Picornaviral 3C protease inhibitors and the dual 3C protease/ coronaviral 3C-like protease inhibitors

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Importance of the field: Picornaviruses are small non-enveloped RNA viruses with genomic RNA of 7500 – 8000 nucleotides, whereas coronaviruses (CoV) are RNA viruses with larger genome of 27 – 32 kb. Both types of viruses translate their genetic information into polyprotein precursors that are processed by virally encoded 3C proteases ($3C^{pro}$) and 3C-like proteases ($3CL^{pro}$), respectively, to generate functional viral proteins. The most studied human rhinoviruses (HRV) belonging to picornaviridae family are the main etiologic agents of the common cold. Due to lack of effective drugs, $3C^{pro}$ has served as an excellent target for anti-viral intervention and considerable efforts have been made in the development of inhibitors. Interestingly, the inhibitors of $3C^{pro}$ cannot inhibit $3CL^{pro}$ potently without modification due to subtle differences in their active-site structures, but a group of common inhibitors against $3C^{pro}$ and $3CL^{pro}$ were found recently.
Areas covered in this review: The inhibitors against $3C^{pro}$ reported in the literatures and patents, with a focus on those inhibiting HRV and the dual picornaviral $3C^{pro}$/coronaviral $3CL^{pro}$ inhibitors, are summarized in this review.
What the readers will gain: Readers will rapidly gain an overview of the individual and dual $3C^{pro}$ inhibitors and the structural basis for discriminating them.
Take home message: In the future, more selective potent inhibitors against each protease and dual inhibitors against both proteases can be further developed to treat the diseases caused by picornaviruses and CoV.

Keywords: 3C protease, 3CL protease, coronavirus, inhibitor, picornavirus, rhinovirus

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1. Introduction

1.1 Pathogens of respiratory infective diseases

Respiratory infections are recognized as the major cause of acute morbidity in individuals of all ages worldwide [1]. The morbidity associated with respiratory infections in developing countries are as severe as those in industrialized countries, and these infections are the leading cause of death in children under the age of five [2]. Viruses are the most frequently identified pathogens of respiratory infective diseases. The primary viral pathogens associated with acute respiratory infections include picornaviruses, coronaviruses (CoV), adenoviruses, parainfluenza viruses, influenza viruses and respiratory syncytial viruses [3,4]. In terms of causing acute respiratory infection in humans, the CoV that emerged recently to cause severe acute respiratory syndrome (SARS) and the picornaviruses are the two main culprits [5,6]. CoV are the positive-stranded RNA viruses with larger genome of 27 – 32 kb, which typically cause respiratory and enteric diseases, pneumonia,

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exacerbation of asthma, neurological symptoms and myocarditis in humans and domestic animals. The outbreak of SARS, caused by a novel human CoV, was spread from China to 29 countries in 2003, infecting a total of ~ 8000 people and killing ~ 800 patients [7-10]. On the other hand, picornaviridae are small non-enveloped RNA viruses with a single strand of genomic RNA of 7500 – 8000 nucleotides [11-17]. The members of picornaviridae include rhinoviruses (RV), enteroviruses (EV), coxsackieviruses (CV), polioviruses, echoviruses, encephalomyocarditis viruses, meningitis virus, foot and mouth viruses and hepatitis A virus. Among them, human RV (HRV) are the major cause of the common cold [18], whereas EV and CV infection can cause hand, foot, and mouth diseases in human and animals [3]. In severe cases, EV can damage the CNS leading to viral meningitis, encephalitis and severe myocarditis, as well as fatal pulmonary edema [19-21]. CV strain B3 is a major human pathogen that causes meningitis and mycocarditis leading to heart failure in young adults and congestive heart failure [22].

HRV is a genus of the picornaviridae family implicated in 50 – > 80% of upper respiratory tract infections [23]. After attachment to the host cell, the viral genomic RNA takes off its coat from the viral capsid. The positive-stranded viral RNA is translated to viral proteins essential for viral gene replication and production of new viral particles. Genome replication and mRNA synthesis occur in small membranous vesicles, induced by several viral proteins. The viral replication speed relies on many factors, such as virus strain, temperature, pH, host cell type and multiplicity of infection. Typically, a single replication cycle ranges from 5 to 10 h. In addition to the common cold, HRV cause a number of other respiratory tract infections and complications such as acute otitis media, acute sinusitis, acute exacerbations of chronic obstructive pulmonary disease, and asthma exacerbations in children and adults [24].

1.2 Difficulties of developing inhibitors for picornaviridae

Researchers have faced several challenges in attempting to develop anti-viral agents to treat infections caused by HRV [25,26]. First, there are > 100 serotypes of HRV, which cause vaccine development to be impractical whilst complicated efforts must be made to develop effective anti-viral treatments with broad activity across all serotypes. Second, to make an anti-HRV compound effective, good oral bioavailability and tissue distribution are essential to reach sufficient drug quantity at the infected site. Third, as all acute viral illness treatment must be given at a critical time point following infection for optimal effect, and most of the symptoms occur within the first 3 days of illness, the drug must be capable of reducing the severity of symptoms within the first 24 h of management [27]. Finally, as the clinical manifestation of HRV infection in otherwise healthy individuals is typically an upper respiratory infection, the drug must have an excellent safety profile to ensure an appropriate risk:benefit ratio [28].

The absence of effective vaccines for most viral infections highlights an urgent necessity for the design and development of effective anti-viral drugs. Due to the advancement in virology since the late 1980s, several key events in the viral life cycle have been well delineated and a number of molecular targets have been validated, culminating in the emergence of a few new anti-viral drugs in recent years. Inhibitors against infectious viruses have been currently under active investigation. To date, numerous compounds with significant *in vitro* activity against HRV have been found [29]. However, the majority of these compounds bind to the viral capsid and inhibit either viral attachment/adsorption or subsequent uncoating [26]. The key roles of Cys proteases in the life cycles of infectious agents such as protozoa and viruses have turned into new important targets for anti-infective drugs [30]. Thus, the effective inhibition of pathologically relevant Cys proteases has raised increasing interests in drug development [31]. Anti-picornavirus agents were designed to target the 3C protease ($3C^{pro}$), which is highly conserved among different viral serotypes, and have exhibited great potential in therapeutic utility.

