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MEDICINAL CHEMISTRY RESEARCH

REVIEW ARTICLE

An update on the discovery and development of reversible covalent inhibitors

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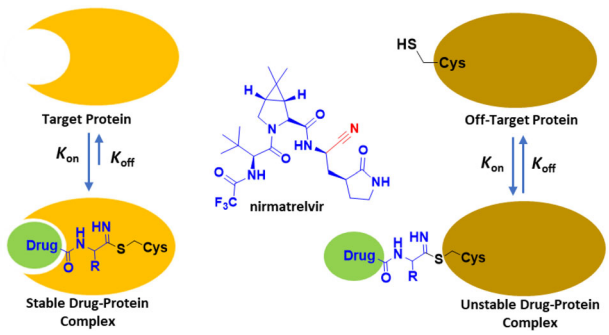
Abstract

Small molecule drugs that covalently bind irreversibly to their target proteins have several advantages over conventional reversible inhibitors. They include increased duration of action, less-frequent drug dosing, reduced pharmacokinetic sensitivity, and the potential to target intractable shallow binding sites. Despite these advantages, the key challenges of irreversible covalent drugs are their potential for off-target toxicities and immunogenicity risks. Incorporating reversibility into covalent drugs would lead to less off-target toxicity by forming reversible adducts with off-target proteins and thus reducing the risk of idiosyncratic toxicities caused by the permanent modification of proteins, which leads to higher levels of potential haptens. Herein, we systematically review electrophilic warheads employed during the development of reversible covalent drugs. We hope the structural insights of electrophilic warheads would provide helpful information to medicinal chemists and aid in designing covalent drugs with better on-target selectivity and improved safety.

Graphical Abstract



Keywords Reversible covalent drugs · Nitriles · α-cyanoacrylamide · Aldehydes · Boronic acids · Ketones

Abbreviations

COX:	Cyclooxygenase
COVID-19:	Coronavirus disease of 2019
SARS-CoV-2:	Severe acute respiratory syndrome-corona virus—2
PDB:	Protein data bank
BTK:	Bruton's tyrosine kinase
EGFR:	Epidermal growth factor receptor
JAK3:	Janus kinase 3
TYK2:	Tyrosine kinase 2
RSK2:	Serine/threonine kinase 2
EV71:	Enterovirus 71
CVA16:	Coxsackievirus A16
CVB3:	Coxsackievirus B3
CPB:	Cysteine protease B

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TgCPL:	Toxoplasma gondii cathepsin protease L
(MALDI)	Matrix assisted laser desorption ionization
TOF MS:	mass spectrometry
FGFR4:	Fibroblast growth factor receptor 4
TR-FRET:	Time-resolved fluorescence resonance energy transfer
SPR:	Surface plasmon resonance
NADPH:	Nicotinamide adenine dinucleotide phosphate hydrogen
ATP:	Adenosine tri-phosphate
ERB-BR:	Epidermal growth factor receptor
BLK:	B-Lymphoid tyrosine kinase
Cbz:	Carbobenzoxy
RSK2:	P90 ribosomal S6 kinase 2
DPP-4:	Dipeptidyl peptidase-4
IP:	Intraperitoneal
BCR-ABL:	Breakpoint cluster region- Abelson

Introduction

The history of covalent drugs spans more than one hundred years. The covalent inhibition mechanism of early covalent drugs, such as acetylsalicylic acid (aspirin), was not elucidated until much later [1]. It turns out that aspirin covalently modifies the cyclooxygenase (COX) enzyme by acetylation of Ser530 near its active site. This covalent modification blocks proper binding of the native substrate and leads to irreversible inhibition. Before 2000, it was generally perceived that covalent warheads such as acrylamides would lead to non-specific irreversible inhibition, resulting in idiosyncratic toxicities. However, covalent drugs have garnered much attention in drug discovery in the last two decades and the approach has now become a common modality for protein target inhibition.

Currently more than 40 covalent drugs are in the market and a myriad of them are in clinical trials. Unlike small molecule non-covalent drugs, covalent drugs bind reversibly or irreversibly via a covalent bond to the target protein, which results in slow off rates and sustained inhibition [2, 3]. This tight binding affinity (slow K_{off}) of an irreversible covalent drug for its target protein allows for targeting sites with low ligand ability (e.g., protein with shallow, or highly charged binding sites). Irreversible covalent inhibitors exhibit prolonged duration of action as compared to non-covalent drugs, which could lead to lower and less frequent dosing. Exquisite selectivity can be achieved within a target protein family if a poorly conserved cysteine is specifically targeted with a covalent warhead from a drug [2, 4]. Despite of all these advantages, one of the key challenges of irreversible covalent drugs is the potential for immunogenicity or toxicity [5]. Overly reactive electrophilic warheads can react non-specifically with nucleophilic residues on other proteins, which could result in off-target toxicities and allergies. Furthermore, irreversible covalent inhibitors tend to undergo extrahepatic clearance more readily than non-covalent inhibitors, which can result in lower bioavailability [6].

Compounds containing an electrophilic warhead which reversibly binds to the target protein may offer a solution to the aforementioned challenges of irreversible covalent inhibitors [7]. Reversible covalent inhibitors are not permanently bound and can be released from off-target proteins, thereby reducing the chances of undesirable activation of the immune system and off-target toxicity. A number of reversible covalent inhibitors are marketed drugs (Fig. 1). Saxagliptin (**1**) is a reversible covalent drug containing a nitrile group as an electrophile warhead which target Ser630 residue of dipeptidyl peptidase-4 (DPP-4) [8]. It was approved by the FDA in 2009 for the treatment of diabetes mellitus (Type 2). Telaprevir (**2**) and boceprevir (**3**) are α-ketoamide-based reversible covalent drugs (both approved by FDA in 2011) which react with the catalytic serine residue of hepatitis C NS3 serine protease [9]. Narlaprevir (**4**) is a potent second-generation inhibitor of hepatitis C virus (HCV) NS3 protease with a Ki of 7 nM [10]. It is α-ketoamide-based reversible covalent drug developed by R-Pharm and was approved for the treatment of hepatitis C in Russia in 2016. The first-in-class 26S proteasome inhibitor, bortezomib (**5**) contains a boronic acid moiety as the electrophilic warhead which targets the N-terminal threonine of 26S proteasome. It was approved by the FDA in 2003 for the treatment of multiple melanoma [11].

Another 26S proteasome inhibitor ixazomib (**6**) is an orally bioavailable, reversible covalent inhibitor binding to the β5 subunit of the 20S proteasome. It was approved by the FDA in 2015 for use in combination with dexamethasone and lenalidomide to treat patients with multiple myeloma [12]. Voxelotor (**7**) was approved by the FDA in 2019 to treat sickle cell disease. Its aldehyde warhead reversibly binds to the N-terminal valine of hemoglobin via imine formation [13]. Nirmatrelvir (**8**) is also a reversible covalent inhibitor with a nitrile warhead targeting a cysteine in the main protease (M^{pro}) of SARS-CoV-2. It was approved by the FDA in 2022 for the treatment of COVID-19 [14].

The successful application of reversible covalent bond formation in drug discovery inspired medicinal chemists to expand this research area to find new electrophilic warheads which reversibly bind to cysteine and non-cysteine amino acid residues. One of the goals in the development of reversible covalent inhibitors is the extension of the residence time of the covalent inhibitor with the target protein to improve the potency. For this purpose, the medicinal chemists are tuning the reactivity of the reversible covalent warhead and making structural modifications around the

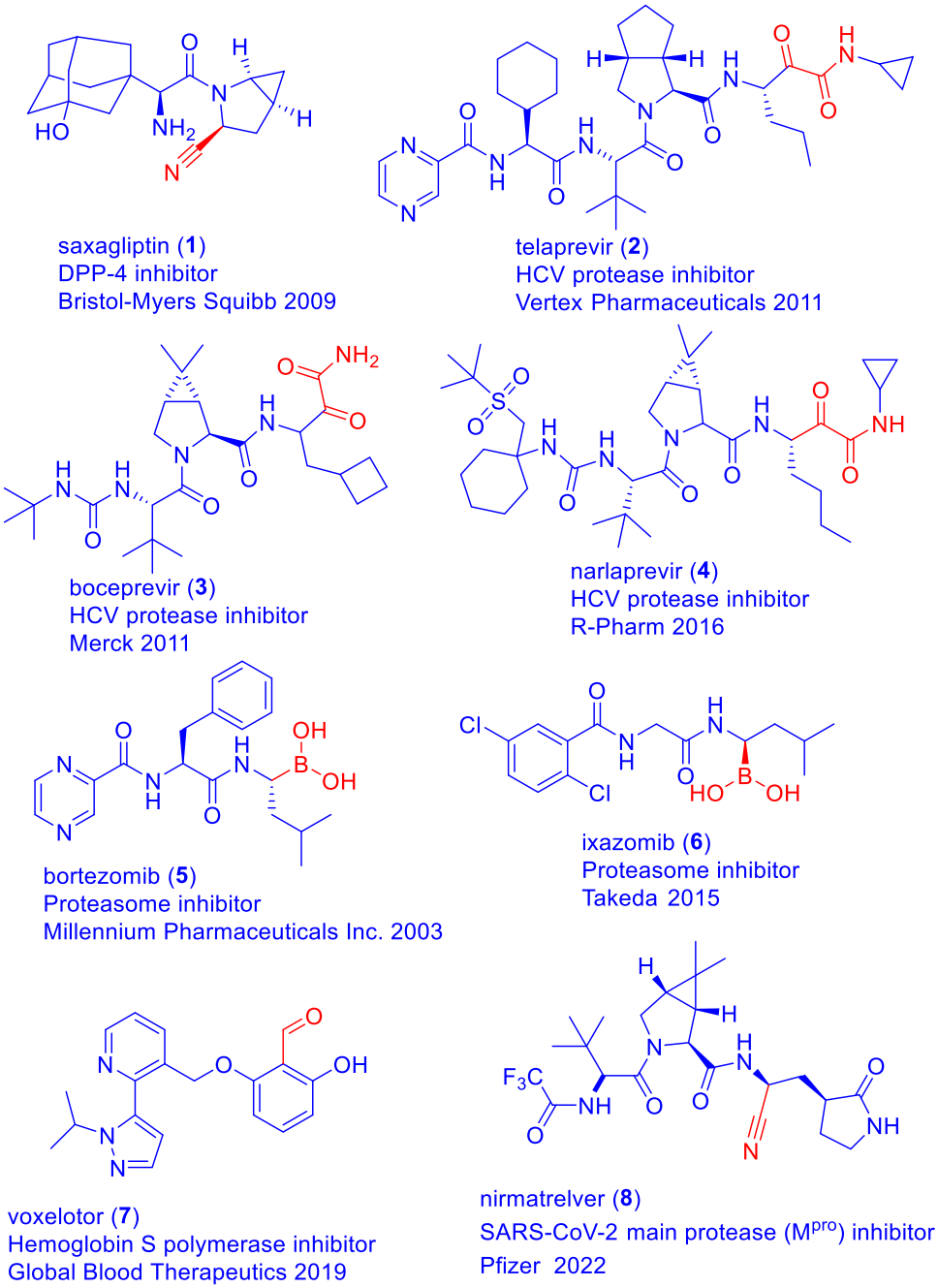

Fig. 1 Examples of reversible covalent drugs on the market

electrophilic warhead to stabilize the reversible covalent adduct in order to extend its half-life. Herein we will discuss the recent progress made in the search of reversible covalent inhibitors.

α-Cyanoacrylamide

Incorporation of an electron-withdrawing nitrile group at the α-position of acrylamide greatly accelerates the thia-Michael addition and the acidic proton at the α-position facilitates a retro-Michael reaction. This retro-Michael reaction will decrease the on-target residence time of reversible covalent inhibitors in comparison to irreversible covalent inhibitors. The proposed reaction mechanism of nucleophilic Cys481 of Bruton's tyrosine kinase (BTK) reacting with the electrophilic α-cyanoacrylamide of compound **9** (PDB ID: 4YHF) [15] is shown in Fig. 2.

