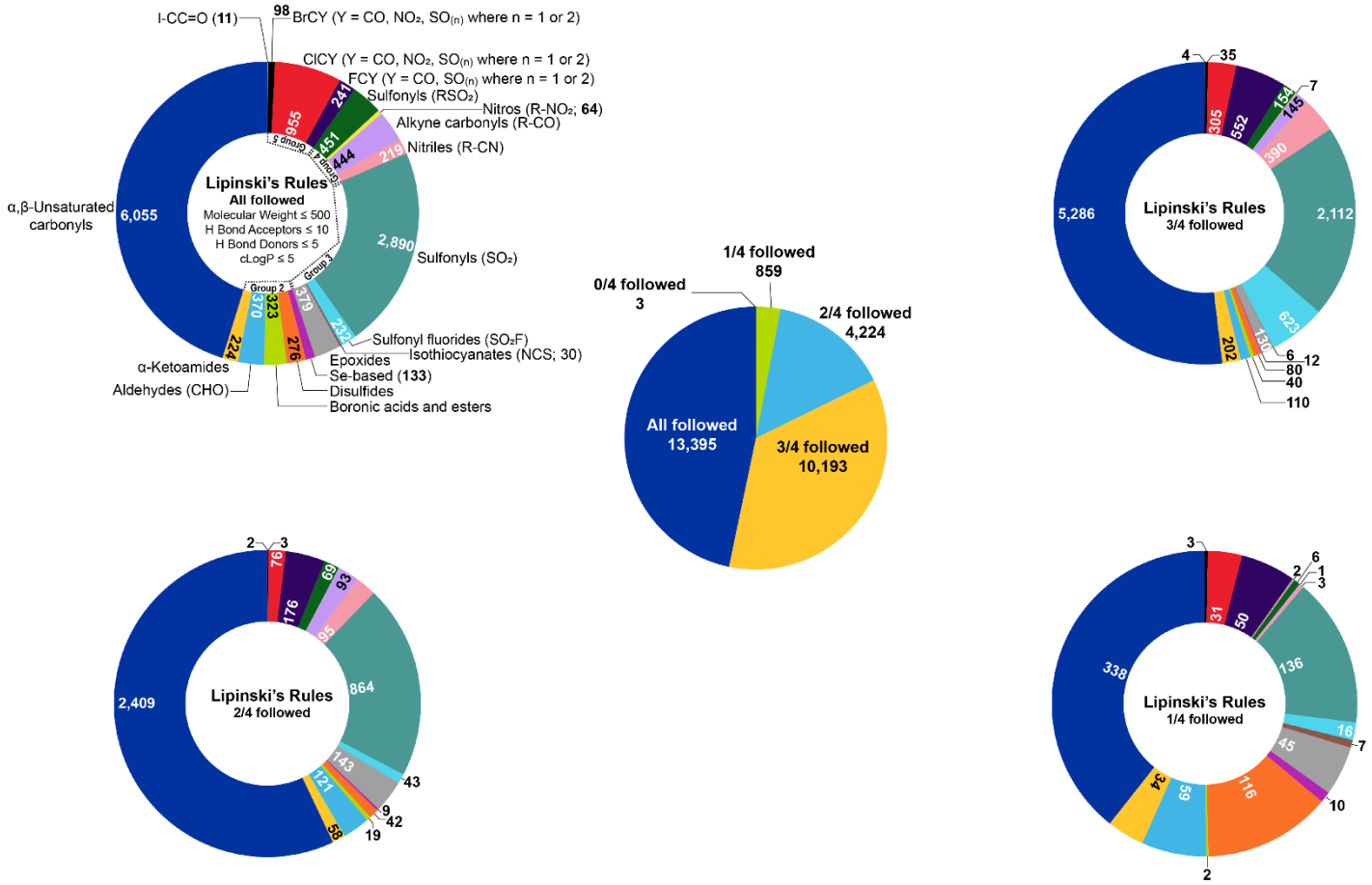

Figure 7. Warhead distribution in the context of Lipinski's rules (molecular weight, H bond acceptors, H bond donors and cLogP). The pie chart in the center shows the overall distribution of warheads containing substances in the CAS Content Collection as per the number of Lipinski's rules that are followed. The four donut charts show in-depth distribution of warheads across the four scenarios i.e. ranging from when all of the parameters are followed (top left panel) to when 1 out of the 4 parameters (bottom right panel) are followed.

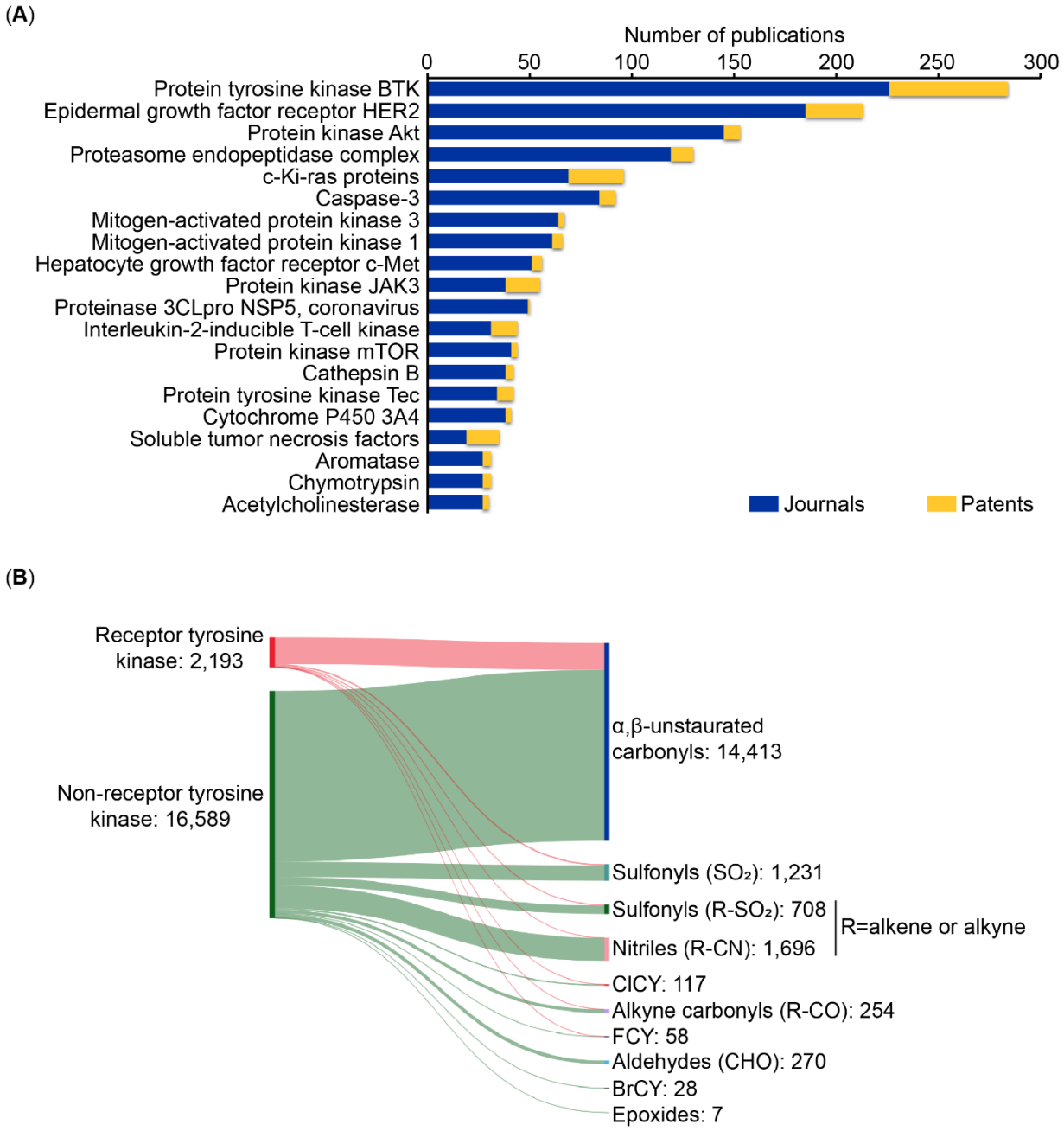

Figure 8. (**A**) Top 20 most targeted proteins by covalent inhibitors in terms of publications (journals and patents in the CAS Content Collection for the period 2001-2023. (**B**) Sankey graph showing association of individual warheads with the two types of protein tyrosine kinases.