1.3 3C and 3C-like proteases as anti-viral targets

In picornaviridae, the roles of proteases as protein degrading and protein processing enzymes both in physiological and pathological processes of mammals are well known [32-35]. Upon entry into susceptible host cells, the viral RNA of HRV is translated into a long polyprotein of approximately 250 kDa. HRV requires the translation of a polyprotein precursor cleaved by virally encoded proteases into the proteins that make up the viral capsid and replication machinery. This single polyprotein undergoes proteolysis by the virus-encoded proteases 2A and 3C into 11 final products (4 structural and 7 non-structural proteins) [36,37]. Cleavage of the Tyr-Gly pairs which connect coat precursors P1 to P2-P3 and 3C/3D in EV is accomplished by viral protease 2A, but the cleavage of 3C/3D by protease 2A is not essential for viability of the virus. The remaining cleavage in P2-P3 at the Gln-Gly pair is executed by viral protease 3C, which is essential for EV replication. Members of the $3C^{pro}$ family are Cys proteases, where the sulfhydryl group most often cleaves the glutamine-glycine amide bond. $3C^{pro}$ of HRV has been used by Agouron Pharmaceuticals Co. (later merged into Pfizer Pharmaceuticals, Inc.) to develop AG7088 (see below), for the treatment of common cold [38]. It indeed can inhibit a broad spectrum of picornaviruses by inhibiting their $3C^{pro}$ due to their high sequence homology [39], although the drug-resistant $3C^{pro}$ mutants have been found [40]. On the other hand, SARS-CoV contains a 3C-like protease ($3CL^{pro}$) analogous to the $3C^{pro}$ of picornaviridae responsible for processing two overlapping polyproteins, pp1a (486 kDa) and pp1ab (790 kDa). Other members of

human CoV including CoV-229E, CoV-OC43, CoV-HKU1 and CoV-NL63 also require a $3CL^{pro}$ in the maturation of viral proteins [41-43]. Although AG7088 was suggested as a starting point for the design of anti-SARS drugs [44], it was found unable to inhibit SARS-CoV $3CL^{pro}$ [45], suggesting at least subtle differences in the active-site structures of $3C^{pro}$ and $3CL^{pro}$.

2. Inhibitors of $3C^{pro}$

Previously developed $3C^{pro}$ inhibitors including iodoacetamides, β-lactones, Michael acceptors, ketones and pseudoxazolones are summarized in the review article [46]. However, after 2005, no patent of $3C^{pro}$ inhibitors was found probably due to the most potent $3C^{pro}$ inhibitor AG7088 having been discovered and most research effort was focused on the development of SARS-CoV $3CL^{pro}$ inhibitors. In this review, some more $3C^{pro}$ inhibitors, especially those inhibiting both $3C^{pro}$ and $3CL^{pro}$ discovered recently, are described below.

2.1 AG7088 and analogues

AG7088 (now called Rupintrivir) is a potent, irreversible inhibitor of HRV $3C^{pro}$ developed through a series of studies [47-51]. This peptidomimetic inhibitor (see 1, **Figure 1**) contained a lactam ring to mimic Gln at the P1-position, fluoro-phenylalanine at P2, Val at P3 followed by 5-methyl-3-isoxazole, and an α,β-unsaturated ester at P1′ as a Michael acceptor to form a covalent bond with the active site Cys residue. The P1-lactam-containing inhibitors displayed enhanced $3C^{pro}$ inhibition activity along with improved anti-HRV properties relative to the corresponding glutamine-derived molecules. The inhibitors with amide bond connecting P2 and P3 (e.g., 2, **Figure 1**) gave similar anti-HRV 14 activity.

The activity of AG7088 was tested *in vitro* against five representative HRV serotypes and 46 clinical HRV isolates, identified from patients with common colds. The EC_{50} (median effective concentration, effective in 50% of isolates) for the five serotypes was 0.02 μg/ml (range < 0.01 to 0.03 μg/ml). For the clinical isolates from nasopharyngeal washes, the median EC_{50} was 0.01 μg/ml (range < 0.01 to 0.04 μg/ml) [52]. In experimental HRV challenge studies of AG7088 prophylaxis or early treatment regimens in adults, AG7088 prophylaxis or a placebo was administered intranasally 6 h before the viral challenge and continued two or five times a day for 5 days. For early treatment regimens in adults, administration began 24 h after the challenge. In the prophylaxis studies, viral shedding was reduced in the group receiving AG7088 five times a day. The incidence of colds, total symptom scores, respiratory symptoms and nasal discharge were also reduced, with a trend towards greater effects in those treated five times a day. In early treatment studies, viral titers were reduced by 2 – 3 days after viral challenge. Although AG7088 did not prevent experimental HRV infection, it modestly reduced illness severity given before or within 1 day of infection, with administration five times a day. The most common drug-related adverse events (nausea and taste disturbance) were mild in severity.

In 2001, Schmidt *et al.* conducted a research in which AG7088 was evaluated in 868 subjects enrolled in a Phase II of naturally-acquired picornaviral infections. In their study, only 29% of subjects were found to have illness caused by the picornaviruses, no effect was seen on respiratory symptoms and the drug was well tolerated. In a subset of subjects who started treatment within 24 h or when the symptom just occurred, a trend was noted towards reduction of total and respiratory symptoms [53]. The intranasal compound was later reformulated to optimize delivery of the active ingredient to the nasal cavity.

AG7088 exhibits better potency and a broader spectrum of anti-HRV activity than pleconaril, which blocks viral entry towards clinical HRV isolates [54]. The median EC_{50} value determined by microscopic CPE inhibition was slightly better for AG7088 compared to pleconaril, but was indistinguishable by spectrophotometric assay. In the case of clinical HRV isolates, however, the median EC_{50} value determined for AG7088 either microscopically or spectrophotometrically was < 1.0 μg/ml and was > 10.0 μg/ml for pleconaril.