Bradshaw et al. tuned the residence time of the α-cyanoacrylamide-based reversible covalent inhibitors (**9, 10**) of BTK by modifying the size of the β-capping substituent which resulted in sustained BTK occupancy in vivo after

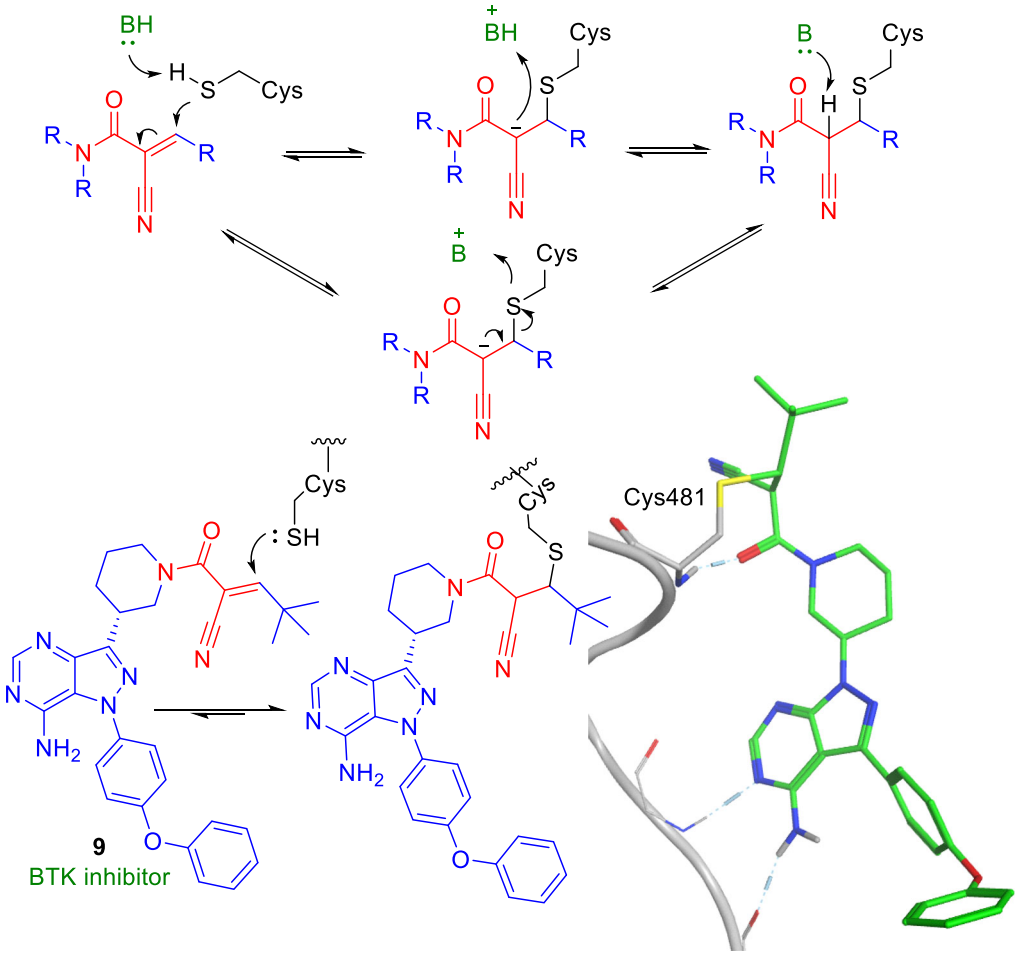

Fig. 2 Proposed reaction mechanisms of cysteine with α-cyanoacrylamide

oral administration in rats (Fig. 3) [15]. The target occupancy for compound **9** with bulky β-capping substituent is 50% at 20 h while the target occupancy for compound **10** is just 5% at 20 h. Rilzabrutinib (PRN1008, **11**) and PRN473 (**12**) are orally bioavailable, reversible covalent inhibitors of BTK [16]. Rilzabrutinib (**11**) is in phase 3 clinical trial for treating pemphigus vulgaris and immune thrombocytopenia. The Phase1–2 clinical trials data of rilzabrutinib (**11**) revealed that it is active and associated with only low-level toxic effects at all dose levels. The dose of 400 mg twice daily was identified as a safe dose for further testing in Phase 3. Overall, rilzabrutinib (**11**) showed a rapid and durable clinical efficacy that improved with length of treatment in patients with immune thrombocytopenia, who had received multiple therapies previously [17, 18]. PRN473 (**12**) with doses ranging from 8 to 21 mg/kg was investigated in a clinical study of canine pemphigus foliaceous. Initially all nine dogs had positive clinical response to treatment, resulting in reduction of lesions by the end of week 2 of treatment. At week 4, four dogs continued to improve while three dogs sustained near complete remission by the end of study. PRN473 (**12**) is well absorbed in dogs in both toxicology and canine clinical studies, but it is poorly absorbed orally in humans [17]. PRN473 (**12**) has completed a Phase 1 clinical trial as a topical agent. Smith et al. reported a series of α-cyanoacrylamide-based reversible covalent inhibitors of epidermal growth factor receptor (EGFR) [19]. They identified compound **13**, which inhibited $EGFR^{L858R}$ and $EGFR^{L858R/T790M}$ with IC_{50} of 10 and 20 nM, respectively and was shown to be over 5-fold more specific for mutant over wild-type EGFR ($IC_{50} = 96$ nM). Thus, inhibitor **13** provides a promising starting point for developing a more selective mutant EGFR inhibitor. London et al. conducted covalent docking of large chemical libraries and discovered a potent Janus tyrosine kinase 3 (JAK3) reversible covalent inhibitor **14** which targets Cys909 ($IC_{50} = 49$ nM) [20]. Besides JAK3, compound **14** also potently binds to multiple off-targets, such as ERB-BR ($IC_{50} = 44$ nM) and B-lymphoid tyrosine kinase (BLK) ($IC_{50} = 22$ nM).

Forster and his coworkers [21] reported two selective reversible covalent inhibitors **15** and **16** of JAK3 (Fig. 4A)

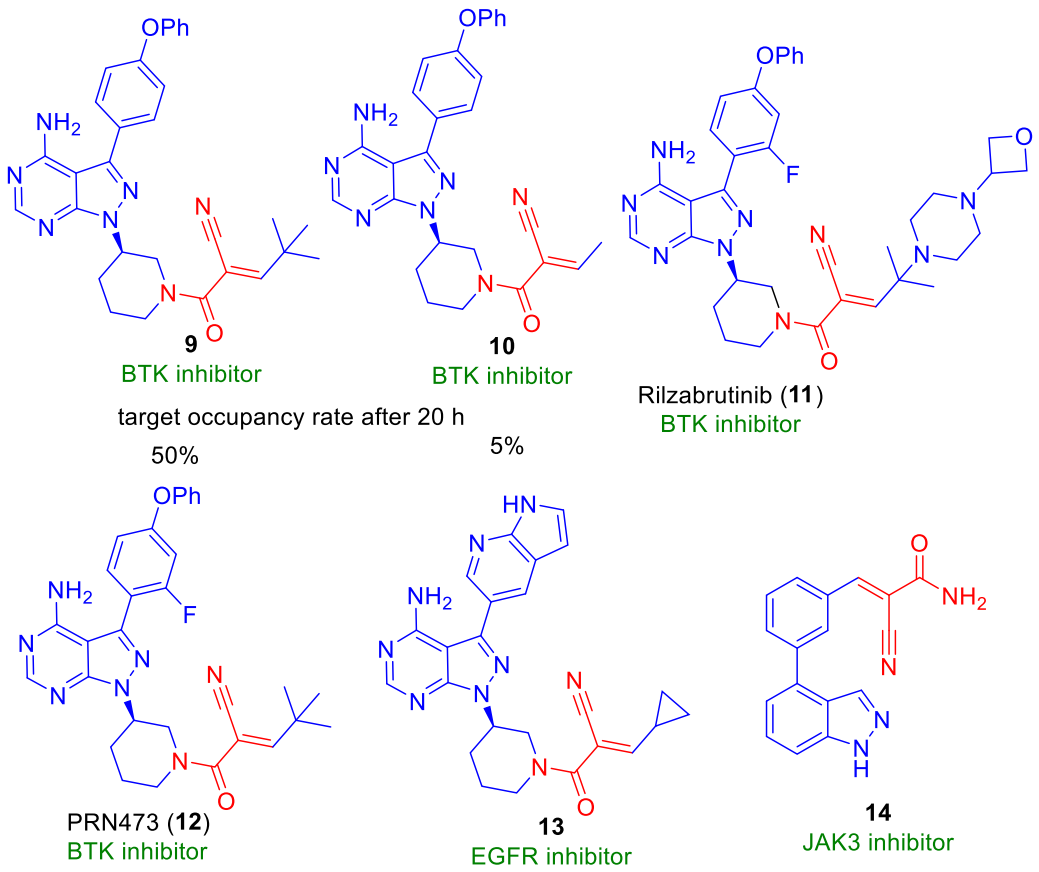

Fig. 3 α-Cyanoacrylamide-based reversible covalent inhibitors

with IC50 of 127 pM and 154 pM, respectively. Compound **15** showed high selectivity of 409-, 2724- and 3614-fold over JAK1, JAK2, and tyrosine kinase 2 (TYK2) respectively, while **16** showed selectivity over JAK1 (416-fold), JAK2 (1753-fold), and TYK2 (5831-fold). They determined the complex crystal structure of **16** with JAK3 and found that the α-cyanoacrylamide electrophilic warhead formed a reversible covalent bond with Cys909. The crystal complex of **16** and the target protein displayed the coexistence of both covalent and the non-covalent binding modes (Fig. 4B). The presence of both binding modes of **16** with JAK3 support the reversible character of the covalent interaction. Serafimova's group [22] working on development of reversible covalent chemical probes also disclosed reversible covalent inhibitor **17** against RSK2. The co-crystal structure of **17** showed that Cys436 on the β2 sheet of RSK2 formed a covalent C–S bond with α-cyanoacrylate warhead of **17** (Fig. 4D). The reversible nature of **17** was confirmed by proteolysis experiments.

Using the electrophilic fragment-based screening strategy, Miller et al. [23] discovered **18** and **19** with strong binding affinity for RSK2 ($IC_{50} = 12$ nM and 15 nM, respectively). The co-crystal structure of **19** with RSK2 shows that covalent bond is formed between the electrophilic α-cyanoacrylamide and Cys436 of T493M RSK2 (Fig. 5B). Targeting focal adhesion kinase (FAK) is a potential approach for the treatment of human malignant glioblastoma. Recently, Li and colleagues [24] reported compounds **20** and **21** (Fig. 5C) as reversible covalent inhibitors of FAK with good binding affinity with IC_{50}(s) ranging from 1.0 to 2.5 nM. Another research group [25, 26] reported cyanoacrylate and Boc-protected cyanoarylamide derivatives **22** and **23** as reversible covalent inhibitors of 3CL protease showing effective antiviral activity against EV71. These two compounds showed broad antiviral effects, acting on 293T, RD and Vero cell lines. Moreover, both **22** and **23** showed remarkable antiviral activities against EV71 A, B, C, CVA16, and CVB3 viral strains.

Nitriles

The human cathepsin K inhibitors, odanacatib (**24**) [27] and balicatib (**25**) [28] containing a nitrile warhead, have advanced into phase III and phase II clinical trials, respectively. In the development of reversible covalent drugs, the proliferation of nitriles as an electrophilic warhead is generally due to their

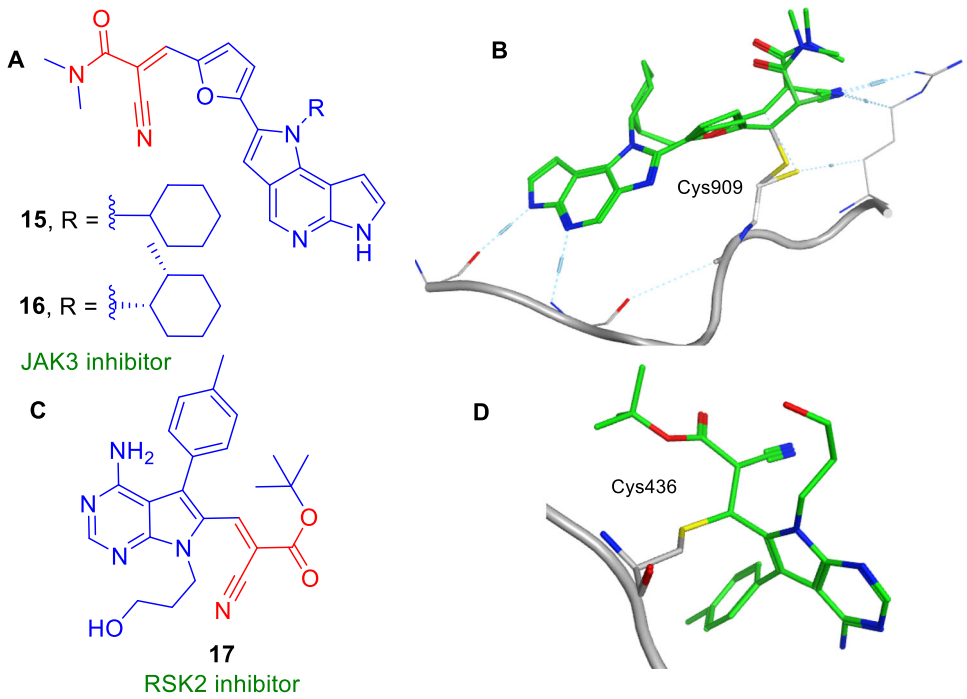

Fig. 4 **A** Reversible covalent inhibitors of JAK3; **B** co-crystal structure of **16** with JAK3 (PDB ID: 5LWN); **C** Reversible covalent inhibitor of RSK2; D. Crystal structure of **17** with RSK2 (PDB ID: 4D9U)