III. Targeted covalent inhibitors in drug discovery

Covalent inhibitors continue to be developed as treatments for a wide variety of human health conditions, with >35 approved in the last 20 years by the US FDA for use in humans. Many protein targets, especially enzymes, have been found to be effectively targeted by covalent

inhibitors for the treatment of human diseases. In this section we focus on some of popular targets for covalent inhibitors, such as the SARS-CoV-2 main protease, RAS proteins, BTK, EGFR and HER2, FGFR, JAK3, and CDK proteins.; We also briefly discuss the use of covalent inhibitors for protein-protein interactions. Structures of compounds discussed in the following section can be found in Supplementary Table S2.

1. SARS-CoV-2 main protease (M^{pro})

SARS-CoV-2 M^{pro} is a prominent target for anti-SARS-CoV-2 drug discovery efforts. It cleaves the overlapping pp1a and pp1ab polyproteins into functional proteins, a critical step during viral replication.[63, 64] Inhibition of its enzymatic activity could thus block viral replication.[65] Based on the analysis of a co-crystal structure, Pfizer successfully discovered PF 00835231, containing an α-hydroxymethylketone as an electrophilic warhead. PF 00835231 demonstrated potent SARS-CoV-2 M^{pro} inhibition in vitro and in vivo.[66] Structure optimization of PF 00835231 to improve its pharmacokinetic properties and oral bioavailability led to the discovery of PF 07321332 (Nirmatrelvir), a reversible covalent SARS-CoV-2 M^{pro} inhibitor possessing high potency and selectivity.[67] Nirmatrelvir is unique amongst the reported covalent SARS-CoV-2 M^{pro} inhibitors because it employs a nitrile group as an electrophilic warhead unlike aldehydes and α-haloacetamides warheads employed by others. The nitrile warhead covalently reacts with Cys145 of SARS-CoV-2 M^{pro}[68] to generate a covalent C-S bond and form a protein-inhibitor complex.[68-71] Besides the nitrile warhead, the remainder of the Nirmatrelvir structure forms crucial hydrogen bonding and hydrophobic interactions at the active site of the protein. Nirmatrelvir has been approved by the US FDA (in combination with ritonavir) under the brand name of Paxlovid as a treatment for COVID-19.[72] Recently, an analog of Nirmatrelvir in which the original nitrile warhead was replaced by a CF_3-capped terminal alkyne was shown to be an effective irreversible inhibitor of SARS-Cov-2 M^{Pro}. Capping the terminal alkyne with an appropriate group increased the potency by ~5-fold. The electrophilic nitrile warhead of Nirmatrelvir yields a more effective inhibitor of isolated M^{pro} than the alkyne-containing analog independent of the scaffold; however, the increased potency of Nirmatrelvir over its alkyne-containing analog may be substantially reduced in live cells.[69]

Another example of recently discovered compounds with potent SARS-CoV-2 3CL protease inhibitory activity are peptidomimetics using an α-acyloxymethyl ketone as their warhead.[70] The most potent of these compounds exhibited low cytotoxicity and good plasma and glutathione stability while possessing activity similar to that of the most potent inhibitors reported. The α-acyloxymethyl ketone warhead reacts irreversibly with the sulfhydryl group of Cys145 to form a covalent adduct. Excellent selectivity for SARS-CoV-2 3CL protease over other Cys proteases such as cathepsin B and S (CatB and CatS) indicates that acyloxymethyl ketones can be incorporated into selective protease inhibitors. The acyloxymethyl ketones also maintained good antiviral potency in multiple coronavirus strains (CoV-229E and CoV-OC43).[70]

2. Rat sarcoma viral oncogene homologue (RAS)

KRAS proteins are small GTPase enzymes that function as molecular switches: they respond to upstream EGFR activation and regulate the downstream mitogen activated protein kinase (MAPK) and PI3K/mTOR pathways, controlling cell proliferation, differentiation, and survival.[73, 74] Oncogenic mutation of KRAS is closely linked to

tumorigenesis.[75-77] For the longest time, KRAS was considered an undruggable target until the Shokat laboratory successfully developed a series of compounds that covalently and irreversibly bound to the cysteine residue of the $KRAS^{G12C}$ (glycine-to-cysteine substitution) mutant. Unfortunately, the most potent compound containing an acrylamide warhead was incapable of engaging $KRAS^{G12C}$ in cells even at a relatively high dose and with long incubation times.[78, 79] Structural optimization of this scaffold led to ARS-853, the first potent KRAS inhibitor shown to selectively and directly inhibit KRAS in cells[78]; however, its low metabolic plasma stability and poor bioavailability stalled further development and prevented its preclinical evaluation. Efforts to improve the physiochemical properties and pharmacokinetic parameters of ARS-853 resulted in a new generation of $KRAS^{G12C}$ inhibitors capable of exploiting an allosteric pocket. The substituted piperazine ring incorporates to scaffolds, like quinazoline core, quinazolinone core, or tetrahydropyridopyrimidine core occupying the allosteric pocket for enhanced potency and provided ADME properties suitable for further optimization. ARS-1620, one of the most potent compounds from the newly developed series was the first $KRAS^{G12C}$ inhibitor with efficacy in vivo in patient-derived xenografts.[80] ARS-1620 is potent, selective, orally bioavailable, and well-tolerated in mice. The compound exhibited both in vitro and in vivo potency and has therapeutic potential as a drug candidate.[80] ARS1620 can lead to the presentation of drug-modified neoantigens by class I major histocompatibility complex (MHC). A bispecific T cell engager that recognizes these neoantigens elicits a cytotoxic T cell response against KRASG12C cells, including those resistant to direct KRAS G12C inhibition.[81] Efforts to identify molecules capable of targeting the GTP-bound, active state of KRAS led to the discovery of a cryptic groove adjacent to the allosteric switch II pocket created by rotation of His95.[82] Adagrasib (MRTX849) and sotorasib (AMG510) were designed to fit into this binding pocket and engage in interaction with His95 in order to maximize potency.[83-85] Approved by the US FDA in 2021, sotorasib became the first therapy to treat KRAS-mutant cancers, particularly $KRAS^{G12C}$ mutant non-small-cell lung cancer (NSCLC). The structurally novel covalent $KRAS^{G12C}$ inhibitor, JDQ443, resulted from a preliminary in silico screening and was developed from a distinct pharmacophore – a unique 5-methylpyrazole core with a spiro-azetidine linker. JDQ443 demonstrated potent and selective antitumor activity in cell lines and in vivo models and unlike other $KRAS^{G12C}$ inhibitors only weakly interacted with His95.[86] Chloro and methyl substituents on the indazole ring optimally fill the hydrophobic region, while the rigid spirocyclic linker orients the acrylamide warhead towards Cys12 allowing the amide carbonyl to form H-bond interactions with the side chain of Lys16. JDQ443 is currently in clinical development as a monotherapy and in combination with either the SH2 containing protein tyrosine phosphatase-2 (SHP2) inhibitor TNO155, the anti–PD-1 monoclonal antibody tislelizumab, or both in advanced duodenal cancer.[87, 88] To date, a total of twelve irreversible inhibitors of $KRAS^{G12C}$ have entered in clinical trials, including adagrasib (MRTX849, Mirati Therapeutics), sotorasib (AMG510, Amgen), JNJ-74699157 (Janssen), LY 3499446 and LY 3537982 (Eli Lilly), divarasib (GDC- 6036; Genentech), D-1553 (InventisBio), JDQ443 (Novartis), BI1823911 (Boehringer Ingelheim), JAB-21822 (Jacobio Pharmaceuticals Group), MK- 1084 (Merck) and RMC- 6291 (Revolution Medicines) with adagrasib[89] and sotorasib[90] gaining FDA approvals in 2022 and 2021, respectively.

