Recent development of 3C and 3CL protease inhibitors for anti-coronavirus and anti-picornavirus drug discovery

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Abstract

SARS-CoV (severe acute respiratory syndrome-associated coronavirus) caused infection of ∼8000 people and death of ∼800 patients around the world during the 2003 outbreak. In addition, picornaviruses such as enterovirus, coxsackievirus and rhinovirus also can cause life-threatening diseases. Replication of picornaviruses and coronaviruses requires 3C^{pro} (3C protease) and 3CL^{pro} (3C-like protease) respectively, which are structurally analogous with chymotrypsin-fold, but the former is a monomer and the latter is dimeric due to an extra third domain for dimerization. Subtle structural differences in the S2 and S3 pockets of these proteases make inhibitors selective, but some dual inhibitors have been discovered. Our findings as summarized in the present review provide new potential anti-coronavirus and anti-picornavirus therapeutic agents and a clue to convert 3CL^{pro} inhibitors into 3C^{pro} inhibitors and vice versa.

Introduction

Viral infections are renowned as a major leading cause of acute morbidity in individuals of all ages worldwide [1]. Respiratory viral infective disease is the major threat to all ages, particularly children under 5 years of age [2]. The primary viral pathogens associated with acute respiratory infections include picornaviruses, CoVs (coronaviruses), adenoviruses, parainfluenza viruses, influenza viruses and respiratory syncytial viruses [3,4]. Recently, a human CoV emerged as life-threatening and highly transmissible disease causing SARS (severe acute respiratory syndrome) and thus the virus was named SARS-CoV. SARS-CoV is a positive-stranded RNA virus with a relatively large genome of ∼30 kb. The genome constitutes five major open reading frames, namely replicase polyproteins, nucleocapsid proteins, spike, envelope and membrane glycoproteins. SARS-CoV typically causes respiratory and enteric diseases, pneumonia, exacerbation of asthma, neurological symptoms and myocarditis in humans and domestic animals [5–7]. The initial outbreak of SARS was first identified in Guangdong Province, China, in November 2002. This outbreak spread to many countries and had a significant impact on health and economies. The mortality rate is nearly 10% [8]. The resulting structural and functional studies of the coronaviral life cycle has provided a number of significant targets for stopping viral replication. During SARS-CoV replication, the replicase polyproteins undergo extensive processing by two viral proteases, namely a chymotrypsin-like protease called 3CL^{pro} (3C-like protease) since it is analogous to 3C^{pro} (3C protease) in picornavirus and PL^{pro} (papain-like protease),

which reside within the polyprotein. They catalyse their own release from the polyprotein and the maturation of other NSPs (non-structural proteins) to initiate virus-mediated RNA replication [9]. These proteases, especially 3CL^{pro}, are attractive targets for developing anti-SARS agents [10].

Analogous to CoV, *Picornaviridae* family members such as CV (coxsackievirus), EV (enterovirus) and RV (rhinovirus) also contain positive-sense single-stranded RNA, but with smaller genomes (∼7.5 kb) [11]. These picornaviral infections are responsible for causing fever, headache, sore throat and gastrointestinal distress, as well as chest and muscle pain, known as pleurodynia or Bornholm disease in many areas. In some cases, the symptoms progress to myocarditis or pericarditis, which can result in permanent heart damage or death. Similarly to SARS-CoV, CV contains a virally encoded chymotrypsin-like protease named 3C^{pro} responsible for viral replication [12].

To date, no effective antiviral drugs for CV and SARS-CoV infections are available except for over-the-counter drugs for symptomatic relief. Also the existing drugs approved for other viral infections have failed against CV and SARS-CoV. Hence it is necessary to find new leads or drugs to meet medical needs. From our efforts, several classes of inhibitors of 3CL^{pro} have been identified (see below), among which one class of compounds also inhibit 3C^{pro}. Crystal structures of both 3C^{pro} and 3CL^{pro} with the same inhibitors have been solved and compared to reveal the subtle differences in the active sites. This provides an opportunity to convert 3CL^{pro} inhibitors into 3C^{pro} inhibitors and vice versa.