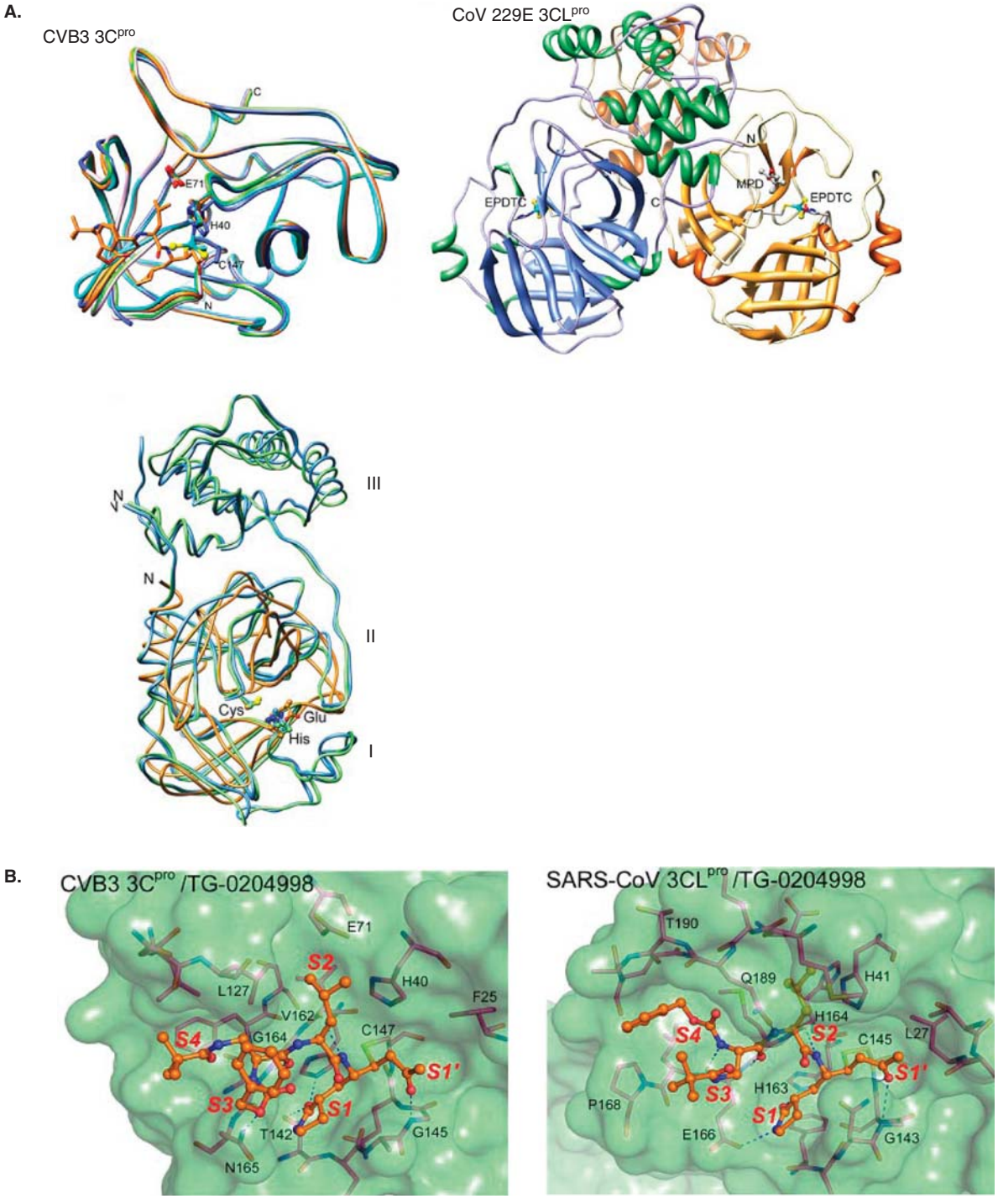
Symptom severity in patients with HRV-induced respiratory illness correlated with elevated levels of inflammatory cytokines IL-6 and -8. AG7088 was tested for its anti-viral activity and ability to inhibit IL-6 and -8 production in a human bronchial epithelial cell line, BEAS-2B. Infection of BEAS-2B cells with HRV-14 resulted in the production of both infectious virus and the cytokines IL-6 and -8. Treatment of HRV-14 infected cells with AG7088 resulted in a dose-dependent reduction in the levels of infectious virus as well as IL-6 and -8 in the cell supernatant. AG7088 is able to inhibit the replication of the virus in BEAS-2B cells [55].

Tian *et al.* from Agouron Pharmaceuticals, Inc. obtained an US patent entitled 'Efficient synthetic routes for the preparation of rhinovirus protease inhibitors and key intermediates' for the application of the AG7088-like compounds with Michael acceptor to inhibit the HRV $3C^{pro}$. They proposed a formula as potential $3C^{pro}$ inhibitors (18, **Figure 2**) without providing their inhibition data [56].

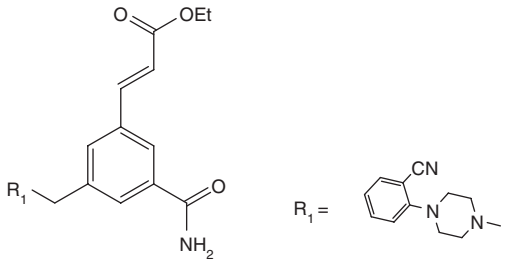
Based on comparative computer modeling, because an Asn residue delineating the S2′ pocket in HRV $3C^{pro}$ was replaced by a Tyr residue in CVB3 $3C^{pro}$, AG7088 was modified by substitution of the ethyl group at the P2′ position with various hydrophobic aromatic rings in order to interact preferentially with the Tyr residue in the S2′ pocket of CVB3 3CP [57]. The resulting derivatives showed dramatically increased inhibitory activities against CVB3 $3C^{pro}$. In addition, one of the derivatives effectively inhibited the CVB3 proliferation *in vitro*.