3. Bruton tyrosine kinase (BTK)

BTK is intimately involved in multiple signal-transduction pathways regulating survival, activation, proliferation, and differentiation of B-lineage lymphoid cells.[91-93] BTK has become a target of interest for treating chronic lymphocytic leukemia owing to its crucial role downstream of the B cell receptor critical for proliferation and survival of leukemic cells, indicating its relevancy as a target for B cell malignancies.[94] Since it was first described in 1993, multiple BTK inhibitors (BTKi) have since been developed. The irreversible BTK inhibitor ibrutinib was discovered in the early 2000s and became the first FDA-approved BTKi in 2013.[95] Ibrutinib is associated with high response rates in relapsed/refractory chronic lymphocytic leukemia (CLL), Waldenstrom macroglobulinemia (WM), mantle cell lymphoma (MCL) and in chronic graft versus host disease.[96-104] Ibrutinib binds to a non-conserved cysteine residue (Cys481) located adjacent to the ATP-binding site in BTK and its selectivity for BTK is derived from the presence of Cys481 since few kinases possess a homologous cysteine. Several other covalent BTK inhibitors have been approved or are in clinical trials, containing a variety of Michael acceptors as alternatives to the acrylamide warhead in ibrutinib.[105-107] Acalabrutinib and zanubrutinib were approved by the US FDA in 2019[108] and 2023[109]; they showed improved selectivity and exhibited fewer adverse effects than ibrutinib. [105-107]

Acalabrutinib contains a butyramide electrophile instead of an acrylamide. The butyramide electrophile is less reactive than the acrylamide warhead of ibrutinib, which is proposed to account in part for its improved selectivity for BTK and the reduced number of adverse cardiovascular events.[110-112] Acalabrutinib was approved in 2017 for MCL and in 2019 for CLL.[113, 114] With higher selectivity than ibrutinib, acalabrutinib inhibits only BTK and has no effect on other kinases such as TEC, BMX, and TXK.[112, 113] Interestingly, ACP-5862, the major metabolite of acalabrutinib, also covalently inhibits BTK while exhibiting two-fold lower potency than acalabrutinib but similar selectivity towards BTK. Current data suggest that ACP-5862 may be clinically relevant to the efficacy of acalabrutinib therapy while retaining its high BTK selectivity.[112, 115] JS25 is a 2nd generation covalent BTK inhibitor with nanomolar potency against BTK (5.8 nM) derived from structural modification of BMX-IN-1, a recently discovered inhibitor of BTK.[116] JS25 binds covalently to BTK at Cys481 and selectively inhibits BTK. JS25 presented a broad spectrum of activity in myeloid and lymphoid B-cell cancers and demonstrated improved therapeutic efficacy versus ibrutinib in patient-derived diffuse large B-cell lymphoma (DLBCL) models, as well as in xenograft models of B-cell lymphoma (BL) and CLL.[116] JS25 also possesses the potential to treat metastatic forms of blood cancers in the brain because of its permeability of blood−brain barrier. JS25 is likely to be a therapeutically relevant BTKi, with demonstrated antiproliferative effects and an improved selectivity profile, for clinical use against hematological cancers and autoimmune diseases).[116]

4. Epidermal growth factor receptor (EGFR) and human epidermal growth factor receptor 2 (HER2)

EGFR is considered a key therapeutic target in oncology because it is overexpressed in several types of cancer. For example, overactivity of EGFR drives the progression of NSCLC.[117] Erlotinib, gefitinib, and lapatinib are reversible non-covalent tyrosine kinase inhibitors (TKIs), which have been approved for the treatment of NSCLC and HER2 receptor positive breast cancer patients.[118-120] However, kinases have acquired resistance to the reversible TKIs over time. To circumvent resistance, second generation TKIs-covalent inhibitors were strategically designed with acrylamide Michael acceptors to react with the cysteine residue (Cys797) in EGFR.[9, 118] These TKIs inhibit phosphorylation of

EGFR irreversibly, providing prolonged suppression of EGFR signaling and showing greater efficacy than reversible first-generation inhibitors. Afatinib and dacomitinib are successful examples of such second generation irreversible covalent TKIs. Afatinib has been approved for clinical use in adult patients with advanced or metastatic NSCLC with the L858R mutation.[121] The third generation of EGFR inhibitors that selectively target the T790M mutant over wild-type EGFR has been developed. They include WZ4002[122], osimertinib[123, 124], rociletinib (CO-1686)[2, 125] and the active clinical candidates nazartinib[126, 127] and avitinib.[128] These agents generally bind to the ATP-binding site of mutant EGFR in a U-shaped conformation, positioning an acrylamide group to form a covalent bond with Cys797.[129]

Replacement of the quinazoline moiety in first- and second-generation compounds with a pyrimidine yielded compounds with high selectivity for mutant EGFR (T790M) over wild-type EGFR.[130] The higher affinity of third-generation EGFR inhibitors for T790M over wild-type EGFR not only results in efficacy in cancers with the EGFR gatekeeper mutation but also contributes to an improved safety profile and enables a higher recommended dose for osimertinib than for afatinib.[131] These novel EGFR and HER2 inhibitors may offer a novel therapeutic option for patients with mutant EGFR NSCLC.

Mutant Her2 (Neu/ErbB2) is a driver of non-small cell lung cancer (NSCLC) in 4% of the patients[132] or mediates resistance toward the inhibition of its family member epidermal growth factor receptor with small-molecule inhibitors.[132, 133] Afatinib is also under investigation as a monotherapy in patients with HER2-positive breast cancer who had progressed despite trastuzumab treatment.[134] Neratinib (Puma) potently inhibits HER2 by covalently binding Cys805 (a cysteine residue homologous to Cys797 in EGFR) and was approved by the FDA for treatment of $HER2^+$ breast cancer in 2017.[135, 136] Dacomitinib (Vizimpro®) is an orally administered, small-molecule irreversible inhibitor of HER1 (EGFR), HER2 and HER4 that was developed by Pfizer Inc. In September 2018, dacomitinib received its first global approval by US FDA, for use in the first-line treatment of patients with *EGFR*-mutated metastatic NSCLC.[137] Data from Phase II clinical trials indicate that poziotinib, a covalent HER2 inhibitor developed by Spectrum Pharmaceuticals, may be a useful treatment for metastatic lung cancers harboring HER2 exon 20 insertion mutations.[138] [138] However, in November 2022 the US FDA denied approval for the use of poziotinib based on the current clinical trial data and asked for data from additional trials.[139] The most recently reported covalent HER2 inhibitors were pyrrolopyrimidines discovered by focused compound screening and structure-based drug design (SBDD). One of the identified compounds inhibited H1781 cancer cells with an IC_{50} value of 161 nM. The compound showed favorable physicochemical and pharmacokinetic profiles comparable to that of neratinib and established the pyrrolopyrimidine core as a suitable scaffold for covalent inhibition of Her2.[140]

5. Fibroblast growth factor receptor (FGFR)

Fibroblast growth factor receptors (FGFR) are a family of four receptor tyrosine kinases (FGFR1-4) essential for cell proliferation and differentiation.[141-143] FGFRs have been implicated in the development of colorectal, lung, and renal cell cancers as well as hepatocellular carcinoma[143] and are therefore considered attractive targets for cancer

therapy).[144] FIIN-2 and FIIN-3 are the first effective inhibitors of the proliferation of cells dependent upon the gatekeeper mutants of FGFR1 or FGFR2, which confer resistance to first-generation clinical FGFR inhibitors such as NVP-BGJ398 and AZD4547.[145] FIIN-2 and FIIN-3 are pyrimidopyrimidinone and pyrimidine analogs of FIIN-1, which in turn was based on a noncovalent inhibitor PD173074 resulting from rational design.[146] Both FIIN-1 and FIIN-2 use a 4-acrylamidobenzyl group as the warhead. Unlike other dual covalent inhibitors of EGFR and VEGFR, FIIN-3 exploits a single acrylamide group that can access two spatially distinct cysteine residues, Cys797 in EGFR and Cys477 in FGFR4, respectively.[122, 147, 148] BLU554 (fisogatinib), an aminoquinazoline-based selective irreversible FGFR4 inhibitor, was designed by covalent targeting of a unique cysteine residue (Cys552) in the hinge region of FGFR4.[149] In contrast, the other analogous residue in other family members, FGFR1/2/3. BLU554 is currently in clinical studies for the treatment of FGFR4-driven hepatocellular carcinoma.[150, 151] Structural modification of the pan-FGFR inhibitor BGJ398 (infigratinib)[152] led to discovery of the potent FGFR4-selective irreversible inhibitor H3B-6527 which utilizes an acrylamide moiety in the ortho-position of the aniline ring as the warhead. H3B-6527 is currently under clinical evaluation for the treatment of hepatocellular carcinoma.[153, 154] Furthermore, the increased activity observed with H3B-6527 in combination with CDK4/6 inhibition provides a rationale for exploration of this combination in early clinical trials. In addition, other reported selective FGFR4 inhibitors are in the early stages of development.[155-160] An irreversible FGFR inhibitor, PRN1371, was developed to target a distinct cysteine residue (Cys488) found in FGFR1-4 but not in related receptor tyrosine kinases such as VEGFR2, PDGFRα, or PDGFRβ, conferring selectivity for FGFR. PRN1371 uses an acrylamide Michael acceptor as warhead and has shown promise in early clinical trials for the treatment of advanced solid tumors.[161]