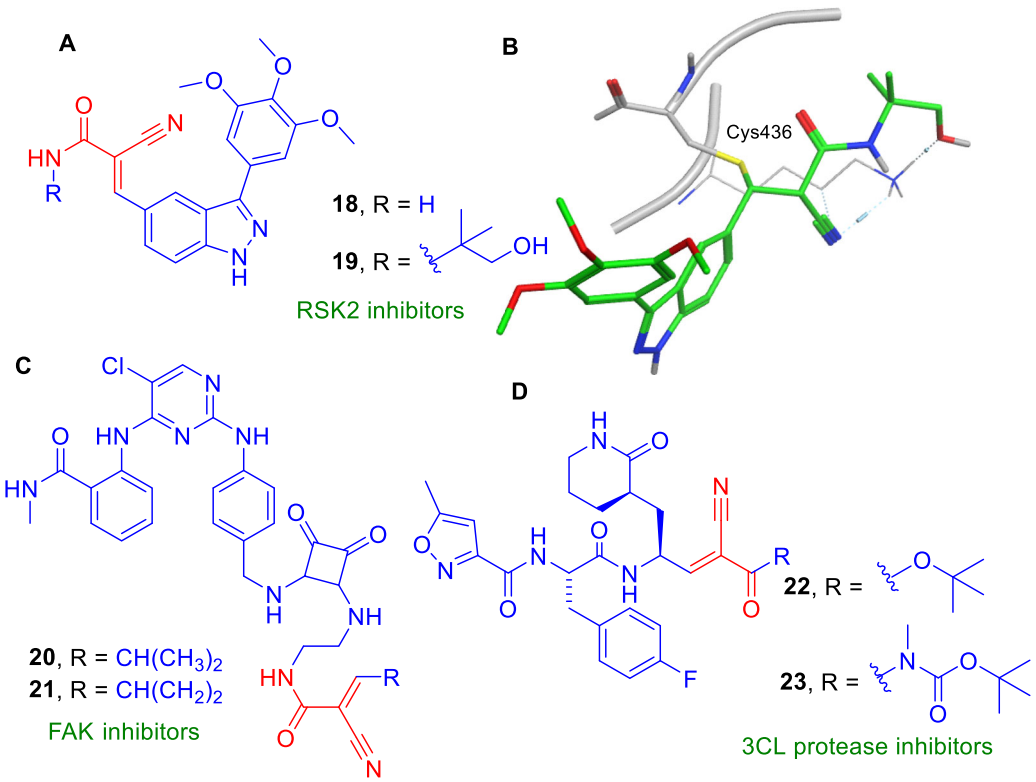

Fig. 5 **A** Reversible covalent inhibitors of RSK2; **B** Crystal structure of **19** with RSK2 (PDB ID: 4JG8); **C** Reversible covalent inhibitors of FAK; **D** Reversible covalent inhibitors (**22, 23**) of 3C protease

lower electrophilicity relative to other more reactive groups, reducing the possibility of unwanted reactions with off-target proteins that would hinder the development of safe drugs. The proposed reaction mechanism [29] of a nitrile-based inhibitor such as nirmatrelvir (**8**) (PDB ID: 7TLL) [14] reacting with SARS-CoV-2 M^{pro} Omicron P132H is shown in Fig. 6. The

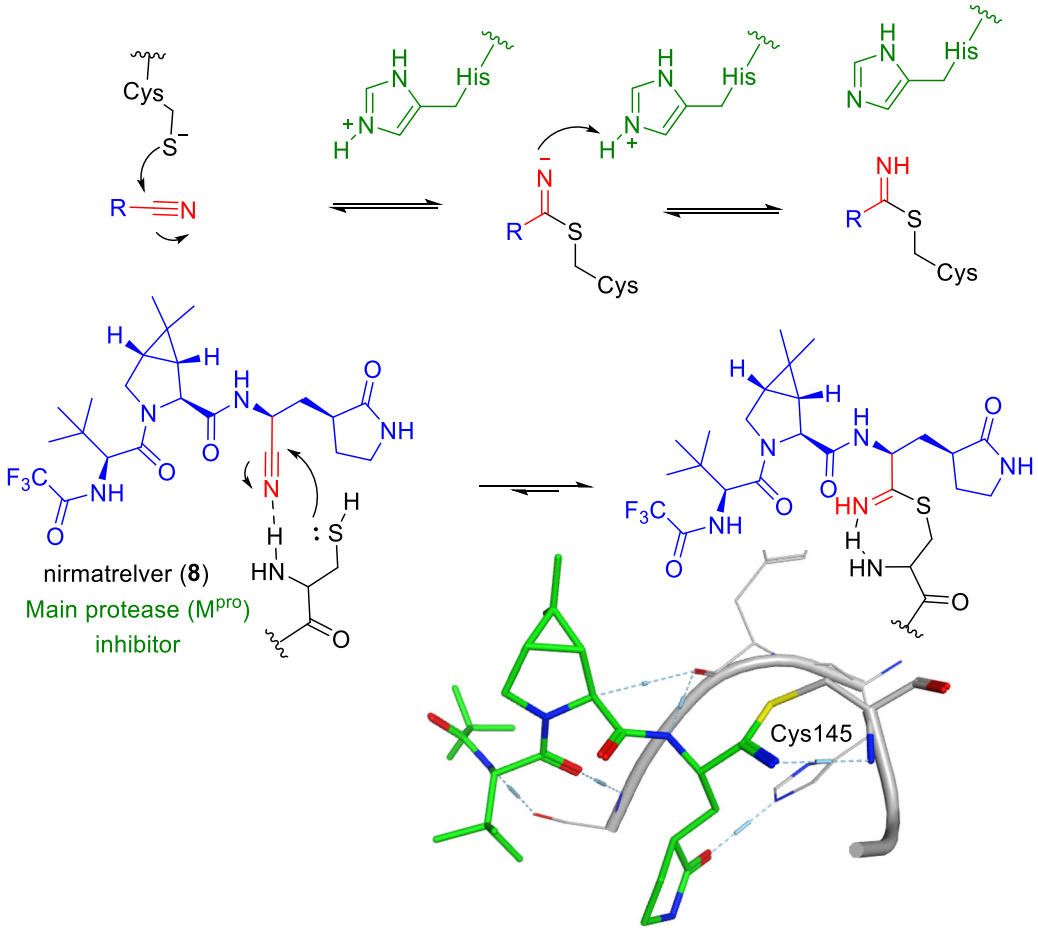

Fig. 6 Proposed reaction mechanisms of cysteine reacting with nitrile-based inhibitors

nitrile moiety of nirmatrelvir (**8**) acts as the electrophilic warhead targeting Cys145 in the main protease (M^{pro}) of SARS-CoV-2.

Herein we further highlight the potential use of nitriles in the discovery of reversible covalent inhibitors. Cianni et al. reported two odanacatib (**24**) derivatives Neq0659 (**26**) and Neq0820 (**27**) [30], as potent reversible covalent inhibitors of cruzain (Cz). Cz is the major cysteine protease expressed in the *Trypanosoma cruzi* parasite, which is the etiological agent causing Chagas disease. Furthermore, they designed and synthesized a different series of nitrile-based reversible covalent inhibitors of Cz represented by **28** ($K_i = 6.8$ nM) with nano-molar range binding affinity (Fig. 7) [31]. In another example, Cortez et al. [32] discovered a bicalutamide derivative (**29**) as a reversible covalent androgen receptor (AR) antagonist, via introduction of a nitrogen atom into the 4-cyano-benzene ring of bicalutamide to activate the nitrile group. The resulting activation allowed the formation of a reversible covalent bond with the proximal conserved Cys784 of AR. Interestingly, 5N-bicalutamide (**29**) ($K_i = 0.15$ nM) binds to AR with ~150-fold greater affinity than the parent compound bicalutamide ($K_i = 22.3$ nM). Benson et al. [33] at Pfizer also reported the use of an azanitrile warhead in their reversible covalent inhibitor PF-303 (**30**) (Fig. 7), which was employed as a chemical probe to investigate the phenotype of BTK inhibition in mice. PF-303 (**30**) is a highly potent ($IC_{50} = 0.64$ nM) and orally bioavailable inhibitor of BTK.

In 2021, Bridenbach and colleagues [34] reported an azanitrile-based reversible covalent inhibitor **31** for M^{pro} of SARS-CoV-2. Compound **31** was shown to covalently bind ($K_i = 24$ nM) to the catalytic cysteine of M^{pro}. Meanwhile, Bai et al. [35] also discovered a series of inhibitors against the 3CL protease of SARS-CoV-2. The typical example **32** ($IC_{50} = 24$ nM) is shown in Fig. 7. These inhibitors also covalently bind to the M^{pro} catalytic cysteine. Cysteine protease B (CPB) is an attractive biological target for therapeutic intervention in the treatment of leishmaniasis. CPB can also be inhibited by reversible covalent inhibitors. Matos and co-workers [36] designed a series of dipeptidyl nitrile derivatives against CPB. They discovered that the most potent CPB inhibitor **33** ($pK_i = 6.82$) was also selective for human cathepsin B ($pK_i < 5$). Nitrile-based inhibitors like compound **34** (Fig. 8) showed promising activity against CPB of

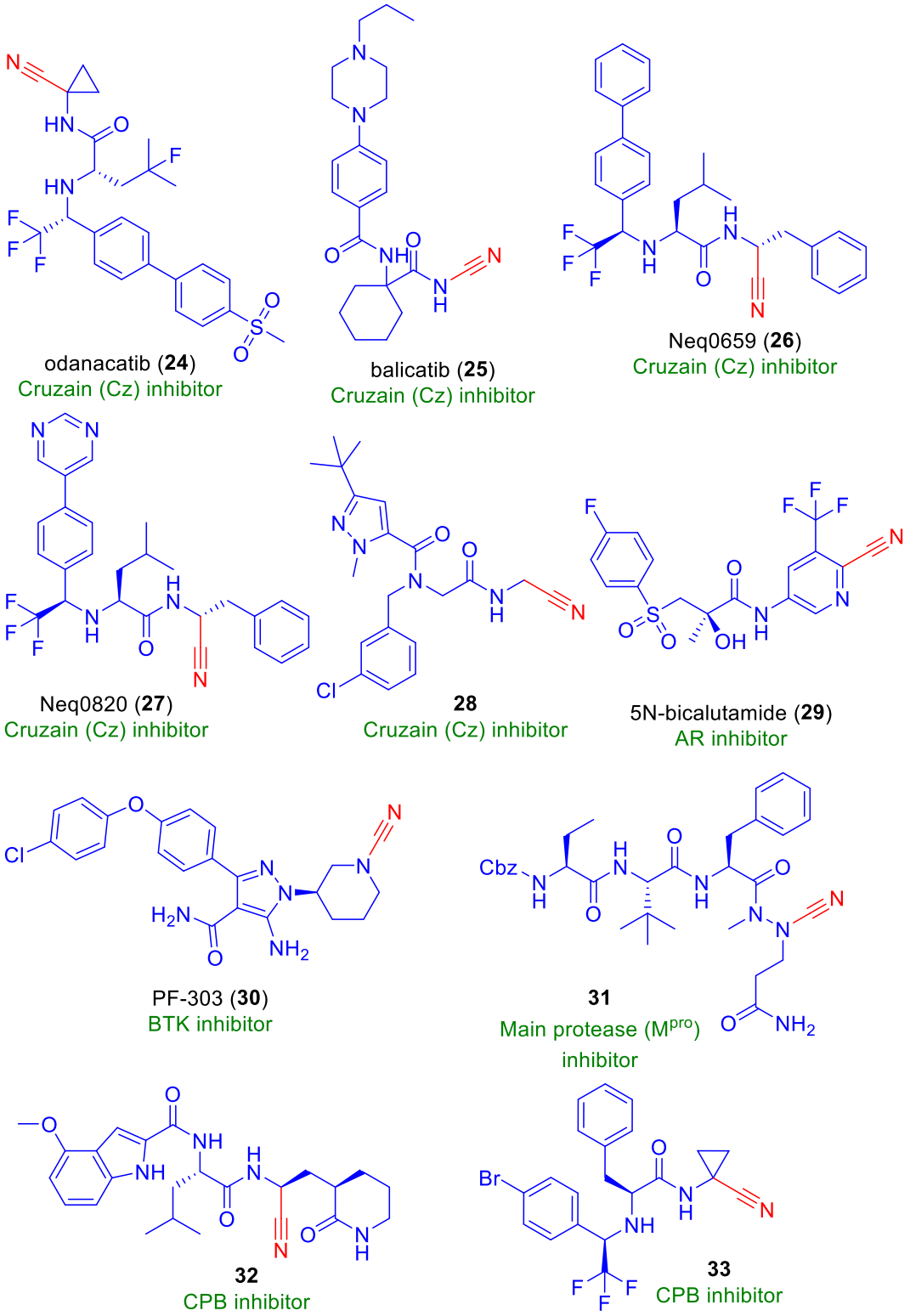

Fig. 7 Nitrile-based reversible covalent inhibitors

L. mexicana. Further optimization of **34** resulted in compound **35** is shown in Fig. 8 [37]. The electrophilic nitrile reacts with the catalytic residue Cys26 at the S1 subsite and forms a covalent adduct. Another group in the same year reported **36** [38] with almost similar structure of **34** (Fig. 8) showing similar inhibition of LmCPB.