2.2 Tripeptide aldehydes against HRV $3C^{pro}$

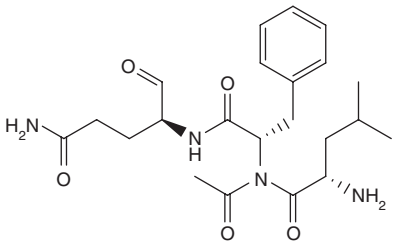
Because peptide aldehydes have been successfully used as inhibitors for Cys and Ser proteases, as well as shown to form reversible covalent adducts, the modified tripeptide aldehydes were designed and synthesized as inhibitors for



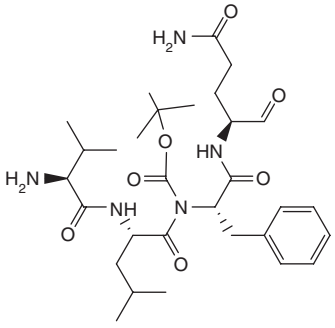
HRV 3C^{pro} [58]. Molecular models based on the crystal structures of HRV-14 3C^{pro} and other trypsin-like serine proteases were constructed to approximate the binding of peptide substrate, generating transition state models of P1–P10 amide cleavage. Because glutaminal derivatives exist predominantly in the cyclic hemiaminal form, several isosteric replacements for P1 carboxamide side chain were designed and incorporated into the tripeptide aldehydes. The synthesized compounds were found to be potent inhibitors of purified HRV-14 3C^{pro} with K_i ranging from 0.005 to 0.65 μM. For example, as shown in 3, **Figure 1**, this compound has low micromolar anti-viral activity, low toxicity and reasonable therapeutic index. Along this line, structure-based design of ketone-containing tripeptidyl HRV 3C^{pro} reversible inhibitors were also reported [59]. Another compound with the 5-methyl-3-isoxazole group at P4-position shown in 4, **Figure 1**,



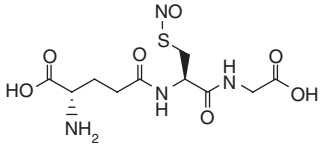
9 (k_{obs}/[I] = 139 $M^{-1}s^{-1}$, EC_{50} = 0.6 μM, CC_{50} = 79 μM)



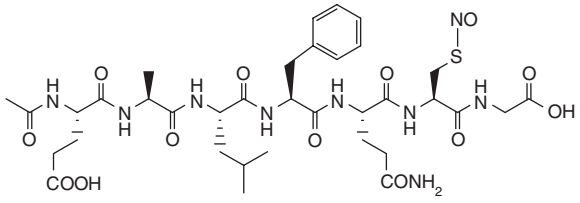
10 [1.4[a], ++[b] (100 μg/mL)]



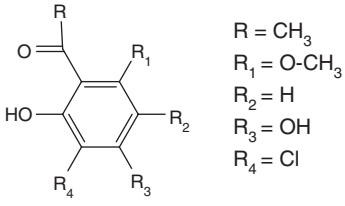
11 [0.2-0.8[a], ++++[b] (5-10 μg/mL), ++[c] (500 μg/mL)]



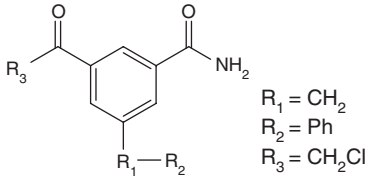
12 GSNO (0.237[a], 0.36[b], 656[c])



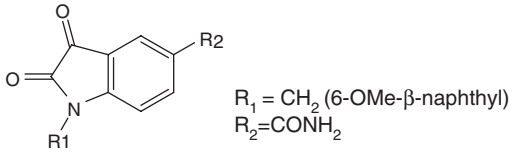
13 EALFQCG-SNO (0.107[a], 0.02[b], 5360[c])



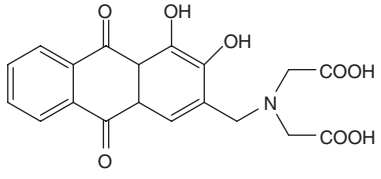
14 (EC_{50} = 11 mM, TD_{50} = 22 mM)



15 (14[a], 79[b], 91[c])



16 (K_i = 3 nM)



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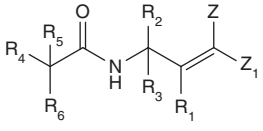

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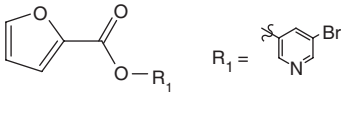
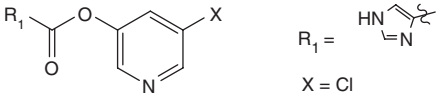


19 (IC_{50} = 0.08 μM)



20 (IC_{50} = 0.29 μM)

Figure 2. Non-peptidomimetic inhibitors of $3C^{pro}$. The symbols (++++) and (++) indicate inhibition > 75% and 50 ~ 25%, respectively. [a]IC_{50} (μM), [b]translation assay and [c]whole cell anti-viral assay.

displayed potent $3C^{pro}$ inhibition activity and *in vitro* antiviral property when tested against HRV serotype-14. Analogues of tripeptide aldehyde (e.g., 5, **Figure 1**) were also synthesized to inhibit picornavirus $3C^{pro}$ [60]. The K_i for the synthesized molecules ranged from 0.0045 to 1.7 μM.

A class of HRV-14 serotype $3C^{pro}$ inhibitors containing a tripeptide as well as a Michael acceptor moiety capable of binding irreversibly to the active site Cys of $3C^{pro}$ was described as agents against RV (e.g., 6, **Figure 1**). The α,β-unsaturated ketone of ethacrynic acid, a diuretic drug, was found to be an appropriate electrophilic moiety. Analysis of the HRV-2 $3C^{pro}$ X-ray crystal structure [61] revealed that only the *trans* P1 Gln amide hydrogen atom interacted with the protease, while the *cis* NH was found to be exposed to the solvent because of methylation at this location.