To avoid the potential undesirable side effects of irreversible inhibitors and to address the rapid FGFR4 resynthesis rate in hepatocellular carcinoma cells (less than 2 h), the reversible-covalent inhibitor, FGF401 (roblitinib) was developed.[7, 162] Roblitinib has entered phase I/II clinical trials for the treatment of hepatocellular carcinoma and other solid tumors harboring abnormal FGFR4 signaling.[163] Unlike irreversible inhibitors, the aldehyde group of FGF401 forms an unstable hemi-thioacetal adduct with Cys552, a distinct non-conserved cysteine of FGFR4, to achieve reversible-covalent inhibition of FGFR4 with prolonged residence time[7, 162] Roblitinib has entered phase I/II clinical trials for the treatment of hepatocellular carcinoma and other solid tumors harboring abnormal FGFR4 signaling.[163 7, 164] Another promising reversible covalent inhibitor currently under development is a 5-formyl-pyrrolo[3,2-b]pyridine-3-carboxamide derivative exhibiting selective single-digit nanomolar activity against both wild-type FGFR4 as well as the FGFR4V550L/M gatekeeper mutants, while sparing FGFR1/2/3. Due to its high potency and both in vitro as well as in cell-based assays, the pyrrolopyrimidinecarboxamide serves as a promising lead compound for the treatment of hepatocellular carcinoma (HCC) involving FGFR4).[165] Recently, a dual warhead covalent inhibitor of FGFR4, CXF-009, containing two acrylamide moieties at the two ends of the molecule have been developed.[166] CXF-009 appears to be capable of interacting with two crucial cysteine residues (Cys477 and Cys552) and represents the first dual warhead inhibitor of FGFR4.[166] Despite the successful design of covalent inhibitors (irreversible and reversible) targeting Cys552 of FGFR4, no FGFR4-selective inhibitors have been approved by the

FDA to date. There remains an urgent need to develop new selective FGFR4 inhibitors to provide a solid foundation for the effective treatment of hepatocellular carcinoma.

6. Janus kinases 3 (JAK3)

Janus tyrosine kinase 3 (JAK3), a non-receptor protein tyrosine kinase, is one of the four family members of Janus kinase, the other three being JAK1, JAK2 and TYK2. Expressed in lymphoid cells and involved in the signaling of T cell functions, JAK3 have been identified in humans as a cause of severe combined immunodeficiency disease (SCID), which manifests as a depletion of T cell, B cell, and natural killer (NK) cells with no other defects.[167, 168] Consequently, selective JAK3 inhibition has been shown to be an attractive therapeutic strategy for autoimmune diseases such as rheumatoid arthritis (RA)[169] ([167, 168] Consequently, selective JAK3 inhibition has been shown to be an attractive therapeutic strategy for autoimmune diseases such as rheumatoid arthritis (RA)[169] (and alopecia areata (AA)).[170] Tofacitinib is the first oral Janus kinase inhibitor indicated for treatment of moderate to severe RA. Tofacitinib demonstrated efficacy and safety comparable to other disease-modifying antirheumatic drugs as indicated by achievement of the ACR20, ACR50 and ACR70 criteria defined by the American College of Rheumatology.[171] A high level of selectivity toward JAK3 is achieved by the covalent interaction of PF 06651600 (Ritlecitinib) with a non-conserved cysteine residue (Cys909) in the catalytic domain of JAK3, whose identity is a serine residue in the other JAK isoforms. [172, 173] In addition to its high selectivity, PF 06651600 also shows good oral bioavailability and favorable pharmacokinetic properties, making it an attractive drug candidate. It has been demonstrated to be effective for treatment of moderate-to-severe RA that does not respond to methotrexate.[174, 175] RB1, is a 4-aminopiperidine-based compound which is highly selective for JAK3 inhibition, with an IC_{50} of value of 40 nM. Reasonable pharmacokinetics properties, good oral availability, and favorable results of toxicology studies suggest that RB1 has the potential to be an efficacious treatment for RA and other immune-related diseases.[176] The highly selective JAK3 inhibitor Z583 is a promising candidate with significant therapeutic potential for autoimmune diseases. Z583 is an attractive novel drug candidate for RA due to its potent efficacy and lack of side effects linked to systemic suppression making it worthy of further evaluation in clinical study.[169, 177]

7. Cyclin-dependent kinases (CDKs)

Cyclin dependent kinases, or CDKs, are an intensively investigated family of evolutionarily conserved protein kinases that orchestrate the cell cycle and gene transcription.[178, 179] One of the first irreversible CDK inhibitors reported is THZ1, which inhibits CDK7 by docking in the active site and covalently modifying the nearby Cys312 residue.[180, 181] Subsequent studies with THZ1 revealed the therapeutic potential of targeting CDK7 in many aggressive cancers, including MYCN-amplified neuroblastoma[182], small-cell lung cancer[183], and triple-negative breast cancer.[184] At higher concentration, THZ1 also demonstrates some activity against closely related kinases CDK12 and CDK13.[180] Based on the THZ1 scaffold, newer covalent inhibitors, SY-1365 and THZ531, with better selectivity profiles have been identified. SY-1365 is currently being investigated for the treatment of ovarian and breast cancers.[185, 186] THZ531 is a covalent inhibitor selective for CDK12 and CDK13, sparing CDK7 activity.[187] Treatment of Jurkat T-ALL cells with THZ531 diminished pSer2 and decreased the expression of DDR and super enhancer-

associated transcription factor genes. Neuroblastoma and lung cancer cells develop resistance to THZ1 and THZ531 through upregulation of the ABCB1 or ABCG2 drug transporters, implying that these compounds are substrates for these proteins.[178, 179]

Efforts to overcome drug resistance to THZ1 and THZ531 led to the discovery of another generation of irreversible CDK inhibitors, compound E9.[181, 188] Compound E9 is not a substrate for ABC proteins and hence escapes drug efflux, thus rendering it an attractive candidate to overcome THZ1 resistance. Compound E9 was more potent than other inhibitors, including ribociclib[189,181, 188 189], palbociclib[190], and AZD5438[191], showing the most potent antiproliferative activity in $THZ1^R$ NB and lung cancer cells, with IC_{50} values ranging from 8 to 40 nM.[181] Compound E9 is a trisubstituted pyrazolopyridine with structural similarity to dinaciclib (which inhibits CDKs 1/2/5/9/12), except that compound E9 contains an acrylamide moiety that targets cysteine residues in CDKs 7, 12, and 13.

A highly selective CDK7 covalent inhibitor, YKL-5-124, triggered cell-cycle arrest without global effects on transcription.[192] Also, in contrast to THZ1, YKL-5-124 treatment only modestly affected bulk Pol II (RNA polymerase II) phosphorylation on CTD residues Ser2, Ser5 and Ser7. YKL-5–124 resulted from fusing the structure of THZ1 with PF 3758309, a PAK4 inhibitor and optimizing the resultant compound to maximize interactions at CDK7.[192] Interestingly, the biochemical and cellular effects of YKL-5-124 more closely resemble those produced by allele-specific inhibition of CDK7 as in human colon cancer-derived cells.[193, 194 192 192193, 194] Another combinatorial strategy for triggering cancer cell death – inducing transcriptional dependency with agents that activate p53 while selectively inhibiting CDK7– recently emerged in studies with *CDK7*$^{as/as}$ colon-cancer-derived cells and was recapitulated in wild-type tumor cells with another CDK7-selective covalent inhibitor, YKL-1-116.[195]

Genetic depletion of cyclin-dependent kinase 12 (CDK12) or selective inhibition of an analog-sensitive CDK12 reduces the expression of DNA damage repair genes. MFH290, a highly selective covalent inhibitor of CDK12/13, was generated by combining structural features of CDK12/13 covalent inhibitor THZ531 with the previously reported noncovalent pan-CDK inhibitor SNS032.[196] MFH290 forms a covalent bond with Cys1039 of CDK12, exhibiting excellent CDK12 selectivity and inhibits the phosphorylation of Ser2 in the C-terminal domain of Pol II thereby reducing the expression of key DNA damage repair genes.[197] MFH290 demonstrated a sustained CDK12 inhibition-induced phenotype including strong antiproliferative effect on cancer cells, a transcriptional defect for DDR genes, and a combinatorial effect with PARP inhibition.[197] BSJ-01-175, another CDK12/13 covalent inhibitor, resulted from structure-activity relationships (SAR) efforts on THZ531 and is reported to possess a higher degree of selectivity for CDK12/13 as compared to its parent THZ531 even at as high concentrations as 5 μM. Additionally, and most importantly BSJ-01-175 shows potent in vivo efficacy.[198]