SARS-CoV 3CL^{pro} inhibitors

Using a fluorigenic peptide as a substrate for assay [13], we have reported various SARS-CoV inhibitors obtained from screening of compound libraries and rational design

based on the crystal structures of the product-bound form of 3CL^{pro} [14]. These inhibitors, as shown in Figure 1, include zinc-conjugates (**1**) [15,16], C_2-symmetric diols (**2a** and **2b**) [17,18], peptidomimetic α,β-unsaturated esters (**3**) [19], anilides (**4**) [20], benzotriazole (**5**) [21], *N*-phenyl-2-(2-pyrimidinylthio)acetamide (**6**) [22], biphenyl sulfone (**7**) [23], natural products including tea polyphenols (**8**) [24] and plant terpenoids and lignins (**9** and **10**) [25], as well as glutamic acid and glutamine peptides possessing a trifluoromethylketone group (**11**) [26], pyrimidinone (**12**) [27], four hits (**13–16**) from a chemical library [28], and pyrazole analogues (**17–19**) that also inhibit 3C^{pro} (dual inhibitors), as described in more detail below [28,29]. Their IC$_{50}$ or K_i values against either only 3CL^{pro} or both 3C^{pro} and 3CL^{pro} are shown below their structures in Figure 1.

Dual 3CL^{pro} and 3C^{pro} inhibitors

Among the 3CL^{pro} inhibitors identified so far, we identified pyrazoles **17** and **18** (Figure 1) as dual inhibitors for 3C^{pro} of CV-B3, EV-71 and RV-14 as well as 3CL^{pro} of SARS-CoV and CoV-229E [28]. Compound **18** showed better inhibition against 3CL^{pro} (IC$_{50}$ 2.5 μM) and 3C^{pro} (IC$_{50}$ 1.7 μM) than **17** (IC$_{50}$ 8.0 μM and 7.7 μM for inhibiting 3CL^{pro} and 3C^{pro} respectively), indicating that the lengthy butyl-benzimidazole group in compound **17** did not provide any other additional interaction with the proteases and that the additional interaction is provided by the imidazole ring of **18**. The diphenyl 4,5-dihydro-1*H*-pyrazole moiety of **17** fits well at the S1′ and S2 sites in the SARS-CoV 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 is evident from its strong inhibition by AG7088 which has a P2-fluorophenylalanine (see Figure 2A for its structure).

With the help of further modelling studies, we designed and synthesized some pyrazolones as dual inhibitors of 3CL^{pro} and 3C^{pro} [29]. Compound **19** showed inhibitory activity against both SARS-3CoV 3CL^{pro} (IC$_{50}$ 8.4 μM) and CV-B3 3C^{pro} (IC$_{50}$ 9.6 μM) with no cytotoxicity at 200 μM [29]. The SARS-CoV 3CL^{pro}-inhibiting activity of the above analogues is dependent on the carboxy group bound at the S3 site and a nitro group at S1 site to form hydrogen bonds with Gln^{192} and Gly^{143} respectively. The C3 phenyl ring forms a hydrophobic interaction at the S2 site. The same compound was docked with CVB3 3C^{pro} and shows that the C3 phenyl ring of **19** is pointed to the S1 site and the carboxyl benzylidene group is relocated to S2 in order to form the hydrogen bond in 3C^{pro} due to the subtle differences between the structures of 3CL^{pro} and 3C^{pro} [30].

Structural differences between 3C^{pro} and 3CL^{pro}

Peptidomimetic inhibitors [31] and zinc-conjugated inhibitors [15] have been co-crystallized with both 3C^{pro} and 3CL^{pro}

to reveal the structural differences of the two proteases, as a basis for rationalizing inhibitor specificities [30]. As mentioned above, unlike 3CL^{pro}, which is dimeric containing three domains in each subunit, 3C^{pro} is a monomer with only two domains forming catalytic site. However, domains I and II from both proteases superimpose well, despite subtle differences. On the basis of structure-based sequence alignment, 3CL^{pro} has a large loop between β-strands C1 and D1, whereas 3C^{pro} has smaller loops inserted between E1 and F1 and between B2 and C2 [30]. The CVB3 3C^{pro} E1–F1 loop makes the S2 site more shallow and open. Therefore AG7088 with planar fluorophenylalanine at P2 and the smaller valine at P3 designed for inhibiting 3C^{pro} failed to inhibit SARS-CoV 3CL^{pro} [19] and TG0204998 with the non-planar leucine at P2 and larger t-butylcarboxamide at P3 shows remarkably improved inhibitory activity against 3CL^{pro} (Figure 2A). As shown in the left-hand panel of Figure 2(B), AG7088 binds well to CV-B3 3C^{pro} as expected, since it was originally developed as potent inhibitor against 3C^{pro} of human RV, but TG0204998 binds poorly to CV-B3 3C^{pro} as compared with its binding with SARS-CoV 3CL^{pro}, as shown in the right-hand panel of Figure 2(B). Thus the bulky P3 residue of TG0204998 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 group facing the bulk solvent (see left-hand panel of Figure 2B). AG7088 fails to bind SARS-CoV 3CL^{pro} due to the steric hindrance exerted by the fluorophenylalanine group against Arg^{188} in the S2 pocket and by the isoxazoyl moiety against the hydrophobic residues if overlaying AG7088 with TG0204998 in the right-hand panel of Figure 2B. These two Figures rationalize the different inhibitor specificities of 3C^{pro} and 3CL^{pro}.