Dragovich *et al.* (Pfizer) demonstrated animal models of HRV infections to correlate human symptomatology with *in vitro* anti-HRV activities and revealed the significance of observed differences between dog and monkey [50] (e.g., 7 in **Figure 1** showed EC_{50} = 0.058 μM, 7 h dog plasma level = 0.248 μM and 7 h monkey plasma level = 0.057 μM). They illustrated these peptidomimetics, 2-pyridone-containing irreversible HRV $3C^{pro}$ inhibitors, as orally bioavailable, potent, anti-HRV and broad spectrum agents. These compounds containing an α,β-unsaturated ethyl ester fragment and either an ethyl or propargyl P2 moiety presented the most potent combination of $3C^{pro}$ inhibition (k_{obs}/[I] 170,000 – 223,000 M^{-1} s^{-1}), antiviral activity (EC_{50} = 0.047 – 0.058 μM against seven HRV serotypes) and pharmacokinetics following oral administration (7 h dog plasma levels = 0.248 – 0.682 μM; 7 h CM-monkey plasma levels = 0.057 – 0.896 μM).

A group of tripeptide aldehydes with the replacement of α,β-unsaturated ester warhead of AG7088 with aldehyde group were synthesized and evaluated on inhibiting the $3C^{pro}$ of EV strain 71 (EV71) and the viral replication [62]. Compared to the complicated procedure to synthesize AG7088, which requires synthesis and assembly of three moieties, potent AG7088 analogues can be easily synthesized and used as anti-EV71 agents. In contrast to the HRV $3C^{pro}$ inhibitors, which contain Gln or unnatural amino acid at P1, Phe at P2 and Cbz-Leu at the P3-position, showing moderate EC_{50} in the micromolar range [58], compound 8 (**Figure 1**) with the lactam ring at the P1-position, Phe at P2, cinnamoyl derivatives at P3 and aldehyde group exhibited great inhibitory activities in enzymatic and anti-viral assays (EC_{50} = 0.018 μM) without cytotoxicity (CC_{50} > 25 μM) [62]. Therefore, the P1-lactam group is important and Leu or Val at the P3-position seems not important for the anti-virus activity. However, structural features of P1-lactam, P2-Phe and P1′-α,β-unsaturated ester do not guarantee potent $3C^{pro}$ inhibition. Addition of the cinnamoyl group at P3 and simultaneous replacement of P1′-α,β-unsaturated ester with aldehyde yield potent inhibition as rationalized by the computer modeling [62]. This study provides potent EV71 $3C^{pro}$ inhibitors as effective as anti-EV71 agents and facilitates the combinatorial synthesis of derivatives for further improving the inhibitory activity.

2.3 α,β-Unsaturated keto benzamides

In order to have more favorable pharmacokinetic properties and to develop orally taken $3C^{pro}$ inhibitors, certain substituted benzamides as non-peptide inhibitors of HRV $3C^{pro}$ were invented [63]. α, β-Unsaturated keto benzamides showed good inhibitory property; yet, 5-substituted benzamides were found to be more active (e.g., 9, **Figure 2**).

2.4 Glutamine aldehyde derivatives

Hammond *et al.* (Eli Lilly and Co.) synthesized a series of glutamines derivatives to test anti-HRV activity, titled 'Anti-picornaviral agents in United States Patent 5,821,331' [64]. As described in the patent, Hela cells were incubated overnight at 37°C in 5% CO_2 atmosphere, and then infected with HRV. After allowing the virus to be adsorbed into the cells for 1 – 2 h, a medium containing serial dilutions of inhibitor or medium alone was added to the wells. The concentration of inhibitor required to inhibit the development of a viral-induced cytopathic effect by 50% (IC_{50}) (μg/ml) was then determined from the linear portion of each dose response curve. Their inhibitors 10 and 11 are shown as examples in **Figure 2** with the assay results ([a]IC_{50} (μM), [b]translation assay and [c]whole cell anti-viral assay).

2.5 S-nitrosothiols analogues

HRV $3C^{pro}$ was also inactivated by a series of S-nitrosothiols [65], which exhibited inhibitory activities in a time- and concentration-dependent manner with second-order rate constants (K_{inact}/K_I) ranging from 131 to 5360 M^{-1} min^{-1} (e.g., 12, 13 are shown in **Figure 2**, [a]K_{inact} (min^{-1}), [b]K_i (μM), [c]K_{inact}/K_i (min^{-1} M^{-1})). As the inactivated enzyme was shown to be reactivated by DDT, GSH and ascorbate, the inactivation process was through an S-transnitrosylation process.

2.6 Benzene analogues

Arad and coworkers from the Cytoclonal Pharmaceutics, Inc. obtained a United States Patent 6,888,033 B1 [66], entitled 'Anti-viral compounds' for a series of the benzene analogues. The ability of benzene derivatives in inhibiting viral $3C^{pro}$ activity was compared with the ability of the compounds in inhibiting the viral life cycle (e.g., 14, **Figure 2**, which showed EC_{50} of 11 μM and TD_{50} of 22 μM). Interestingly, many of the compounds displayed greater *in vivo* anti-viral activity (H1-HeLa cells) than inhibition of the $3C^{pro}$ activity, indicating that the compounds may target more sites than the $3C^{pro}$. The inhibition of cytopathic effect ICE_{50} (the concentration in which 50% of the cells remain viable after viral infection) by inhibitors was measured. Compounds were tested at concentrations lower than 50% toxic dose (TD_{50}) previously found.