FMF-04-159-2, a CDK14-specific covalent inhibitor, and its reversible analog were used to characterize the cellular consequences of covalent CDK14 inhibition, including an unbiased investigation using phospho-proteomics. This investigation suggested that CDK14 plays a supporting role in cell-cycle regulation, particularly mitotic progression, and

identified putative CDK14 substrates. Together, these results represent an important step forward in understanding the cellular consequences of inhibiting CDK14 kinase activity.[199]

8. **Euchromatic histone lysine methyltransferase 2/lysine methyltransferases (G9a-like protein) (G9a/GLP)**

G9a (also known as euchromatic histone lysine methyltransferase 2; EHMT2) and its closely related paralogue GLP (G9a-like protein, also known as euchromatic histone lysine methyltransferase 1; EHMT1) can catalyze the methylation of both histone and non-histone substrates. G9a overexpression is associated with proliferation and metastasis in several types of cancer including brain, breast, ovarian, lung, bladder, melanoma, and colorectal cancer.[200-206] Moreover, it has been shown that G9a is involved in embryonic stem cell maintenance[207, 208] and T-cell differentiation and is implicated in other diseases such as Alzheimer's disease (AD)[209, 210], sickle cell disease[211] and Prader−Willi syndrome.[212] MS8511[213] the first G9a/GLP covalent inhibitor reported, reduced histone H_3 lysine 9 (H3K9me2) methylation levels and showed enhanced antiproliferative activity over related noncovalent inhibitors. The acrylamide warhead of MS8511 is thought to interact with Cys1098 and Cys1186 in G9a and GLP, respectively. There appears to be a slight preference for G9a over GLP, due to faster modification/kinetics, by MS8511. Overall, MS8511 is a highly potent, selective, and cell-active covalent inhibitor of G9a and GLP which could serve as a useful chemical tool for investigating the physiological and pathophysiological functions of G9a and GLP.[213]

9. Other target proteins

A. Eukaryotic translation initiation factor 4E (eIF4E)

The eukaryotic translation initiation factor 4E (eIF4E) is a protein which binds to mRNA and stimulates its translation. It is frequently overexpressed in human cancers, with expression positively correlated to cancer progression[214] and drives cellular transformation, tumorigenesis, and metastatic progression in experimental models. Yet, despite the apparent attractiveness of targeting eIF4E (and the eIF4F complex), until recently there has been little to no development of eIF4E-specific therapies. Despite lacking a cysteine residue in the vicinity of the eIF4E cap binding site, a proximal lysine residue in eIF4E (Lys162, which interacts with the β-phosphate of the cap) was hypothesized/considered as a potential site for covalent attachment. Using a virtual docking approach, a library of approximately 88,000 arylsulfonyl fluorides was screened for candidates that could interact with Lys162 and bind within the eIF4E cap binding pocket. Based on the results of in silico screening, two viable compounds were identified which were further optimized to take advantage of a deep lipophilic pocket close to the cap binding site. Following structural optimization, an aminoquinazolinone-substituted arylsulfonyl fluoride was identified and used in cell-based assays to inhibit cap binding by eIF4E and suppress cap-dependent translation in cells.[215, 216]

B. Acetylcholine Esterase (AChE)

Acetylcholine is a neurotransmitter that stimulates cholinergic receptors at chemical synapses in the central nervous system. Patients suffering from Alzheimer's disease (AD) have decreased levels of cholinergic receptors and the most common treatment option is to reduce the breakdown of acetylcholine by inhibiting the enzyme acetylcholinesterase (AChE).[217] Rivastigmine (FDA 2000, Exelon®)[218] and metrifonate (BAY-A-9826, ProMem, 1997)[219, 220] are classified as pseudo-irreversible inhibitors because they react with the critical active site serine residue to form a covalent carbamoyl-AChE complex that temporarily prevents the hydrolysis of acetylcholine in the active site [218] However, metrifonate was abandoned as a treatment for AD because it produces severe muscular and life-threatening respiratory paralysis in some AD patients, a sign of organophosphate-induced delayed neuropathy.[220, 221] Irreversible AChR inhibitors are likely to have selectivity for central nervous system (CNS) because of the reduced rate of resynthesis of CNS AChE. The structural requirements for irreversible AChE inhibitors are thus relaxed; an AChE inhibitor must form an inhibitor-enzyme inactive complex that does not undergo spontaneous hydrolysis. These research findings confirmed the use of methanesulfonyl fluoride (MSF) for the treatment of AD).[222] Sulfonyl fluorides such as MSF, induce AChE inhibition with long-term disease-modifying benefits, perhaps by enhancing acetylcholine-dependent stimulation of neurotrophin nerve growth factor production and release and associated basal forebrain survival processes. [223-229]

C. Tau

Tau proteins help to maintain the structure of microtubules in nerve cells.[230] Aggregation of a mutant form of tau is observed in Alzheimer's disease (AD) and may be responsible for some of the observed brain damage. Inhibition of tau aggregation is thus a potential treatment for AD. Among covalent tau aggregation inhibitors (TAIs), oleocanthal, a natural product, reacts with the epsilon amino groups of lysine residues[231, 232] including residues residing in the microtubule binding repeat region, to form imines. In addition, other natural polyphenols are covalent TAIs, such as oleuropein aglycone[233], abundant in extra virgin olive oil, or green tea-derived (−)-epigallocatechin gallate (EGCG).[234] Other redox-active compounds, including the non-neuroleptic phenothiazine methylene blue (methylthioninium chloride; MTC), can modulate cysteine oxidation when incubated in the absence of exogenous reducing agents.[235] High concentrations of reduced sulfhydryl groups in the form of glutathione normally maintain a reducing intracellular environment[236], and therefore compounds acting solely through this mechanism could have low potency and efficacy in vivo. In general, covalent mechanisms of tau aggregation inhibition in AD are predicted to have low utility in vivo.[237] However, dimethyl fumarate, an electrophile capable of reacting covalently with cysteine sulfhydryl groups, was approved as an oral treatment for multiple sclerosis[238], providing further evidence that suggesting that electrophiles acting as covalent inhibitors can be useful therapeutic agents including residues residing in the microtubule binding repeat region to form imines.[239]

D. Cathepsins

Cathepsins are a group of cysteine proteases that are involved in proteolysis in lysosome and control various signaling pathways in cells.[240] Among different cathepsins, CatK has been of high interest as it occurs abundantly in osteoclasts and plays an important role in resorption and remodeling of bones. As such it has been a drug target for the treatment of osteoporosis, a condition in which bones decrease their density significantly and

become fragile. Several covalent inhibitors were designed to target CatK for the treatment of osteoporosis but have been discontinued owing to their side effects. Balicatib showed great selectivity for CatK with respect to other cathepsins[241] but was discontinued after its Phase II clinical studies as it led to morphea-like skin lesions in some patients.[242] Among many covalent inhibitors of CatK, odanacatib reached phase-III trials.[243, 244 240 241 242 243, 244] Odanacatib possesses a nitrile group that reacts with Cys25 of CatK to form an iminothioester adduct[51]; unlike other CatK inhibitors, odanacatib does not accumulate in lysosomes, reducing its inhibition of other cathepsins and thus its side effects. Although odanacatib was efficient in increasing bone mineral density and reducing hip or vertebrae fractures, its phase III trial was prematurely terminated on the grounds that it increased the likelihood of cardiovascular complications such as stroke in patients.[245] A CatK inhibitor that is currently in the phase II clinical trial stage for osteoporosis and osteoarthritis is MIV-711.[242, 245-248 242, 246-248]

Among other cathepsins, CatS has been found to play a unique role in mediating the immune response in dendritic and B cells. Hence inhibition of CatS can be useful for combatting hyperactivation of immune systems against host antigens in several auto-immune diseases such as rheumatoid arthritis, bronchial asthma etc.[249-251] Although no CatS specific covalent inhibitor has been approved by the US FDA, several compounds have been discovered and investigated in vitro and in vivo to inhibit CatS specifically, such as Balicatib; Odanacatib; JPM-OEt; and JPM-565.[242, 252-258]