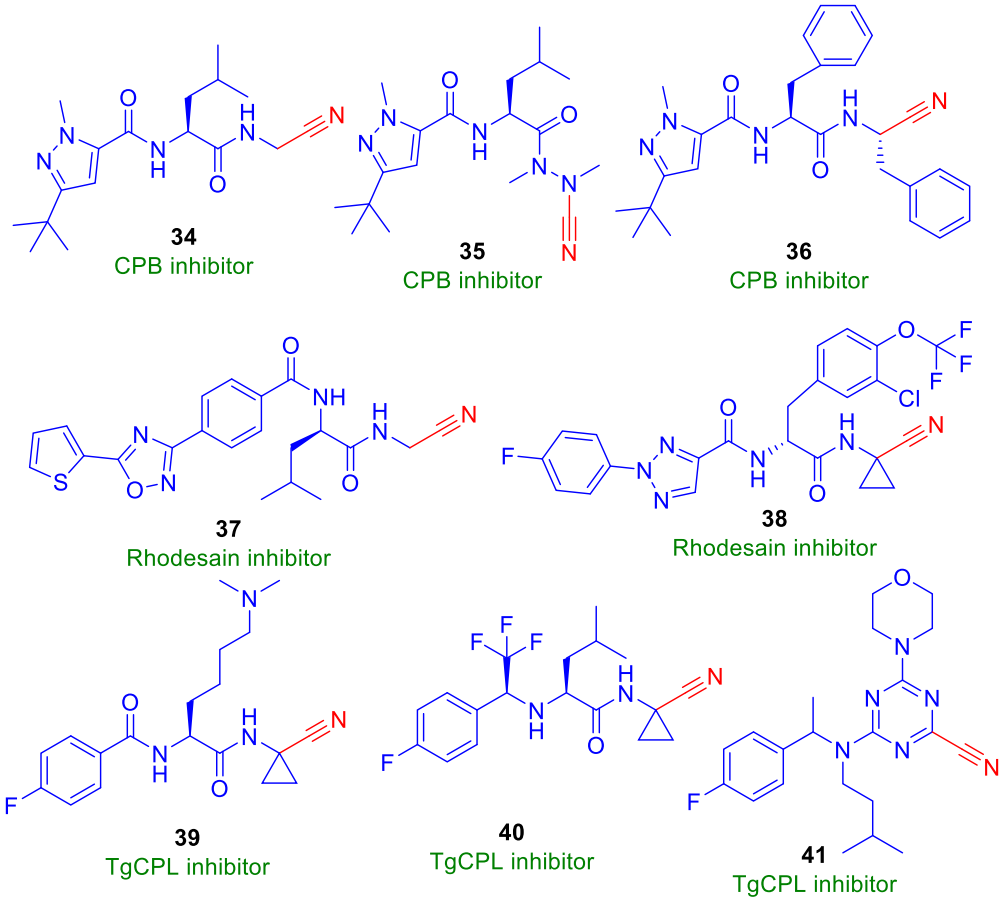

Fig. 8 Nitrile-based reversible covalent inhibitors

Rhodesain, a cysteine protease of *Trypanosoma brucei rhodesiense* which can cause Human African Trypanosomiasis, has been validated as a drug target [39]. A series of dipeptide nitriles were synthesized by Schirmeister's group and evaluated for inhibition of rhodesain. From their study they identified compound **37** ($K_i = 5.3$ nM) as a potent reversible covalent inhibitor of rhodesain [40]. Giroud et al. [41] also reported dipeptide nitrile-based reversible covalent inhibitor **38** ($K_i = 7.4$ nM) that exhibited both potent and selective activity against rhodesain in vivo against the parasite with acceptable pharmacokinetic properties. The cathepsin protease L (TgCPL) of *Toxoplasma gondii*, which causes chronic infection in the CNS, is critical to parasite survival during the chronic phase [42, 43]. The currently available inhibitors are ineffective against the chronic form. Therefore, there is an urgent need to develop effective and brain-penetrant inhibitors which could lead to development of new therapeutics to treat this parasitic infection. Zwicker et al. reported nitrile-based inhibitors **39** and **40** of TgCPL [44]. In further development of their work, the peptidomimetic scaffold with a triazine ring resulted in the synthesis of compound **41** which exhibits good brain exposure in mice after IP administration and showed efficacy in an in vitro model of bradyzoite stage parasites [45].

Ketones

The ketone moiety activated by an adjacent electron-withdrawing group also works as a reversible electrophilic warhead and shows broad-range reactivity toward nucleophilic amino acid residues, such as cysteine and serine. Ketone-based peptidomimetics serve as reversible covalent inhibitors of serine and cysteine proteases mimicking a tetrahedral transition state through the formation of hemiketal and thiohemiketal complex respectively. For instance, the α-ketoamide-based inhibitors telaprevir (**2**) and boceprevir (**3**) react with the catalytic serine (Ser1139) of hepatitis C virus NS3/4A. The proposed reaction mechanism and the X-ray crystal structure of **2** in complex with the NS3/4A protease (PDB ID: 3SV6) [46] is illustrated in Fig. 9.

The successful application of the ketone group as a reversible covalent warhead inspired many pharmaceutical companies to develop the antiviral agents for the pathogen

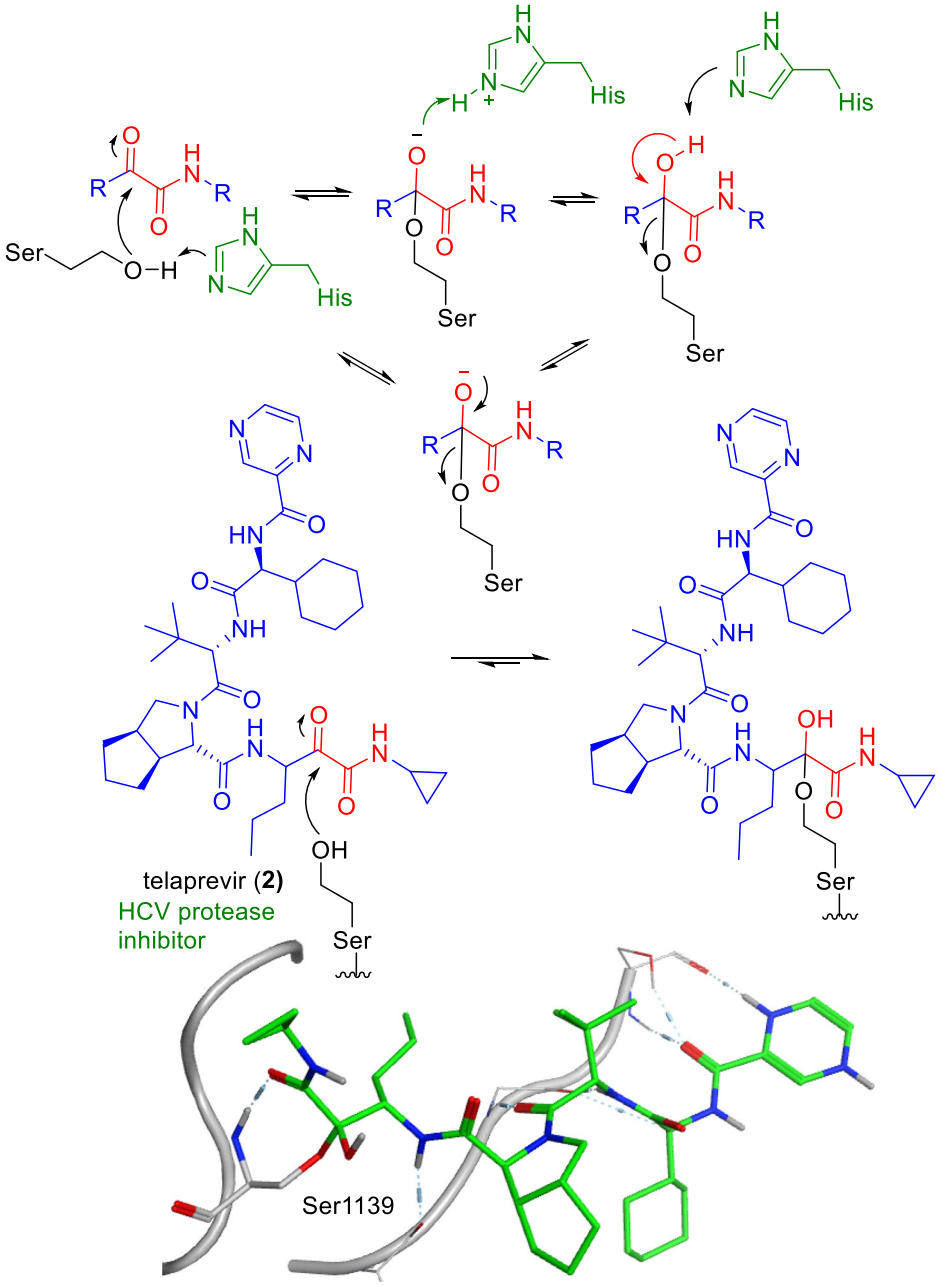

Fig. 9 The proposed reaction mechanism and the X-ray crystal structure of **2** complex NS3/4A protease (PDB ID: 3SV6)

SARS-CoV-2. The α-hydroxymethylketone compound PF-00835231 (**42**) showed potent inhibition against SARS-CoV-2 M^{pro} with an IC_{50} of 6.9 nM [47]. The co-crystal structure (PDB ID: 6XHM) of **42** complexed with SARS-CoV-2 M^{pro} reveals that the carbonyl group of the hydroxymethyl ketone is covalently bonded to Cys145 forming a thiohemiketal (Fig. 10). Further optimization of solubility and the pharmacokinetic profile led to its phosphate prodrug, PF-07304814 (**43**) (Fig. 10) [48].