Maugeri *et al.* used docking studies on the crystallized structure of HRV-2 $3C^{pro}$ to design and synthesize a series of 3,5 disubstituted benzamides (e.g., 15, **Figure 2**, which inhibited 14, 79 and 91% of the $3C^{pro}$ activity at [a]0.1, [b]1, and [c]10 μM) [67]. They also examined 1,3,5-trisubstituted benzamides which contained aromatic substituents to investigate the significance of π–π interaction on the stabilization of the $3C^{pro}$-inhibitor complex. All inhibitors were assayed against HRV-14 $3C^{pro}$. In this report, some 1,3 disubstituted and 1,3,5 trisubstituted benzamides inhibited HRV-14 $3C^{pro}$ > 90% at 10 μM.

2.7 1,5-Disubstituted isatins

A combination of protein structure-based drug design, molecular modeling and structure-activity relationship analysis [68] led to the discovery of a novel series of 2,3-dioxindoles (isatins) as HRV-14 $3C^{pro}$ reversible inhibitors. The isatin C-2 carbonyl was envisioned to react in the active site of HRV $3C^{pro}$ with the Cys responsible for catalytic proteolysis. Molecular modeling using the HRV-14 $3C^{pro}$ crystal structure and a peptide substrate provided the template for building recognition features into P1 and P2 subsites, respectively, from 5- to 1-positions of isatin. The synthesized compounds (e.g., 16, **Figure 2**, which showed a K_i of 3 nM) were found to possess excellent inhibitory properties toward HRV-14 $3C^{pro}$ compared to other proteolytic enzymes, including chymotrypsin and cathepsin B.

2.8 Quinone analogues

Recently, it was claimed that compounds having quinone moiety as well as quinone analogues are useful inhibitors of Cys proteases, in particular, caspases and 3C cysteine proteases [69]. These compounds, as exemplified in **Figure 2**, 17, have been tested against HRVs 1A, 1B and 14 and showed moderate *in vitro* activity with IC_{50} values ranging around sub-micromolar to micromolar. They are assumed to act as active Michael acceptors which are prone to attack by the Cys residue, thereby, disrupting the activity of Cys protease.

2.9 Heteroaromatic esters

Im *et al.* synthesized 31 heteroaromatic esters, non-peptidic inhibitors, to screen against HRV $3C^{pro}$. Substitution of 5-halopyridine with various heteroaromatic rings resulted in the discovery of the 4-quinolinone group as an alternative key structure [70]. Heteroaromatic esters compounds with thiophen-2-carbonyl, benzoyl, phenylpropanoyl groups and cinnamoyl showed lower activities than the 2-furoyl analogues, and the most effective inhibitor with a 5-bromopyridinyl group, having an IC_{50} value of 80 nM (19, **Figure 2**). Substitution of the furan ring C5-group with the aromatic groups retained a high level of inhibition. The aromatic groups could form the π-stacking interaction with histidine (His40) rather than tight binding to S2 pocket. The 2-naphthoyl, 1-naphthoyl and imidazole groups are building blocks showing potent inhibitory activities (IC_{50} = 290 nM for 20, **Figure 2**).

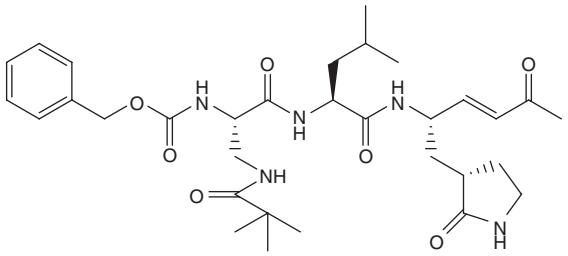
3. Structural basis for different inhibitor specificities of $3C^{pro}$ and $3CL^{pro}$ and their dual inhibitors

As described above, many inhibitors have been developed to inhibit $3C^{pro}$ of HRV and EV and $3CL^{pro}$ of SARS-CoV. However, their inhibitors could not be mutually used without modification. For example, AG7088, a potent inhibitor of picornavirus $3C^{pro}$, failed to inhibit SARS-CoV $3CL^{pro}$ [45] and the AG7088 analogues which showed good inhibition on $3CL^{pro}$ did not significantly inhibit $3C^{pro}$ [71]. The different inhibitor specificity of $3C^{pro}$ and $3CL^{pro}$ indicate at least subtle differences in the active-site structures of these two kinds of proteases. These structural differences to discriminate the inhibitors are described below. However, the dual $3C^{pro}$/$3CL^{pro}$ inhibitors which equally inhibited $3C^{pro}$ and $3CL^{pro}$ have been discovered recently [72] as also summarized below.

3.1 Structural differences of $3C^{pro}$ and $3CL^{pro}$ for discriminating inhibitors

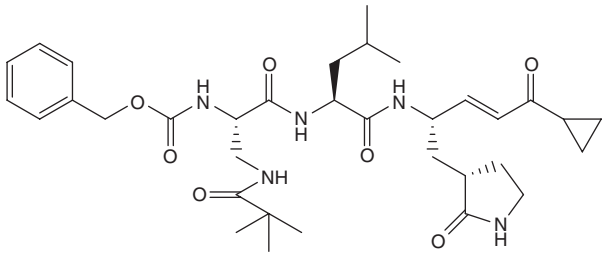
AG7088 only inhibits $3C^{pro}$ strongly, but the compounds TG-0204998 and TG-0205221 retaining the P1′ α,β-unsatured esters but changing P2 and P3 groups show better inhibition against $3CL^{pro}$ than $3C^{pro}$ (**Figure 3A**). First, unlike $3CL^{pro}$, which is dimeric and in which each subunit is composed of three domains I, II and III, $3C^{pro}$ is a monomer with only two catalytic domains I and II (**Figure 4A**). Second, based on structure-based sequence alignment, $3CL^{pro}$ has a large loop between the β-strands C1 and D1, whereas $3C^{pro}$ has smaller loops inserted between E1 and F1 and between B2 and C2 [73]. The C1-D1 loop of SARS-CoV $3CL^{pro}$ secures the S2 hydrophobic pocket for the P2 side chain. The two loops E1-F1 and B2-C2 of CVB3 $3C^{pro}$ are also adjacent to the active site, and they modulate binding of the P3 and P4 residues. For the peptidomimetic inhibitors, both TG-0204998 and TG-0205486 bind to the active site of SARS-CoV $3CL^{pro}$ in similar modes, whereas TG-0204998 binds differently to CVB3 $3C^{pro}$ (**Figure 4B**). The P2-cyclohexyl side chain of an inhibitor, TG0205221, fits well in the S2 site of SARS-CoV $3CL^{pro}$ but is too bulky for CVB3 $3C^{pro}$, whereas the P2-Leu of another inhibitor, TG0204998, fits well in both. The CVB3 $3C^{pro}$ E1-F1 loop makes the S2 site shallow and open. This is consistent with its 46-fold higher K_i of TG-0205221. On the other hand, CVB3 $3C^{pro}$ structure is analogous to that of RV $3C^{pro}$, and a P2-phenyl side chain may be preferred by CVB3 $3C^{pro}$ as evidenced from the tight binding of AG7088 to HRV $3C^{pro}$ [74]. Moreover, the P3 t-butyl group is favored for tight binding to the SARS-CoV $3CL^{pro}$ S3 site, which enhances the inhibition by > 10-fold [72]. Conversely, the AG7088 P3-Val fits HRV $3C^{pro}$ very well, but the additional t-butyl group makes the compounds weaker CVB3 $3C^{pro}$ inhibitors. With the t-butyl group, the bulky P3-residue is actually relocated to the hydrophobic environment in the S4 site formed by the CVB3 $3C^{pro}$ B2-C2 loop, leaving the unbound P4-benzoxy