E. Caspases

Caspases are enzymes belonging to the cysteine protease family, consisting of 12 family members, that play a critical role in apoptosis.[259, 260] Inhibition of caspase activity is potentially beneficial in degenerative disorders such as Alzheimer's disease (AD), Parkinson's, and Huntington's diseases.[261] A tetrapeptide containing the sequence Ile-Glu-Thr-Asp and an aldehyde warhead covalently bonded to Cys360 in the active site of caspase-8.[262] Acetic acid derivatives inhibiting caspase-3 with micromolar IC_{50} values have been identified. Caspase inhibitors are well known in the literature.[261, 263] However, a group of thiol-containing compounds have been recently identified to inhibit caspase activities.[264] Instead of targeting the active site cysteine residue, these compounds, known as "disulfide tethers", bind to an allosteric site[259, 260] and trapping the enzyme in an inactivated (zymogen) state. FICA and DICA, indole and dichlorophenoxy derivatives, respectively, are examples of "sulfur tethers" which binds at the dimeric interface of caspase-7 to form a disulfide linkage with Cys290.[265, 266] Two thiol containing thienopyrazoles were found to inactivate caspase-1[267], and caspase-5, respectively.[268]

IDN-6556 (emricasan), a pan caspase oxamyl dipeptide irreversible inhibitor was in phase II clinical trials for treatment of liver diseases.[269] Emricasan in combination with birinapant, a second mitochondria-derived activator of caspases (SMAC) mimetic has shown great therapeutic efficacy and safety in the treatment of AML.[270] A covalent inhibitor of caspase 8 containing an 2-oxoalkyl dichlorobenzoate moiety was designed to form a covalent adduct with Cys360.[271] A similar strategy has also been applied to design covalent inhibitors for caspase 3 and caspase 6. These inhibitors have a 2-oxoalkyl

tetrafluorophenyl ether moiety that reacts with a nucleophilic cysteine to release tetrafluorophenol and forming a covalent 2-oxoalkyl cysteinyl ether adduct.

F. Metallocarboxypeptidases (MCPs)

Metallocarboxypeptidases (MCPs) excise one amino acid at a time from the C-terminal ends of polypeptides and are important for digestion, as exemplified by bovine pancreatic carboxypeptidases A and B.[272, 273] MCPs are subdivided into the A/B subfamily (M14A according to MEROPS), the N/E subfamily (M14B), the γ-d-glutamyl-*meso*-diaminopimelate peptidase I (M14C), and the complex cytosolic carboxypeptidases, CCPs (M14D).[274, 275] These enzymes are expressed in all tissues and likely play a role in processes such as the maturation of neuropeptides, hormones, and cytokines, in blood fibrinolysis, and in anaphylaxis.[276] MCPs may also be involved in harmful processes such as fibrinolysis and inflammation, and may contribute to the pathology of Alzheimer's disease (AD) and cancer.

Their inhibition has been studied by Testero and co-workers, who tested an extensive series of thiirane and epoxide analogs of (2S, 3R)-2-benzyl-3,4-epoxybutanoic acid (BEBA), in which they modified the original BEBA phenyl ring to alkyl side chains or rings.[277] Mobashery and co-workers have developed thiirane- and epoxide-containing molecules such as (R)-ND-336 which have been shown to inhibit matrix metalloproteinase (MMP) through covalent interaction with nucleophilic residues in the active site.[278] Some recent studies have reported the application of epoxides to the inhibition of bacterial enzymes. Epoxycephalosporins reported by Lebedev inhibited class A β-lactamases; however, their study was inconclusive on the exact mechanism by which the inhibition took place.[279] Fosfomycin, which is the only phosphonate in the clinic, inhibits uridine diphosphate-N-acetylglucosamine enolpyruvyl transferase (MurA), an enzyme involved in peptidoglycan synthesis.[280] Nucleophilic attack of the active site cysteine residue of MurA, Cys115, on the fosfomycin epoxide ring inactivates MurA, disrupting cell wall synthesis and thus killing the bacterium. [281]

G. Glucocorticoid receptors

Glucocorticoid receptors are a type of nuclear receptor that are activated by the binding of cortisol (endogeneous glucocorticoid) resulting in shuttling from the cytoplasm to the nucleus and modulation of gene transcription (either activation or repression). Within the nucleus, activated glucocorticoid receptors bind to DNA via zinc fingers motifs in their DNA-binding domain.[282] Glucocorticoids are administered in autoimmune disorders as well as cancer.[283] Covalent selective glucocorticoid receptor agonists (SEGRA) of glucocorticoid receptors should only trigger repression of transcription and has applications in inflammatory diseases. Noncovalent SEGRA, GSK866, was modified by incorporating chloroacetamide or acrylamide warheads in a bid to obtain covalent agonists. The idea was to target cysteine residues in the vicinity of the ligand binding site, specifically Cys643. Covalent cysteine modification was confirmed by mass spectrometric analysis. While covalent SEGRAs are still in nascent stages, they show great potential in the treatment of inflammatory disorders.[284]

H. Estrogen related receptors (ERRs)

Estrogen related receptors (ERRs) are a subfamily of nuclear receptors consisting of three members ERRα, ERRβ and ERRγ. Named due to their similarity to another family of nuclear receptors, estrogen receptors, ERRs are constitutively active transcription factors. ERRα is known to modulate transcription of genes critical in lipid metabolism making it an attractive target in the treatment of diabetes.[285] Alkylidenyl diaryl ethers identified from high-throughput screening were subject to extensive SAR studies which led to the identification of an arylmethylenethiazolidinedione covalent inverse agonist of ERRα. The methylenethiazolidinedione moiety of the inverse agonist reacted with the sulfhydryl group of cysteine Cys325 in the binding pocket of ERRα. The other components of the agonist played a critical role by engaging in favorable noncovalent interactions with residues within the pocket. Pharmacokinetic studies indicated a very slowly reversible covalent interaction between inverse agonist and EERα with a half-life of ~18 h. Additionally, the identified covalent inverse agonist showed little to no off-target activity. In vivo studies indicated that the identified covalent inverse agonist improved glucose tolerance exhibiting potential as development of treatment for type 2 diabetes.[285]

10. Covalent Inhibitors of Protein−Protein Interactions

The design of effective inhibitors of protein−protein interactions (PPIs) for therapeutic use has been a notoriously arduous and challenging task, making PPIs a largely untapped target space for new therapeutics.[286] The molecular surface area involved in PPI is large enough to make inhibitor design difficult. However, covalent inhibitors have been explored to inhibit PPI. A recent example of a covalent PPI inhibitor is the (arylaminoalkyl)oxabicycloheptadienedicarboxylate COH000. COH000 reacts with a Cys residue located in an allosteric site of SUMO E1, stabilizing the enzyme in an inactive state.[287] Other examples of PPI inhibitors include oridonin, an α-methylenepoxykaurenone small molecule inhibitor of NLRP3/NEK7[288], and TED-347, a chloroketone small molecule inhibitor of TEAD/Yap[289] useful in the treatment of breast cancer and glioblastoma, respectively. Arylsulfonyl fluoride and aryl fluorosulfate warhead-containing small molecule covalent inhibitors of XIAP/BIR3[13], and covalent heterobivalent, inhibitors of the crosslinking of allergen-specific IgE, showed the ability of inducing ovarian cancer cell line SKOV3 apoptosis and prevent IgE-dependent responses to peanut allergen, respectively are other examples of PPI inhibitors.[290]

291-293294, 295

IV. FDA approved covalent inhibitors and CIs in clinical trials

Continued interest and research efforts has led to over 33 covalent inhibitors receiving regulatory approval as therapeutics (Supplementary table S1 and Figure 9A and 9B). While mostly dominated by α,β unsaturated carbonyls, other warheads such as boronic acids and ester, α-ketoamides, nitriles and butynamides have also been used in USFDA approved drugs (Figure 8A). Cysteine continues to be the target residue of choice, with only a handful of approved covalent inhibitors targeting serine, threonine and lysine residues (Figure 9A). A majority of the approved covalent therapeutics are utilized in the treatment of cancer (~66%) followed by autoimmune diseases (such as rheumatoid arthritis and multiple sclerosis) and antivirals (SARS