Zhang et al. reported a broad-spectrum, α-ketoamide-based inhibitor **44** of coronavirus and enterovirus replication [49]. This inhibitor was further optimized to a more selective inhibitor **45** against SARS-CoV-2 M^{pro}, with better pharmacokinetic profile by inserting a pyridone ring and replacing the cyclohexyl and cinnamoyl moieties by a smaller cyclopropyl group and a hydrophobic *tert*-butyloxycarbonyl group, respectively [50]. The crystal structure of SARS-CoV-2 M^{pro} in complex with **45** (PDB ID: 6Y2F) confirmed that the ketoamide moiety of **45** is involved in covalent adduct formation with Cys145 (Fig. 10) [50]. Sacco's group also disclosed α-ketoamide reversible covalent inhibitors of SARS-CoV-2 M^{pro}, UAWJ246 (**46**) with $K_i = 36$ nM and UAWJ248 (**47**) with $K_i = 13$ nM [51]. Voss's research group discovered tripeptidic α-ketoamide **48** as a potent reversible covalent inhibitor of the protease with IC_{50} of 38 nM [52]. Based on compound **48**, Wang

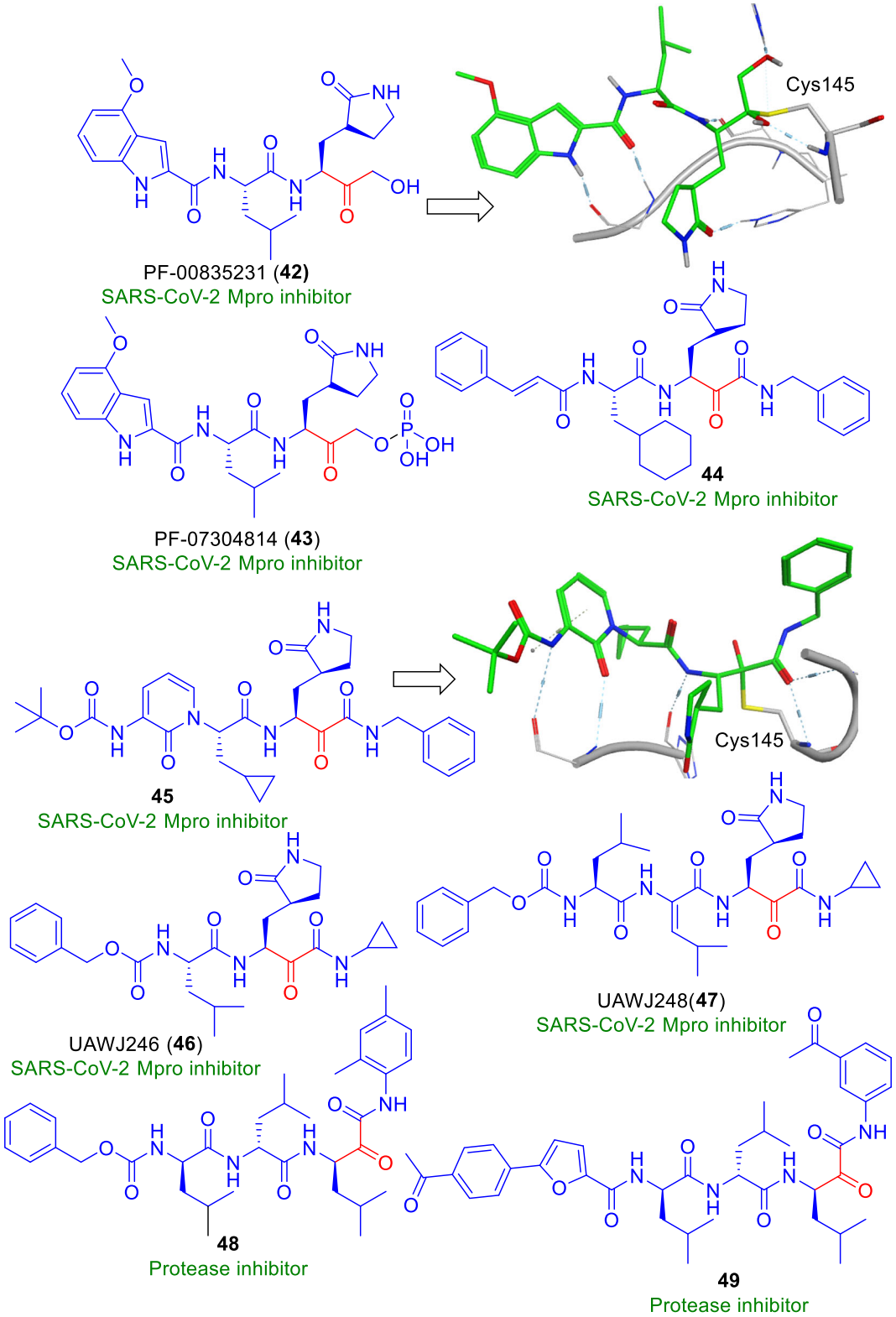

Fig. 10 Ketone-based reversible covalent inhibitors

et al. developed a more potent derivative **49** with IC_{50} of 25.3 nM against the protease together with excellent anticancer activity [53].

The trifluoromethyl ketone moiety was found to be an effective reversible covalent warhead targeting cysteine residues. Zhang et al. discovered a series of selective fibroblast

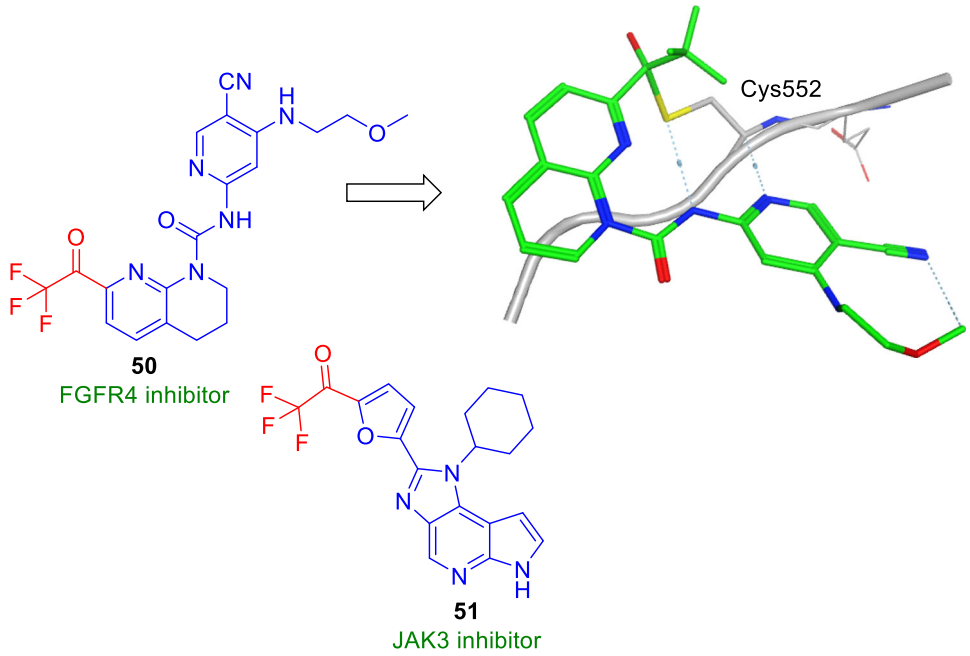

Fig. 11 Trifluoromethylketone-based covalent inhibitors

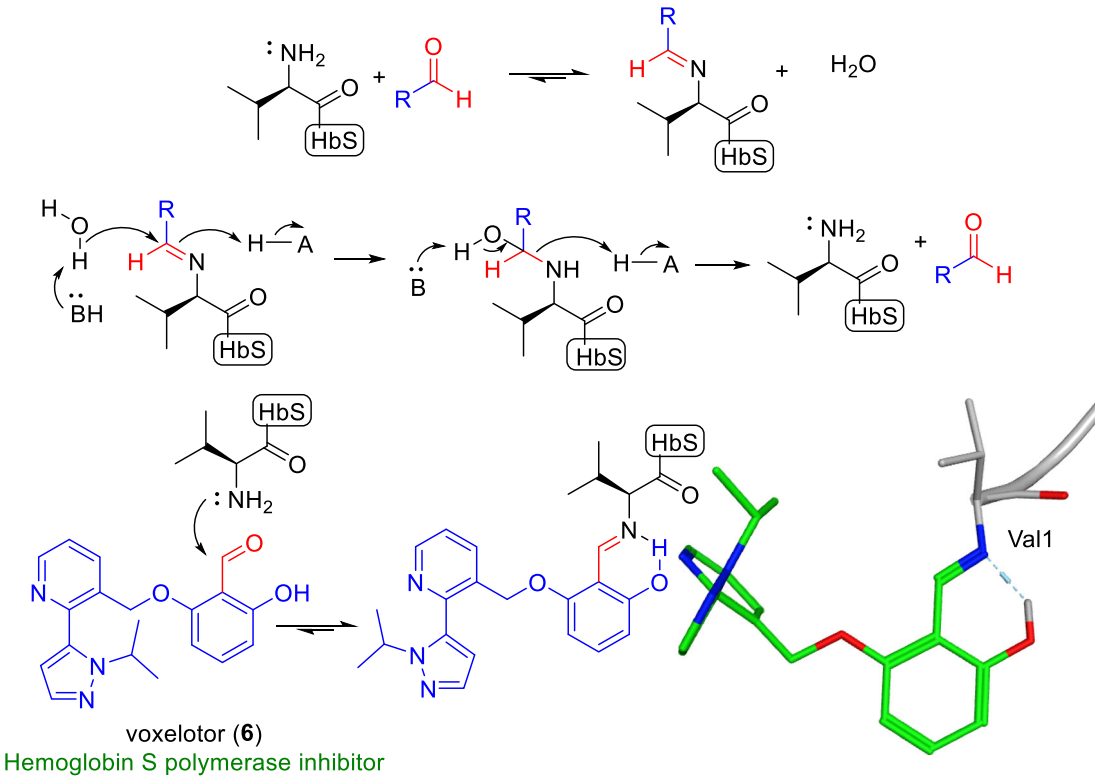

Fig. 12 Proposed reaction mechanism of aldehyde with valine and X-ray crystal structure (PDB ID: 5E83) of voxelotor (**6**)

growth factor receptor 4 (FGFR4) inhibitors with a low nM IC_{50} value. The reversible covalent bonding mode of these inhibitors was confirmed by using MALDI-TOF mass spectrometry and X-ray crystallographic studies. The X-ray crystal structure of compound **50** in complex with FGFR4 (PDB ID: 7VJL) shows that the trifluoromethyl ketone warhead is covalently bonded to Cys552 (Fig. 11). The trifluoromethyl ketone moiety was also successfully used to design new JAK3 inhibitors. One example **51** of a JAK3 inhibitor with $IC_{50} = 87.2$ nM is shown in Fig. 11 [54].

Aldehyde

Analogously to electrophilic ketones, aldehydes can also serve as reversible covalent warheads. The aldehyde moiety reacts reversibly with cysteine or lysine to form the thiohemiacetal or Schiff base, respectively. Generally, the aldehyde moiety should be avoided in drug design due to its metabolic liability and potential toxicity [55]. However, in some cases aldehydes are stable as highlighted in voxelotor (**6**). The Schiff base formed from the aldehyde of voxelotor (**6**) and the amino group of Val1 of hemoglobin is stabilized by a strong intramolecular H-bond as shown in the crystal structure (PDB ID: 5E83) Fig. 12.

In another example of the stabilization of lysine Schiff base, Cal et al. [56] used the strategy of coordination of the nitrogen lone-pair electrons of the imine to a boronic acid in the reversible protein modification. In this strategy, boronic acid is introduced to the ortho-position of a benzaldehyde (**52**) moiety to obtain an iminoboronate upon Schiff base formation where the latter is stabilized by an intramolecular dative bond between the electrophilic boron center and the nucleophilic nitrogen lone-pair electrons. It has been confirmed that this reaction is reversible under physiological conditions [57, 58]. The chemistry of iminoboronates was applied by Akçay et al. [59] to develop the reversible covalent inhibitors of the induced myeloid leukemia cell differentiation protein (MCL-1). The incorporation of 2-carbonylphenylboronic acid with pharmacophores of MCL-1 inhibitors were designed to reversibly target the surface-exposed Lys234 side chain of the target protein. 2-Carbonylphenylboronic acids **53–55** (Fig. 13) showed low nanomolar IC_{50} values in a TR-FRET assay. On the other hand, derivatives lacking either the boronic acid or the *ortho*-carbonyl group were significantly less active. Mass spectrometry analysis showed that compound **53** was approximately 50% attached to the target protein after 1 h, while the reaction with acetophenone **55** was slightly slower. Reversibility of these inhibitors was confirmed by surface plasmon resonance (SPR) experiments.

In 2021, Quach's group [60] also utilized the chemistry of iminoboronates to develop BCR-ABL reversible covalent inhibitors to target the catalytic lysine (Lys271) with excellent potency against both wild-type and mutant ABL kinases. The representative example **56** is highlighted here. As expected, **56** showed time-dependent inhibition of ABL with IC_{50} values from 13 nM at 0 h to 1.7 nM after 12 h. Inhibitor **56** also showed excellent time-dependent potency against both mutants, ABL^{T315I} from 25 nM ($T = 0$ h) to 0.1 nM ($T = 6$ h) and ABL^{E255K} from 43 nM ($T = 0$ h) to 0.5 nM ($T = 12$ h). The X-ray co-crystal structures of **56** with the ABL kinase domain (PDB ID; 7DT2) did not show the expected dative bond between the imine nitrogen and the boron atom. However, from the enzymatic assay data and MALDI-TOF analysis, the boronic acid was believed to still play a significant role in the formation of the adduct. Therefore, they proposed that the obtained cocrystal structure of **56** with the ABL kinase domain (PDB ID; 7DT2) may be the key intermediate during the formation of the iminoboronates.

Furthermore, imine products were found to be stabilized by incorporating a nitrogen atom close to the aldehyde instead of a boronic acid, such as the benzaldehyde derivative of *o*-aminomethyl phenylboronic acid (**57**) [61]. Excitingly, the *o*-aminomethyl phenylboronic acid-derived imines are much more stable with half-lives of approximately 5–11 hours than the imines that were stabilized only by a boronic acid. The *o*-aminomethyl phenylboronic acid group was introduced into the cyclic peptide that noncovalently binds to the sortase A enzyme of *Staphylococcus aureus*, a potential target for combating *S. aureus* infections. The resulting compound **58** (Fig. 13) potently binds to sortase A through covalent modification of a lysine residue (Lys173) in the sortase A enzyme. Thus, they discovered the *o*-aminomethyl phenylboronic acid group as a promising motif for targeting lysine by reversible covalent inhibitors with long residence times.