A.



0.8[a], 0.058[b]

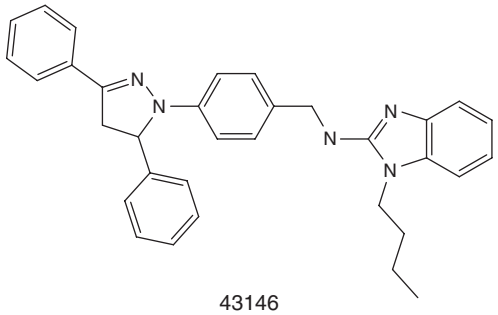
TG-0204998



0.4[a], 0.099[b]

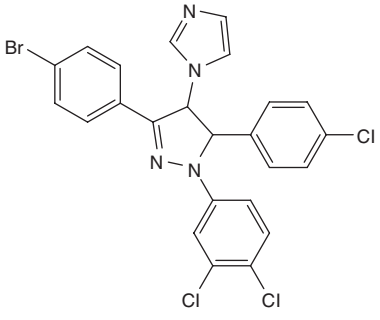
TG-0205486

B.



8.1[a], 10.3[b], 5.4[c], 3.3[d], 5.2[e]

43146



2.5[a], 2.6[b], 1.2[c], 0.5[d], 1.7[e]

45240

Figure 3. Discriminating and dual 3C^{pro}/3CL^{pro} inhibitors. A. Peptidomimetic compounds as better inhibitors against [a]CVB3 3C^{pro} than against [b]SARS-CoV 3CL^{pro}. **B.** 43146 and 45240 as inhibitors of 3CL^{pro} from [a]SARS-CoV and [b]HCoV229E, as well as 3C^{pro} from [c]CVB3, [d]EV71, and [e]HRV14.