Cov-2 and hepatitis C) (Figure 9B). Approved covalent inhibitors are spread across a range of protein targets with the highest number for EGFR followed by BTK (Figure 9B). Notable among the approved covalent therapeutics is BTK inhibitor Ibrutinib (Imbruvica). Used to treat multiple types of myelomas as well as other indications, it was ranked in the top 20 drugs for global sales from 2019-2021 reaching $9.8 billion in global sales in 2021 which is a testament to the potential of covalent inhibitors as profitable drugs. [296, 297] Three companies have each launched 3 covalent inhibitor therapeutics in the past 20 years: Takeda, AstraZeneca and Pfizer. Two of Takeda's drugs, Bortezomib (Velcade)[298] and Ixazomib (Ninlaro)[299], use boronic acid warheads that act on proteosomes to treat mantle cell lymphoma and multiple myeloma, respectively. The third Takeda covalent inhibitor, Mobocertinib (Exkivity), uses an acrylamide warhead to treat non-small cell lung cancer (NSCLC) through action at EGFR.[300] Two of AstraZeneca's covalent inhibitors use α,β-unsaturated amides as warheads: Acalabrutinib (Calquence) has an alkyne that targets BTK and is used to treat various types of lymphomas[110], while Osimertinib (Tagrisso) uses an acrylamide warhead to interact with EGFR creating a viable therapeutic for NSCLC.[24] Meanwhile both of Pfizer's drugs, Tofactinib (Xeljanz) and Nirmatrelvir (Paxlovid) utilize nitriles as warheads. Tofactinib is a JAK inhibitor that is used primarily for rheumatoid arthritis[7] and Nirmatrelvir is an inhibitor of SARS-CoV-2 major protein used to treat COVID-19.[66, 301] Its worldwide sales were $19 billion in 2022.[302] The third Pfizer drug is Dalomitinib (Vizimpro) using a substituted acrylamide as a warhead to serve as an EGFR inhibitor in the treatment of NSCLC.[303]

Several covalent inhibitors are currently in clinical trials (Table 1 and Figures 9C and 9D). Close to half of the covalent inhibitors currently in development are in phase 3, 20% of them have been abandoned and the rest are evenly distributed across phase I and II (Figure 9C). An overwhelming majority of these utilize an acrylamide moiety as the warhead (Figure 9C) and almost all of them target cysteine residues. Similar to FDA approved covalent therapeutics, trends for covalent inhibitors currently in development indicate that cancer (~70%) continue to be the most targeted indication followed by autoimmune diseases (~15%) (Figure 9D). Out of the 18 covalent inhibitors currently in various stages of clinical trials, 50% and 25% target EGFR and BTK, respectively (Figure 9D). Other targets of covalent inhibitors in development include FGFR, JAK and CatK (Figure 9D). Three of those trials are for Novartis compounds. The first two, Remibrutinib and Narzartinib, use an acrylamide warhead to form a covalent bond with the target. Remibrutinib is currently in Phase III clinical trials for use in chronic uticaria through inhibiting BTK.[304] Nazartinib is currently in Phase II clincal trials as an EGFR inhibitor for the treatment of NSCLC.[305] The third Novartis compound, currently in stage I/II clinical studies, is Roblitinib which uses an aryl aldehyde to be a reversible covalent inhibitor of FGFR4 to serve as a treatment for hepatocellular carcinoma and solid tumors.[164, 306] Bristol-Myers Squibb also has multiple covalent inhibitors that act through BTK currently in the clinic. Branebrutinib has an alkyne amide as a warhead and is currently in Phase II trials as a treatment for rheumatoid arthritis.[307] Spebrutinib is currently in Phase I trials as a treatment for large B-cell lymphoma using an acrylamide as a warhead.[308]

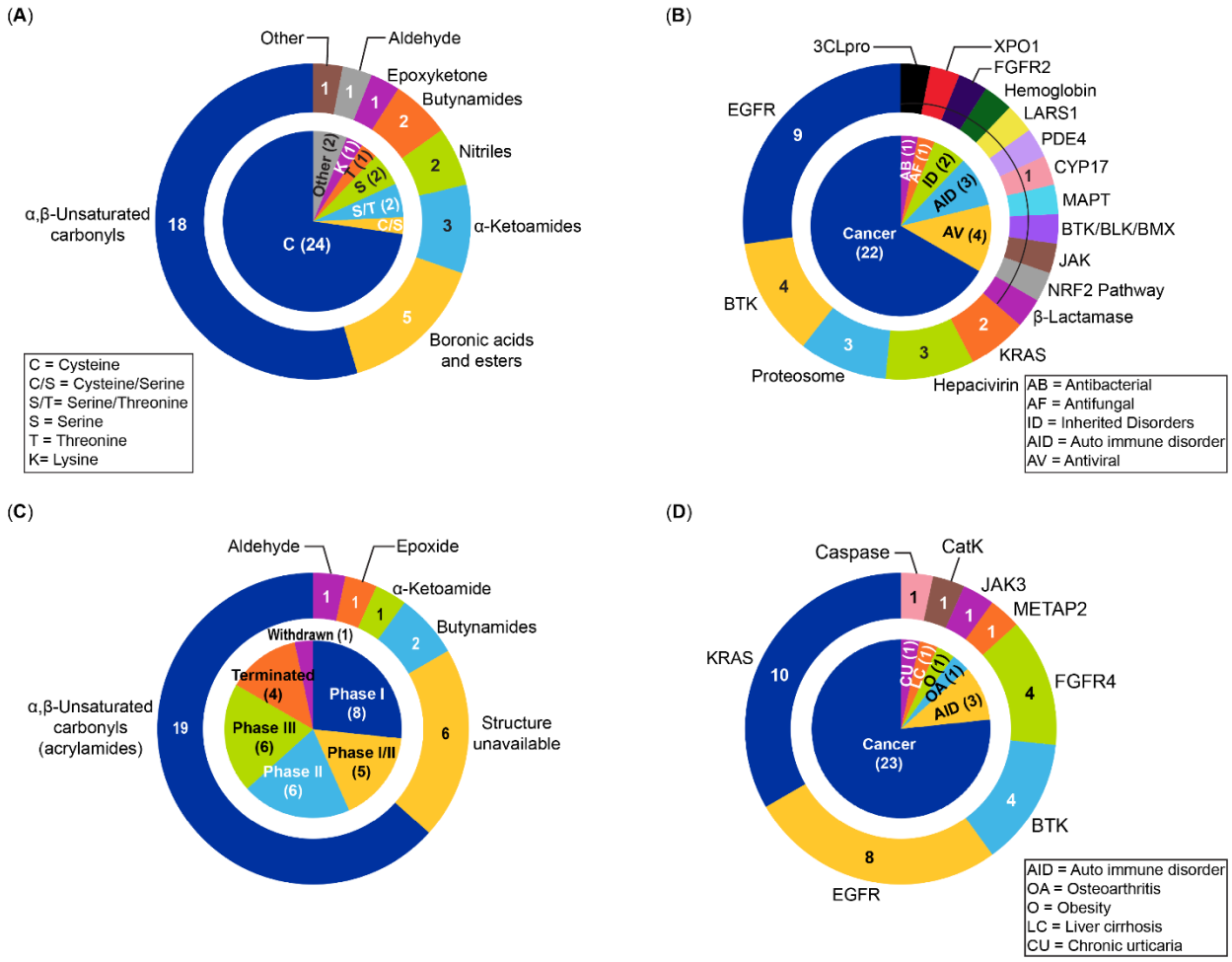

Figure 9. Distribution of covalent inhibitors with US FDA approval (**A** and **B**) and currently in clinical trials (**C** and **D**) across different parameters. (**A**) Warhead distribution for the >30 US FDA approved covalent inhibitors shown in the outer donut chart. Inner pie chart depicts distribution of targeted residue. Standard single letter codes used to represent amino acid residues – cysteine (C), serine (S), threonine (T) and lysine (K). The other here represents abiraterone's interaction with the He complex in CYP17. (**B**) Outer donut and inner pie charts indicating protein targets and indications of covalent inhibitors, respectively. (**C**) Warhead and clinical phase distribution of covalent inhibitors currently in development shown in the outer donut chart and inner pie chart, respectively. Terminated group consists of covalent inhibitors such as rociletinib, canertinib, mavelertinib and PRN1371 whose development were abandoned due to various reasons. (**D**) A majority of covalent inhibitors currently in clinical trials are being development for cancer treatment (~70%).