In a drug discovery program carried out at Novartis, Knoepfel et al. [62] identified 2-formylquinoline amide **59** ($IC_{50} = 57$ nM) as a potent inhibitor of FGFR4 with a good selectivity in the FGFR family. FGFR4 is considered as a potential target for the treatment of hepatocellular carcinoma (HCC) because of its important role in HCC development and progression. Targeting the unique cysteine (Cys522) in the FGFR4 hinge region with the formyl group provided an opportunity for high FGFR family selectivity. Scaffold morphing of quinolone-amide **59** resulted in several 2-formyl tetrahydronaphthyridine urea analogs like **60** with better FGFR4 activity, selectivity, and improved physicochemical properties. Further extensive optimization of the 2-formyl tetrahydronaphthyridine urea series resulted in a drug candidate, roblitinib (**61**) (Fig. 14) which is being evaluated in phase III clinical trials [63, 64]. In a Phase 1–2 study, roblitinib (**61**) demonstrated favorable pharmacokinetic characteristics with evidence of FGFR4 inhibition. Roblitinib (**61**) alone or in combination with spartalizumab had a manageable safety profile with AEs that are considered on-target effects of pathway inhibition. Clinical efficacy was observed in patients with hepatocellular carcinoma.

As previously mentioned, the aldehyde moiety is also susceptible to the nucleophilic addition by the cysteine-SH which leads to the formation of a reversible thiohemiacetal adduct with relatively high stability and a longer residence time. Aldehyde-based reversible covalent inhibitors targeting cysteine have been reported before [65, 66]. This warhead received increased attention during the development of potent inhibitors of SARS-CoV-2 M^{pro} after the WHO

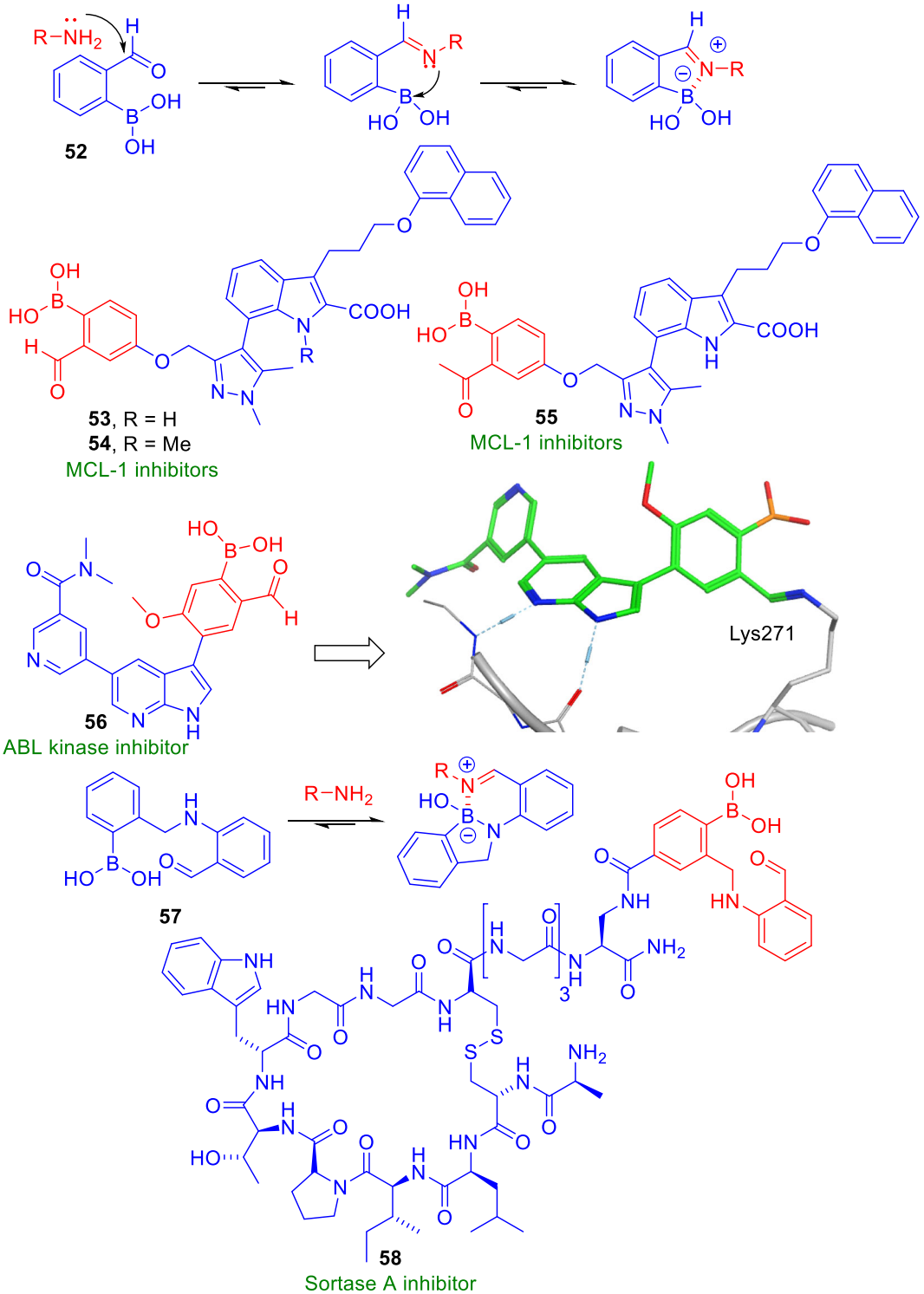

Fig. 13 *o*-Carbonylbenzeneboronic acids-based reversible covalent inhibitors

declared COVID-19 as a global health emergency in 2020. Dai et al. [67] rationally designed two of the first peptidomimetic derivatives **62** and **63**, targeting SARS-CoV-2 M^{pro}. Both exhibited excellent inhibitory activity (IC_{50} of 53 nM and 40 nM, respectively) and potent anti-SARS-CoV-2 infection activity (EC_{50} of 530 nM and 720 nM, respectively). The X-ray co-crystal structure of **62** complexed with SARS-CoV-2 M^{pro} (PDB ID: 6LZE) showed that the aldehyde group is covalently bound to Cys145 of M^{pro} (Fig. 15). Both compounds exhibited favorable

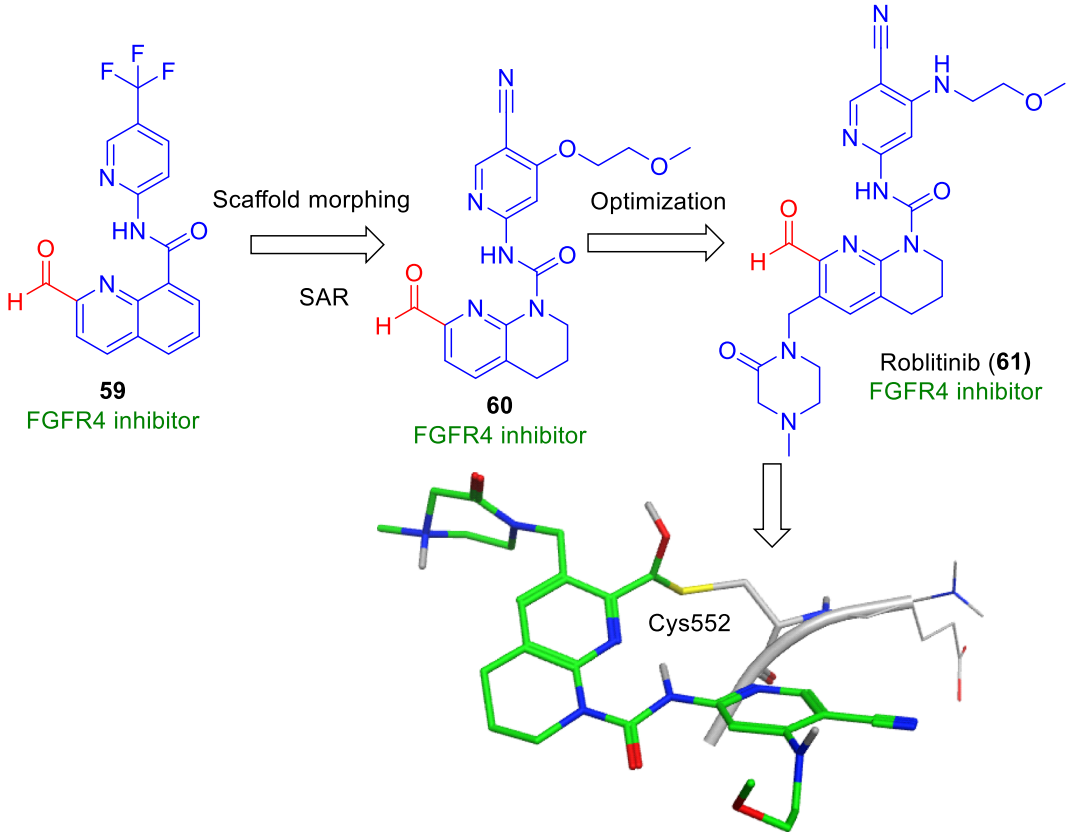

Fig. 14 Discovery of roblitinib (**61**) and its co-crystal structure complex with FGFR4 (PDB ID: 6JPJ)

pharmacokinetic properties in vivo and low toxicity. Qiao et al. [68] synthesized a series of new bicycloproline-containing M^{pro} inhibitors derived from either telaprevir (**2**) or boceprevir (**3**). All synthesized compounds inhibited SARS-CoV-2 M^{pro} activity in vitro, with IC_{50} values ranging from 7.6 to 748.5 nM. The two lead compounds MI-09 (**64**) and MI-30 (**65**) showed excellent antiviral activity in cell-based assays. Both compounds **64** and **65** displayed favorable pharmacokinetic properties and safety in rats and significantly reduced lung viral loads and lung lesions in a transgenic mouse model of SARS-CoV-2 infection. Another research group [69] synthesized UAWJ9-36-1 (**66**) and UAWJ9-36-3 (**67**) with very similar structures to **64**. Both compounds showed comparable inhibitory activity against SARS-CoV-2 M^{pro} to **64**.

GC-373 (**68**) and its prodrug GC-376 (**69**) in which the aldehyde was masked as its bisulfite adduct to increase solubility and allow release under physiological conditions, were initially designed as inhibitors of feline coronavirus (FCoV) 3CL protease for use of veterinary medications [70]. In the search of anti-COVID-19 agents recently, the two compounds **68** and **69** were tested for the inhibition of SARS-CoV-2 M^{pro}. Both **68** and **69** inhibit the SARS-CoV-2 M^{pro} in vitro with IC_{50} of 0.40 µM and 0.19 µM, respectively [71, 72]. The X-ray crystallographic studies of both compounds in complex with SARS-CoV-2 M^{pro} displayed similar binding modes of **68** (PDB ID: 6WTK) and **69** (PDB ID: 6WTJ) which confirms that the bisulfite adduct of **69** rapidly releases the aldehyde (Fig. 16). Further in vitro investigation using a model of SARS-CoV-2 infection in Vero E6 cells showed high inhibitory activity of both **68** and **69** with EC_{50} of 1.5 and 0.92 µM, respectively with low cytotoxicity even at high concentrations.