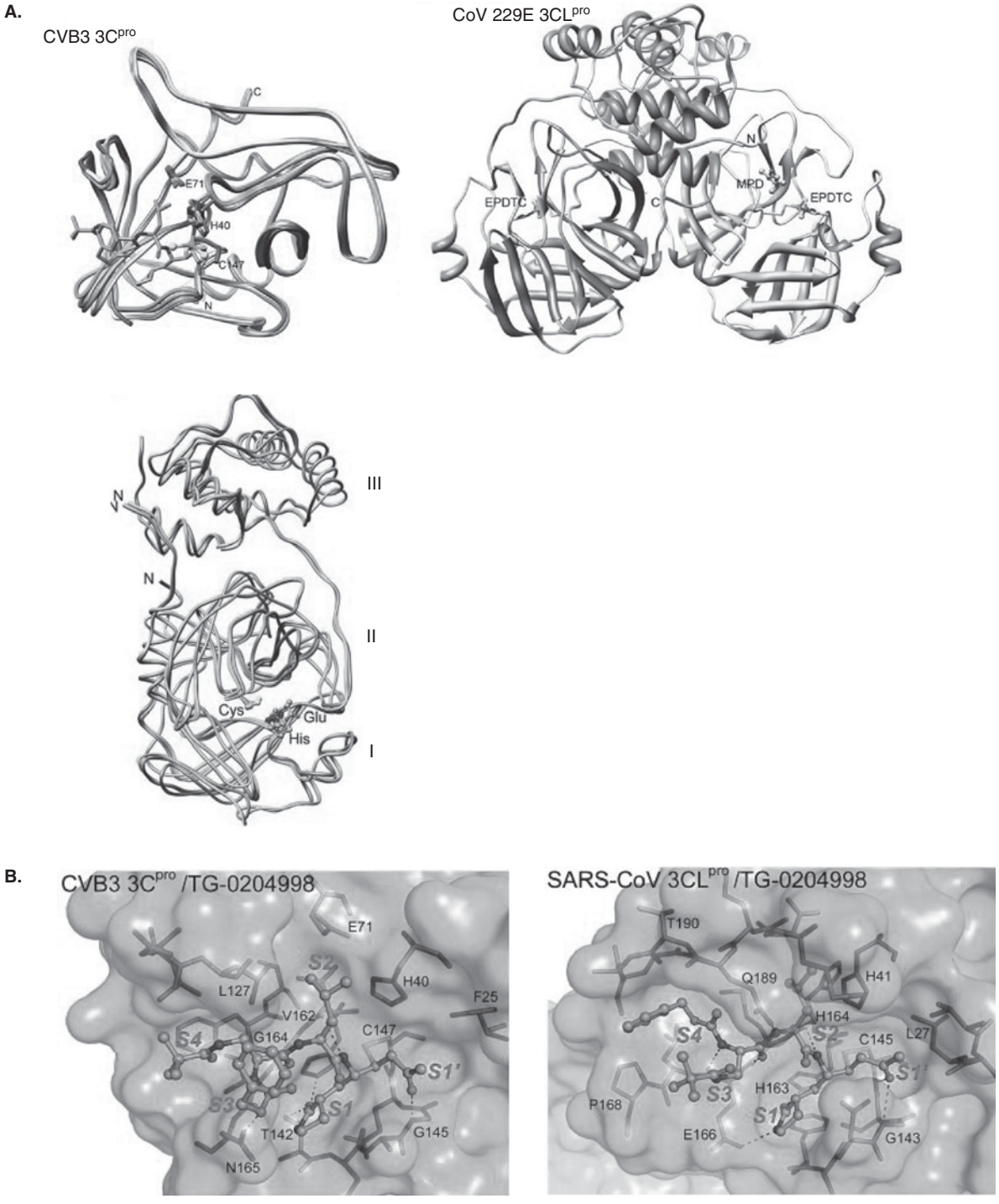


Figure 4. Structural basis of inhibitor specificity of 3C^{pro} and 3CL^{pro}. A. Crystal structures of CVB3 3C^{pro} (upper left) and CoV229E 3CL^{pro} (upper right), and the superimposed structures colored in orange, light blue and green for CVB3 3C^{pro}, SARS-CoV 3CL^{pro} and CoV229E 3CL^{pro}, respectively (bottom). **B.** Co-crystal structures of TG-0204998 bound with CVB3 3C^{pro} and SARS-CoV 3CL^{pro}. (Readers are referred to the full-colour version, available at http://informahealthcare.com/loi/etp)

group facing the bulk solvent [73]. This may also contribute to the higher K_i. Removal of the P3 t-butyl group or the entire P4 residue may improve the inhibitors against CVB3 $3C^{pro}$.

In these inhibitors, the P1 site favors Gln or its mimicking lactam ring, and the lactam ring provides 15-fold better inhibitory activity than Gln as revealed by the previously reported structure-activity relationships. Strong binding of the lactam ring to the proteases is evidenced by the multiple hydrogen-bond formations in the crystal structures [73]. Addition of a cyclopropyl group to the TG-0205486 P1′ residue enhances the inhibition against CVB3 $3C^{pro}$ by almost four-fold as compared to TG-0203770, but it becomes weaker against SARS-CoV $3CL^{pro}$. The triangular group tended to clash with the protein atoms, due to the more limited space of the S1′ site adjacent to the loop C1-D1. In CVB3 $3C^{pro}$, the S1′ site is more open, yet still flanked by the hydrophobic side chain of Phe25.

3.2 Dual $3C^{pro}$/$3CL^{pro}$ inhibitors

Through high-throughput screening on a library of ~ 6800 compounds provided by Korean Chemical Bank (Faejeon, Korea), compounds which can inhibit both $3C^{pro}$ from HCVB3, HEV71 and HRV14 as well as $3CL^{pro}$ from SARS-CoV and HCoV229E were identified [72]. These inhibitors as shown in **Figure 3B** contain a dihydropyrazole ring in the center surrounded by 3 or 4 groups. The diphenyl 4,5-dihydro-1H-pyrazole moiety of 43146 fits well at the S1′ and S2 sites in the SARS $3CL^{pro}$ with the rest of the molecule at the S3 site and beyond. With this binding mode, the compound was predicted to also bind well in the $3C^{pro}$, consistent with the inhibition data. In fact, RV $3C^{pro}$ prefers a phenyl group at the S2 site, as evidenced by its strong inhibition by AG7088 which has a P2-fluorophenylalanine. Thus, it could be rationalized by computer modeling that only 43146 among the five hits can inhibit the $3C^{pro}$ in addition to the $3CL^{pro}$. The analogues of 43146, such as 45240 as shown in **Figure 3B**, bind in the $3C^{pro}$ and $3CL^{pro}$ active sites with similar modes to that of 43146. 45240 showed significantly better inhibition against the $3C^{pro}$ than 43146. Apparently, the lengthy side chain attached to the phenyl group in the compound did not provide additional interaction with the protease, consistent with the binding mode deduced from computer modeling. However, the additional interaction may be provided by the pyridine ring bound near the more open S1′ site in $3C^{pro}$.

4. Expert opinion

In this review, the inhibitors against $3C^{pro}$ are summarized and the structural basis of inhibition specificities of $3C^{pro}$ and $3CL^{pro}$ by peptidomimetic compounds described. The tripeptide aldehyde and the tripeptides with the α,β-unsaturated group as Michael acceptor (e.g., AG7088) for the active-site Cys are the potent inhibitors of $3C^{pro}$. AG7088 has been shown to be effective in inhibiting many $3C^{pro}$ from a broad spectrum of picornavirus, but failed to inhibit SARS-CoV $3CL^{pro}$. The AG7088 analogues with P2 cyclohexyl moiety and additional P3 t-butyl group favor the binding with the $3CL^{pro}$. Crystal structures can be used to elucidate the subtle changes between the active-site structures of $3C^{pro}$ and $3CL^{pro}$ [73-77]. The separate efforts of developing inhibitors against $3C^{pro}$ [78] and $3CL^{pro}$ [79-81] may be merged to provide the rationale to design selective potent inhibitors against each type of the proteases, that is, with suitable modifications, the inhibitors of $3CL^{pro}$ can be converted into the inhibitors of $3C^{pro}$ and vice versa. Moreover, a group of compounds were found to inhibit $3C^{pro}$ and $3CL^{pro}$ almost equally, providing a possibility of developing dual inhibitors against both proteases. These exciting discoveries will lead to more varieties of inhibitors, which can then be potentially used to treat the diseases caused by picornavirus and CoV.

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Declaration of interest

The authors state no conflict of interest and have received no payment in preparation of this manuscript.

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