Future perspectives

No effective therapy has been developed so far, and the recent isolation of strains of SARS-CoV emphasizes the possibility of a re-emergence. Therefore it is still a great challenge to explore new chemical classes of SARS-CoV 3CL^{pro} inhibitors that can be used in anti-SARS therapy in case the disease re-emerges. There is also no anti-picornaviral drug targeting 3C^{pro} in the market yet, although AG7088 (now named rupintrivir), which contains a lactam ring to mimic glutamine at the P1 position and an α,β-unsaturated ester at P1′ as a Michael acceptor to form a covalent bond with the active-site cysteine residue, shows promising results against a broad spectrum of picornaviruses [32]. We have identified several classes of 3CL^{pro} inhibitors and some can also inhibit 3C^{pro}. Structural studies have revealed that 3C^{pro} is a monomer, whereas 3CL^{pro} is a dimeric protein which has an extra third domain for dimer formation, although their active sites are superimposed very well [30]. Moreover, 3C^{pro} has a more open but shallow S2 site, as well as a smaller S3 site, which account for different inhibitory specificity. The information discussed in the present review helps to ultimately develop drugs against infectious CoVs and picornaviruses, which should be continuously pursued.

Figure 1 | Inhibitors of SARS-CoV 3CL^pro and dual inhibitors for 3CL^pro/3C^pro derived from our studies
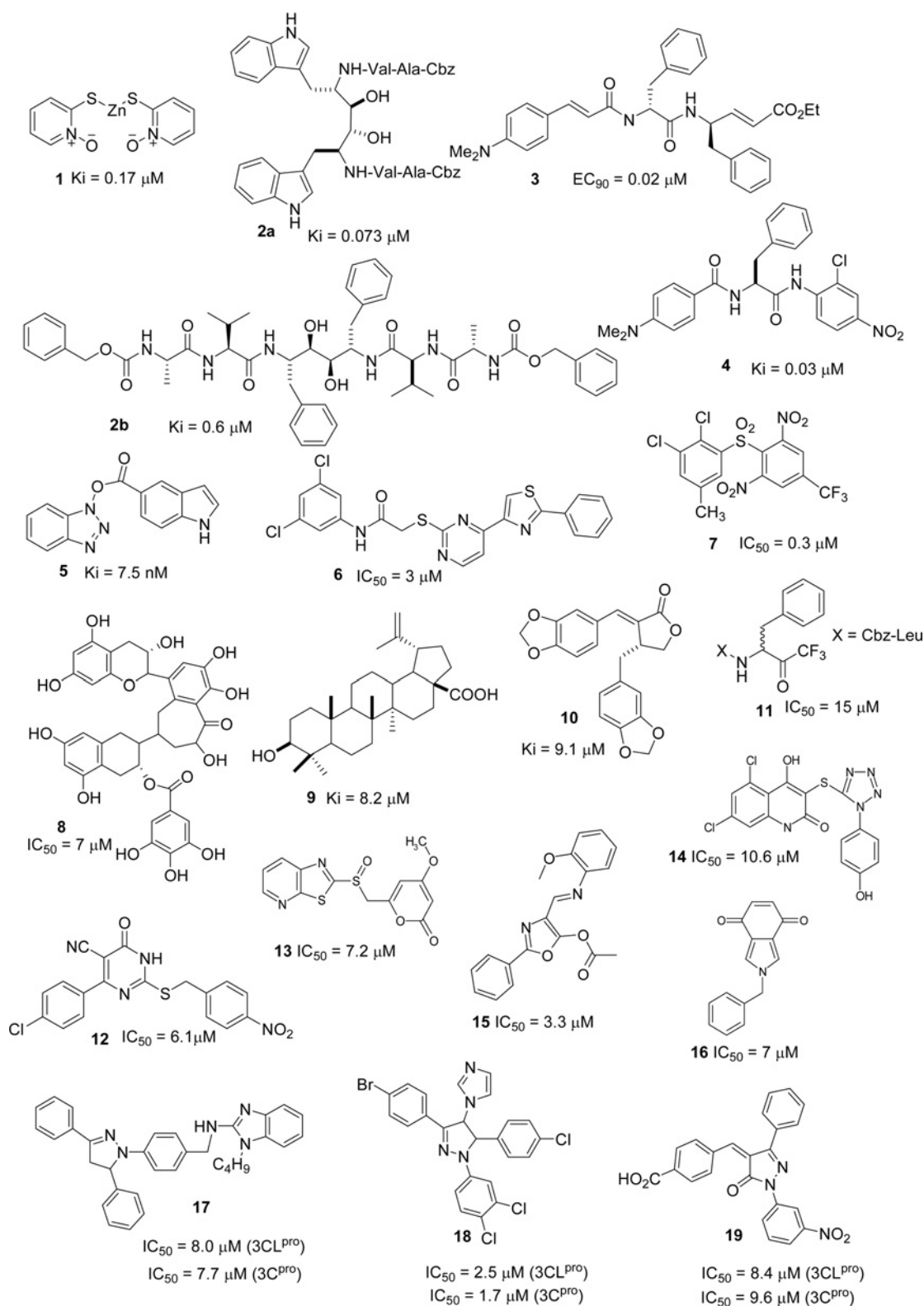
See the text for further details.