Table 1. List of covalent inhibitors currently in various stages of clinical trials

Name of covalent inhibitor	Clinical Phase	Protein target	Indication	Company	Targeted Residue	Warhead
Spebrutinib 1202757-89-8	I	Tyrosine kinase BTK	Large B-cell lymphoma	Bristol-Myers Squibb	Cys	Acrylamide
Branebrutinib 1912445-55-6	II		Atopic dermatitis, Rheumatiod arthritis	Bristol-Myers Squibb	Cys	Butynamide
Rilzabrutinib 1575591-66-0	III		Immune Thrombocytopenia	Sanofi	Cys	Acrylamide
Remibrutinib 1787294-07-8	III		Chronic urticaria	Novartis Pharmaceuticals	Cys	Acrylamide
Poziotinib 1092364-38-9	II	EGFR	NSCLC/ Breast cancer	Spectrum Pharmaceuticals	Cys	Acrylamide
Nazartinib 1508250-71-2	III (withdrawn)		NSCLC	Novartis Pharmaceuticals	Cys	Acrylamide
Abivertinib 1557267-42-1	III		NSCLC	Hangzhou ACEA Pharmaceutical Research Co.	Cys	Acrylamide
Furmonertinib (Alflutinib) 1869057-83-9	III		NSCLC	Allist Pharmaceuticals	Cys	Acrylamide
Olafertinib 1660963-42-7	I/II, III (China)		NSCLC	NeuPharma, Checkpoint Therapeutics	Likely Cys	Structure unavailable
Rociletinib 1374640-70-6	Terminated 2016		NSCLC	Clovis Pharmaceuticals	Cys	Acrylamide
Canertinib 267243-28-7	Terminated 2015		NSCLC	Pfizer	Cys	Acrylamide
Mavelertinib 1776112-90-3	Terminated 2020		NSCLC	Pfizer	Cys	Acrylamide
H3B-6527 1702259-66-2	I	FGFR	Advanced Hepatocellular Carcinoma	H3 Biomedicine Inc.	Cys	Acrylamide

Fisogatinib 1707289-21-1	Ib/II		Hepatocellular Carcinoma (HCC)	CStone Pharmaceuticals	Cys	Acrylamide
Roblitinib 1708971-55-4	II		HCC, solid malignancies	Novartis Pharmaceuticals	Cys	Aldehyde
PRN1371 1802929-43-6	I; Terminated 2020	FGFR1-4	Metastatic Urothelial Carcinoma	Principia Biopharma, a Sanofi Company	Cys	Acrylamide
Ritlecitinib 1792180-81-4	III	JAK3	Alopecia areata	Pfizer	Cys	Acrylamide
MIV-711 2419130-28-0	II	CatK	Osteoarthritis	Medivir	Cys	Structure unavailable
JNJ 74699157 2586053-43-0	I	c-Ki-ras protein	NSCLC	Janssen	Cys	Structure unavailable
LY 3499446 2676847-24-6	I/II		Advanced solid tumors	Eli Lilly	Cys	Acrylamide
LY 3537982 2738368-69-7	Ia/Ib		Advanced solid tumors	Eli Lilly	Cys	Acrylamide
Divarasib 2417987-45-0	I		Advanced solid tumors	Genentech	Cys	Acrylamide
Garsorasib 2559761-14-5	I/II		NSCLC	InventisBio	Cys	Acrylamide
Opnurasib 2653994-08-0	I/II		Advanced solid tumors	Novartis	Cys	Acrylamide
BI 1823911 2756575-54-7	I		Advanced solid tumors	Boehringer Ingelheim	Cys	Structure unavailable
JAB-21822 2775298-75-2	I/II		Advanced solid tumors	Jacobio Pharmaceuticals Group	Cys	Structure unavailable
MK 1084 2896711-14-9	I		Advanced solid tumors	Merck Sharp & Dohme LLC	Cys	Structure unavailable
RMC 6291 2775304-30-6	I/Ib		Advanced solid tumors	Revolution Medicines	Cys	Butynamide?
Emricasan 254750-02-2	II	Caspase	Liver cirrhosis	Conatus Pharmaceuticals Inc.	Cys/Ser	α-Ketoamide
Beloranib 251111-30-5	II	Methionine aminopeptidase 2	Obesity	Zafgen, Inc.	His	Epoxide

V. Perspective

Covalent inhibitors have received significant research and commercial interest in the last ten years. The ability to irreversibly inhibit an enzyme or to reversibly inhibit it for a prolonged period has allowed biologists to understand the function of proteins and has more recently allowed medicinal chemists and doctors to treat diseases previously recalcitrant to treatment. Using the CAS Content Collection, we investigated research about covalent inhibitor drugs, including journal and patent publication frequencies, the types of warheads used, their biological targets, and the diseases they are intended to treat. The interest in covalent inhibitors has increased significantly, with both journal and patent publications per year increasing linearly over time. Larger US academic institutions such as the University of California system are significant contributors to journal publication, while pharmaceutical companies such as Pfizer publish the largest numbers of patents each year. Academic research tendencies in covalent inhibitors may arise from the origins of covalent inhibitor research [for example, the initial development of covalent K-RAS inhibitors from the Shokat group[309] and thus the institutional experience and their subsequent success. Commercial research may be driven by the increased understanding of the chemical and biological behavior of covalent inhibitor; the need for comprehensive understanding of the behavior of covalent inhibitors by both developers and regulators may require significant technical knowledge and infrastructure to obtain.

The most common warheads identified from documents were α,β-unsaturated carbonyl compounds, particularly amides, which are primarily selective for the thiol moieties of cysteine residues in proteins. Aldehydes, sulfonamides and unsaturated sulfones, α-haloacetamides, and nitriles are less common but have received significant interest from scientists, with aldehydes and nitriles being incorporated into US FDA-approved medicines. α,β-Unsaturated amides are less reactive than earlier warheads used for antitumor agents such as β-haloamines but reactive enough to form stable adducts with thiols and to form them selectively. The prevalence of amines in drug intermediates and the availability of amide-forming reactions allows α,β-unsaturated amides to be placed where needed – they can be treated as an inhibitor module in drug design. The selectivity for cysteine residues lowers the scope of covalent drug design but likely reduces the number of potential reactive moieties as well, and the knowledge of their behavior may reduce risk in drug development.

While covalent inhibitors have been used (unintentionally) to inhibit many enzymes, recent covalent inhibitors have primarily targeted kinases. Recent covalent inhibitors have been tried or used as treatments for cancers; some have also been used to treat autoimmune diseases or rheumatoid arthritis. The frequency of cancers, their consequences (severe debilitation and death) and the plethora of cancer types (many of which cannot be addressed by current treatments) provide medical and financial reasons to develop treatments for cancer. If previous treatment modalities have been unsuccessful, then cancers are reasonable indications against which to try novel treatment methods. The severity of many cancers also means that greater side effects may be tolerated in successful cancer drugs than in other drugs that either have less severe symptoms or are chronic and thus require treatment for longer periods of time (although, as noted, covalent drugs may have fewer side effects than the corresponding noncovalent drugs).[17]

What developments are important for covalent inhibitors to expand in importance? While covalent inhibitors reacting with residues other than cysteine exist and have been commercialized, few covalent inhibitors are intended to react with lysine, aspartate, glutamate, serine, threonine, and

histidine residues. The relative paucity of cysteine residues limits the targets for covalent warheads because cysteines may not be present in a target or may not be effectively positioned to inhibit the protein. Having covalent inhibitors capable of binding to other residues would increase the number of proteins and thus the number of diseases addressable with covalent inhibitors. Vinyl sulfones and vinyl sulfonamides, for example, may be able to react with both cysteine and lysine residues.[7] However, covalent warheads that react with common amino acid side chains may be difficult to incorporate into selective covalent inhibitors; multiple reactive residues may be present in different positions on a protein, requiring other interactions with the target protein to determine selectivity. Data on the reactivity and selectivity will likely be necessary as well. In addition, a broader pool of data on the pharmacokinetics and toxicology of covalent inhibitors would be helpful for both the most common warheads such as acrylamides and aldehydes and newer or less-commonly used warheads. Knowledge of the benefits and liabilities of covalent-binding fragments is necessary to expand the scope of indications beyond cancer, particularly for chronic diseases which require long-term administration of drugs.

We hope that this article provides evidence that covalent inhibitors are an increasingly important tool in drug development and that intentional covalency is an important tool not just to understand biological systems, but also to design and create new drugs and to treat diseases previously untreatable.

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