The research group of Vuong [73] also synthesized a series of analogs of GC376 (**69**) and identified compound **70** with excellent inhibitory activity against M^{pro} ($IC_{50} = 0.07$ µM) and antiviral efficacy ($EC_{50} = 0.57$ µM). The improved activity of **70** in comparison to the parent compound **69** is attributed to the more compact cyclopropyl group of **70** which can penetrate deeper into the S2 pocket of the target protein. Additionally, introduction of a fluorine on the Cbz group allows this moiety to move away from the solvent exposed region. Further structural modification of GC-376 (**69**) by replacing the isopropyl moiety with a phenyl group resulted to UAWJ247 (**71**) with IC_{50} of 0.045 µM [51]. Recently, Liu et al. reported a series of analogs of **69**. They discovered NK01-63 (**72**) which is more active than the lead compound with excellent inhibitory activity ($IC_{50} = 16$ nM) against

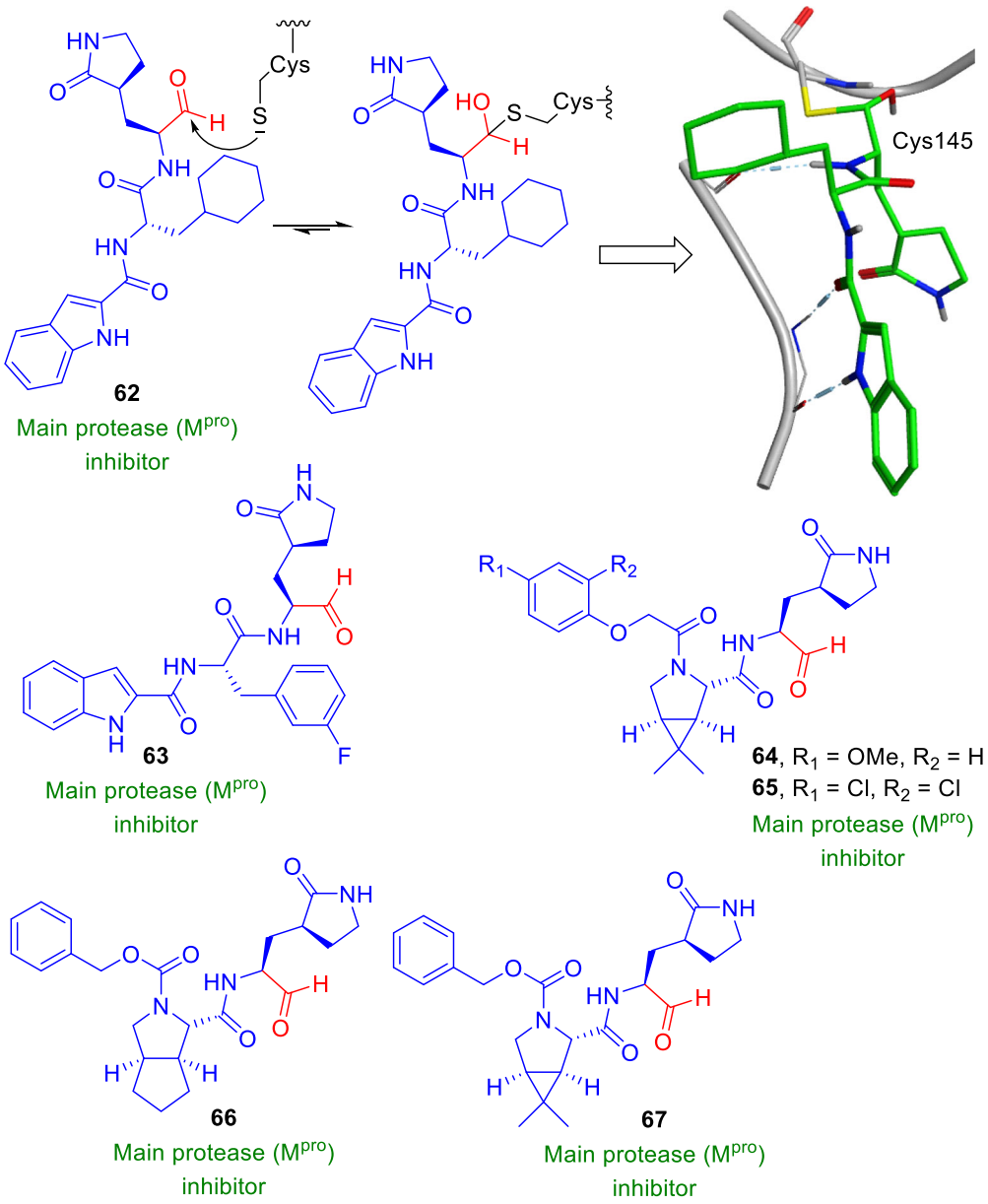

Fig. 15 M^{pro} Cys145 targeting aldehyde-based reversible covalent inhibitors

SARS-CoV-2. Compound **72** also showed excellent antiviral effect in cell assay ($EC_{50} = 6$ nM in Huh-7ACE2 infected cells) with high selectivity against other human proteases [74]. Yang and his colleagues reported tripeptidyl inhibitors MPI3 (**73**) and MPI8 (**74**) (Fig. 16) as SARS-CoV-2 M^{pro} inhibitors with high activity (8.5 and 105 nM respectively). Despite the weaker enzymatic activity of **74** in comparison to **73**, it completely inhibits the SARS-CoV-2 infection in Vero E6 and A549/ACE2 cell lines with an IC_{50} of 0.31 μM [75]. Moreover, other aldehyde-based reversible covalent tripeptidyl inhibitors of SARS-CoV-2 M^{pro} were explored [76–78].

Boronic acids

After approval of bortezomib (**4**) by the FDA in 2003, boronic acids have garnered much attention by the pharmaceutical industry and now various boron-containing drugs are available on the market and many more are currently being evaluated in clinical trials. Comprehensive reviews are available on the use of boronic acids in drug discovery and as covalent inhibitors [79–82]. Here we will briefly describe the application of the boronic acid moiety as a reversible electrophilic warhead in the discovery of reversible covalent drugs. Boronic acids can form covalent

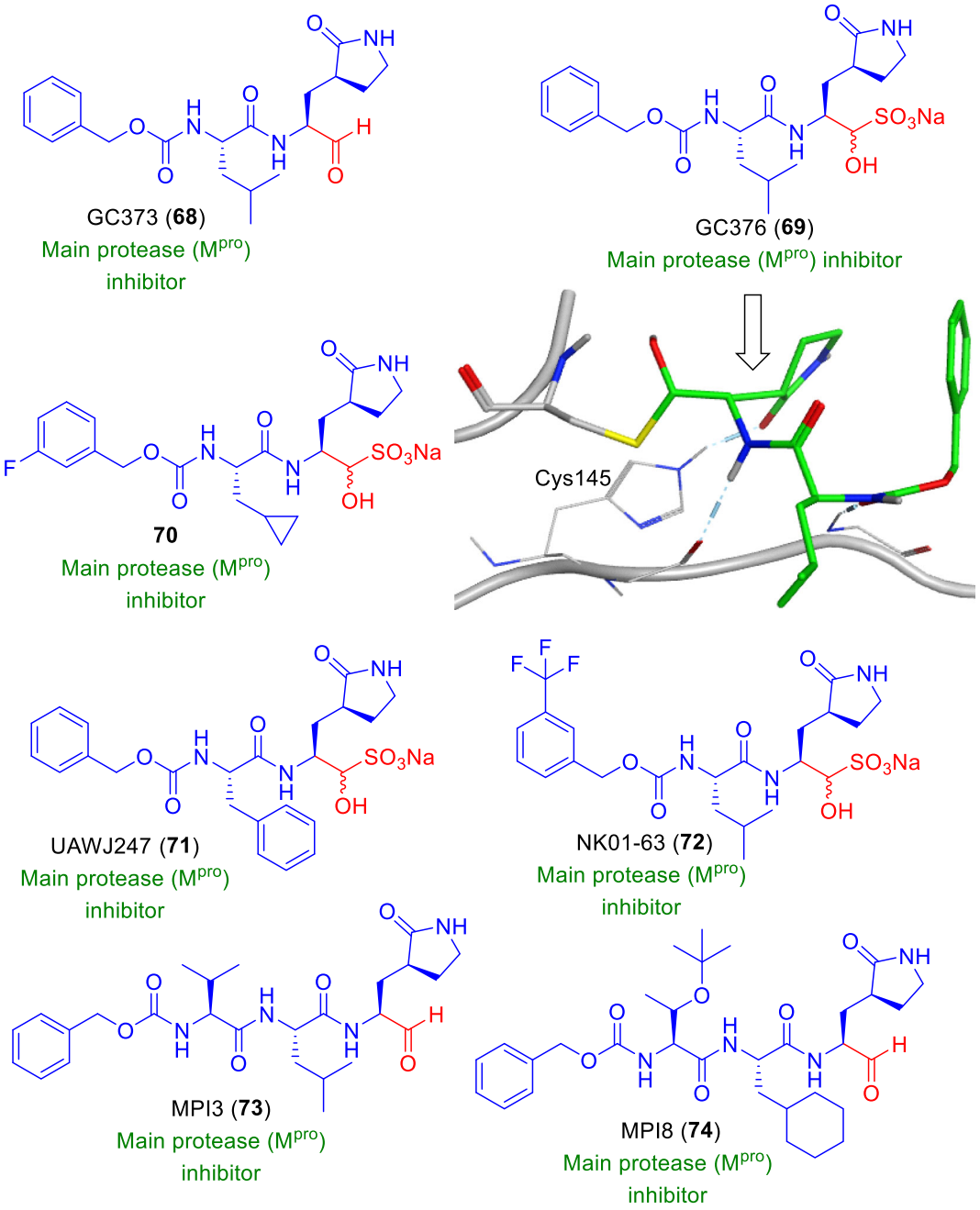

Fig. 16 Cysteine targeting aldehyde-based reversible covalent inhibitors

adducts with nucleophiles such as serine, lysine, threonine, tyrosine, and cysteine residues in target proteins. As described before, bortezomib (**4**), containing a boronic acid as an electrophilic warhead which targets the N-terminal threonine of 26S proteasome, is the first-in-class reversible covalent inhibitor for the treatment of multiple melanomas [10]. The X-ray structure of human 20 S proteasome complex with bortezomib (**4**) (PDB ID: 5LF3) indicated that the boronic acid formed a tetrahedral adduct with a threonine residue of the target protein [83]. Ixazomib (**5**) is a second-generation proteasome inhibitor that also possesses a boronic acid group. It was approved by the FDA in 2015 for the treatment of multiple myeloma. Delanzomib (**75**) composed of a boronic acid as the electrophilic warhead is a novel orally-active inhibitor of the chymotrypsin-like activity of the proteasome that down-modulates the nuclear factor-κB (NF-κB) activity [83]. Vaborbactam (**76**), approved for the treatment of various bacterial infections, consists of a cyclic boronic acid as the electrophilic warhead targeting the serine residue of the β-lactamase as illustrated