Figure 2 | AG7088 and its analogue as a potent 3C and 3CL protease inhibitor respectively

(**A**) The structure and inhibitory constants of AG7088 and TG-0204998. (**B**) Left-hand panel: structures of AG7088 (cyan) and TG-0204998 (green) binding with CVB3 3C^{pro}. AG7088 is modelled in the active site based on the previously solved crystal structure of AG7088 with 3C^{pro} of a human RV (PDB code 1CQQ). Right-hand panel: structure of TG-0204998 (green) binding with CVB3 SARS-CoV 3CL^{pro}. The PDB codes for the crystal structures of TG-0204998 binding with CVB3 3C^{pro} and SARS-CoV 3CL^{pro} are 2ZU3 and 2ZU4 respectively.
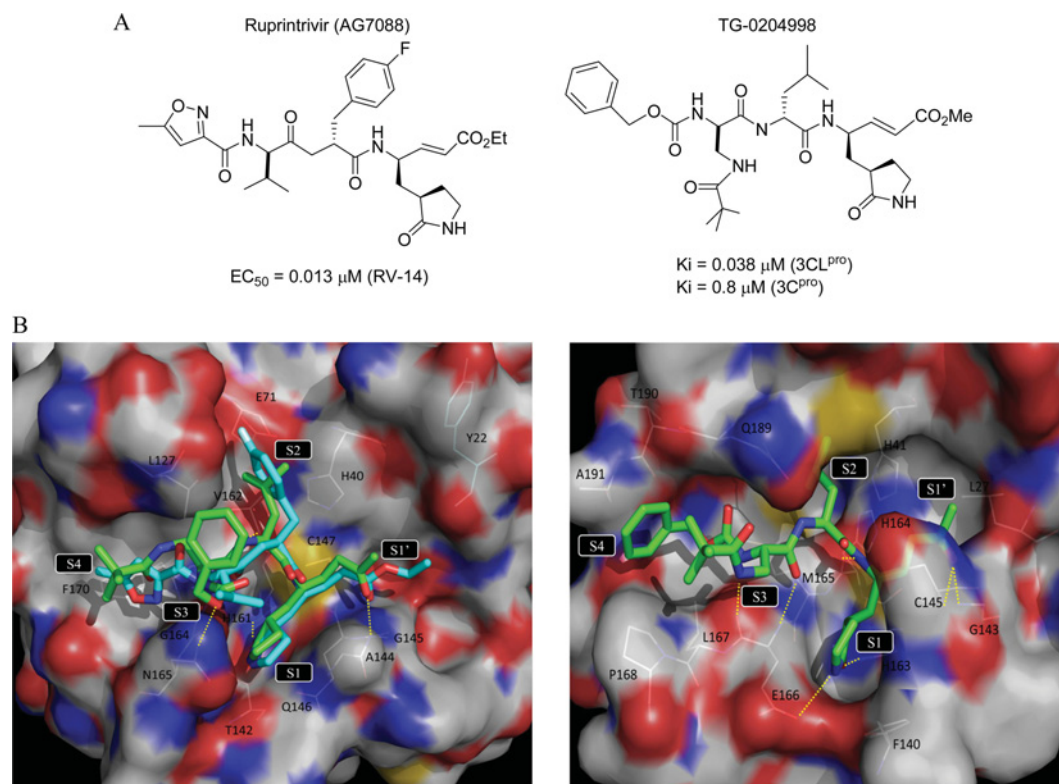
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