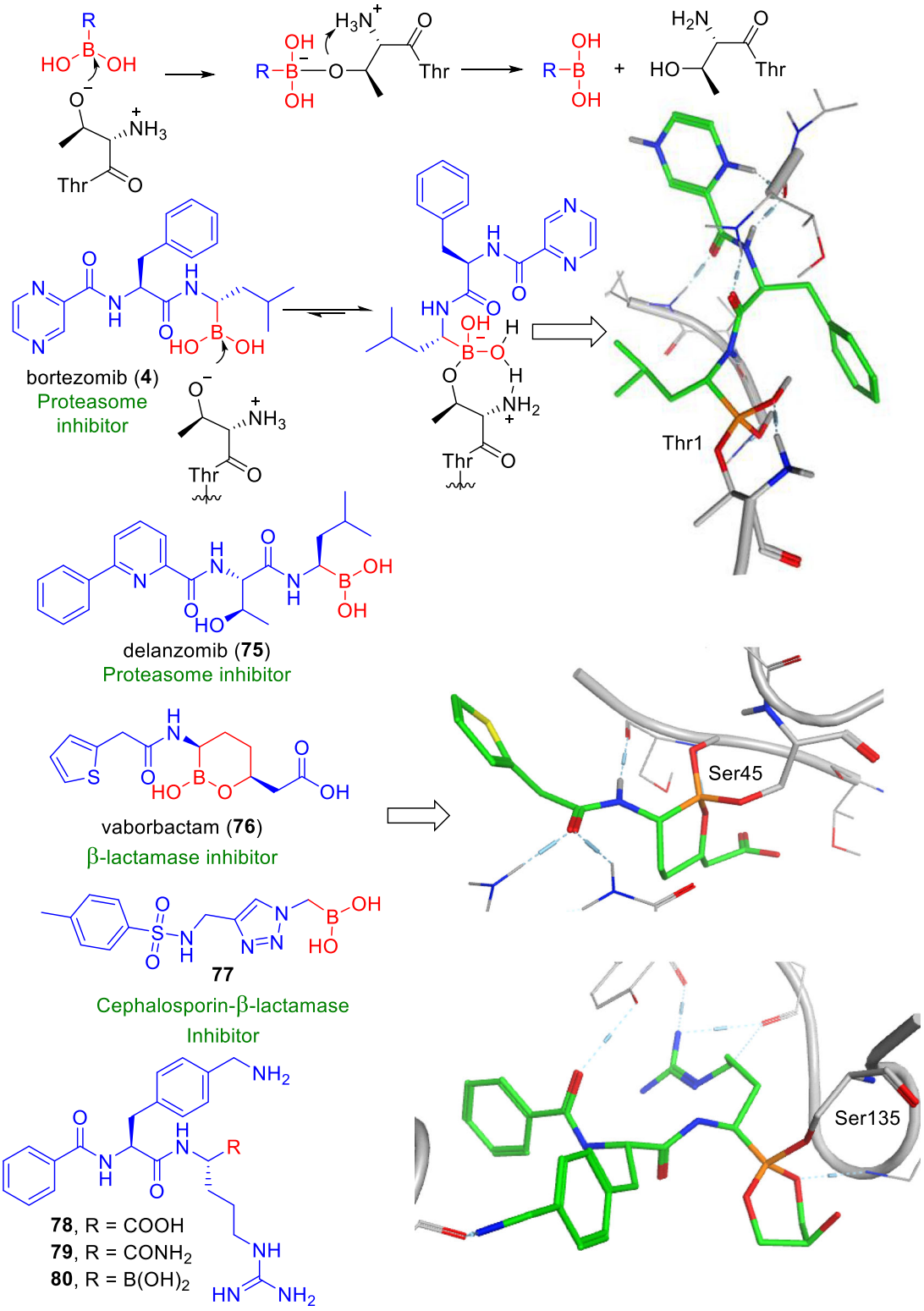

Fig. 17 Boronic acid-based reversible covalent inhibitors

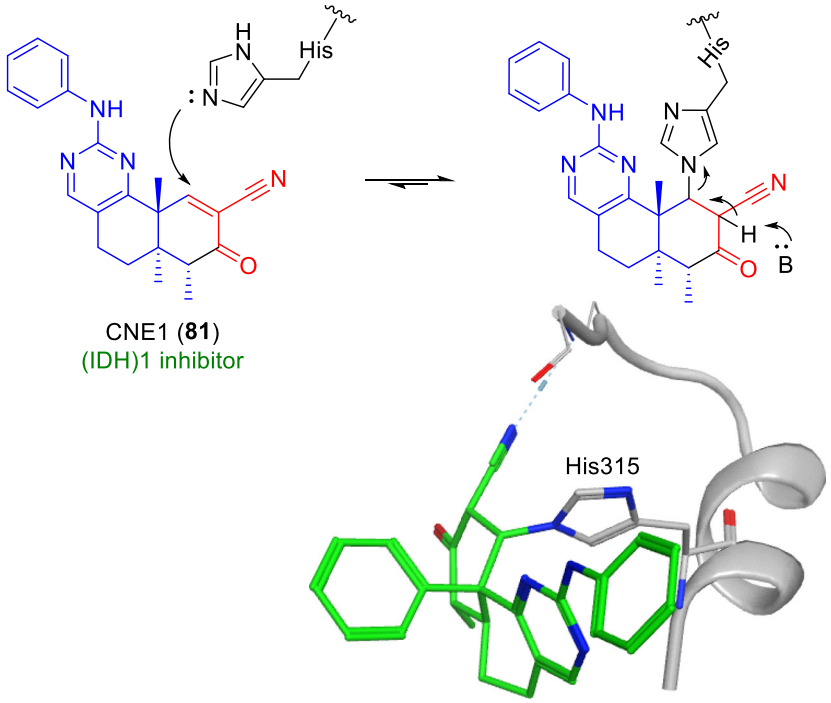

Fig. 18 The X-ray crystal structure of **81** complex in IDH1 (PDB ID: 6BL1)

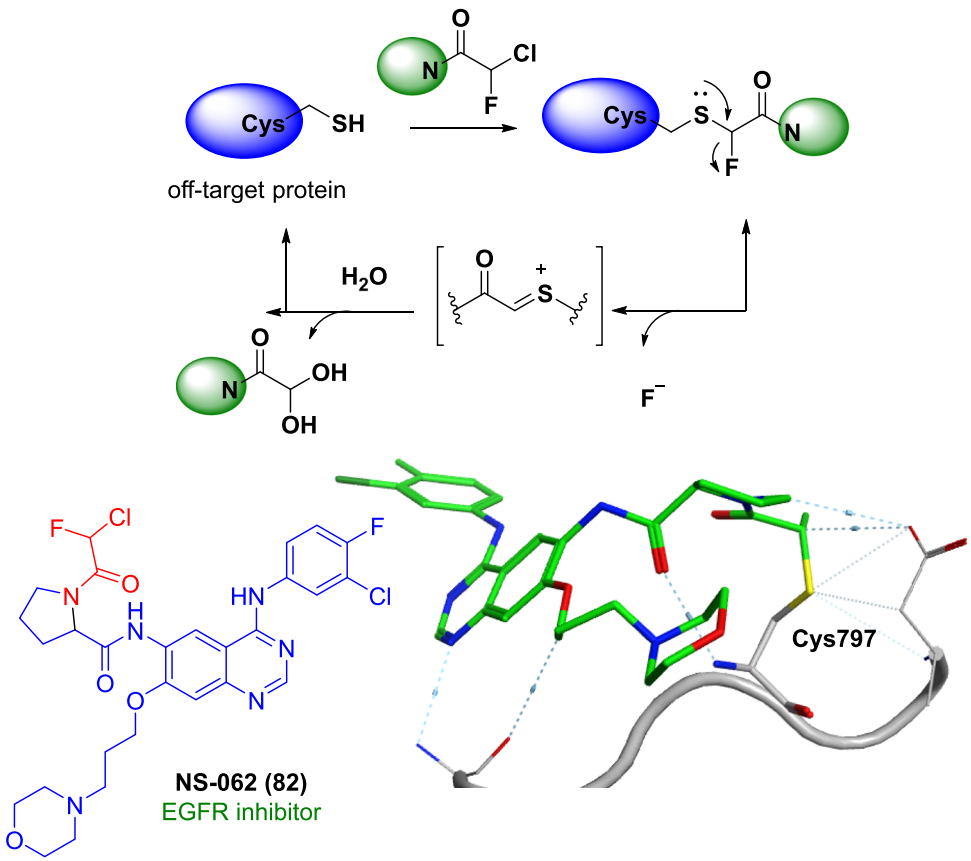

Fig. 19 α-Chlorofluoroacetami de-based reversible covalent inhibitor **82** and its X-ray crystal structure complex in EGFR (PDB ID: 5Y25)

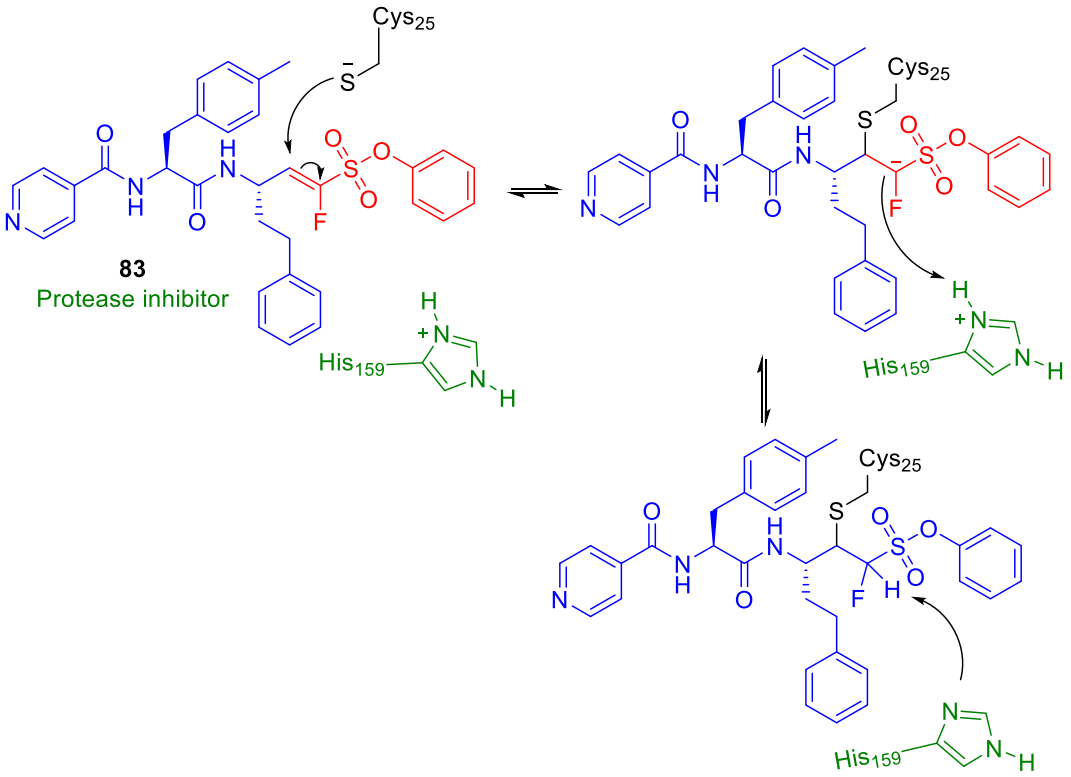

Fig. 20 α-Fluorovinyl-sulfones/-sulfonates based reversible covalent inhibitors

in Fig. 17 (PDB ID: 4XUZ) [84]. Caselli et al. [85] synthesized a library of 26 α-triazolylmethaneboronic acids. These compounds showed K_i values ranging from 0.09 µM to 38 µM against the clinically concerning *Acinetobacter*-derived cephalosporinase, ADC-7. In this series the most potent compound **77** ($K_i = 90$ nM) is covalently bound to the catalytic serine.

It has been reported that the replacement of carboxylic acid (**78**) and amide (**79**) by boronic acid (**80**) at the C-terminal of dipeptidic inhibitors against Zika, West Nile, and dengue viral proteases resulted in a thousand-fold affinity gain [86]. The most active compound **80** had K_i values of 51, 82 and 40 nM for dengue, West Nile and Zika viral proteases, respectively. The crystal structure of **80** in complex with ZIKV NS2B-NS3 protease (PDB ID: 5LC0) [87] showed that a six-membered boronate structure was formed between glycerol and boronic acid, in which the boron atom was covalently bonded to the Ser135 residue of the target protein (Fig. 17).

Miscellaneous warheads

A reversible-covalent approach was recently pursued by Jakob et al. to engage a histidine (His315) in wild-type isocitrate dehydrogenase (IDH)1 [88]. A high-throughput screen and subsequent optimization identified covalent reversible inhibitor **81** with IC_{50} of 110 nM and favorable permeability. Covalent bond formation of the α-cyanoenone moiety with His315 in the binding pocket of NADPH via aza-Michael addition was confirmed by X-ray crystallography. The X-ray crystal structure of **81** complexed in IDH1 (PDB ID: 6BL1) is shown in the Fig. 18. Reversibility of **81** was confirmed by wash-out and jump-dilution experiments.

Shindo et al. [89] reported α-chlorofluoroacetamide as a novel warhead for the development of covalent inhibitors. Regardless of the weak intrinsic reactivity, α-chlorofluoroacetamide-appended quinazoline exhibited high reactivity toward Cys797 of EGFR. For example, NS-062 (**82**) had higher target specificity for EGFR than the corresponding Michael acceptors in a wide range of concentrations (0.1–10 µM) in cells. The covalent adduct formed between cysteine and the α-chlorofluoroacetamide derivative was susceptible to hydrolysis and reversibly released the intact thiol but remained stable in the solvent-sequestered ATP-binding pocket of EGFR. This characteristic environment-dependent hydrolysis can potentially reduce off-target protein modification by α-chlorofluoroacetamide-based drugs. Oral administration of NS-062 (**82**) significantly suppressed tumor growth in a mouse xenograft model (Fig. 19).

In 2016, Schirmeister and co-workers [90] developed α-halovinylsulfones as a new class of covalent reversible

cysteine protease inhibitors. Structure optimization of α-fluorovinylsulfones/-sulfonates by rhodesain-based molecular modeling approaches resulted in compound **83**, the most potent and selective reversible covalent inhibitor in the series with single-digit nanomolar affinity and high selectivity toward mammalian cathepsins B and L (Fig. 20). Enzymatic dilution assays and MS experiments indicated that **83** is a potent tight binder ($K_i = 3$ nM) and slowly reversible inhibitor of trypanosomal cysteine protease rhodesain [91].

Conclusions

Covalent drugs through formation of a covalent bond with their target proteins exhibit a favorable pharmacological profile, such as enhanced potency and prolonged duration of action over non-covalent drugs, leading to a higher possibility of durable efficacy in the treatment of hard-to-treat human diseases. However, one of the key challenges associated with covalent drugs is how to mitigate selectivity and toxicities. The electrophilic warheads could react with nucleophilic residues of proteins other than the intended target protein, which could lead to off-target effects and further idiosyncratic toxicity and haptenization. This issue of covalent therapeutics can be potentially de-risked by tuning the chemistry of electrophilic warhead to form a reversible covalent bond with the intended target protein. This would allow for more sustained target engagement with lower immunogenicity risks and fewer off-target effects by readily releasing from unintended proteins. This strategy has been successfully applied in the development of reversible covalent drugs. Herein we have described the current status of reversible covalent inhibitors. We believe that the cumulative structural data of reversible covalent inhibitors presented in this review will provide a valuable guidance for future research in this area to further explore the chemistry of reversible covalent warheads to develop life-saving drugs.

Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

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