Potential therapies for coronaviruses

Andrea Savarino, Canio Buonavoglia, Sandro Norelli, Livia Di Trani & Antonio Cassone

Department of Infectious, Parasitic and Immune-mediated Diseases, Istituto Superiore di Sanità, Viale Regina Elena, 299 00161 Rome, Italy.

Coronavirus replication offers several attractive targets for chemotherapy. These include: viral entry (inhibited by chloroquine and peptides); viral RNA (targeted by antisense approaches/RNAi); the main protease 3CLpro (inhibited by peptidic molecules such as HIV-1 protease inhibitors and miscellaneous compounds); the accessory protease(s) PLpro(s) (inhibited by zinc ions); RNA-dependent RNA polymerase (inhibited by aurintricarboxylic acid and antisense approaches); and helicase (inhibited by bananins). Chloroquine and HIV-1 protease inhibitors (with well-known toxicity profiles) should be considered for clinical tests if severe acute respiratory syndrome (SARS) re-emerges; however, there are other attractive compounds. Lessons should be learnt from AIDS research for choosing the best strategies.

Keywords: angiotensin-converting enzyme 2, antiretrovirals, aurintricarboxylic acid, canine coronavirus, chloroquine, SARS-CoV, small interfering RNAs

Expert Opin. Ther. Patents (2006) 16(9):1269-1288

1. Introduction

Coronaviruses were isolated from chickens in 1937 and were long considered to be important pathogenic agents in animals, producing seasonal cold or mild gastrointestinal infections in humans. It was not until the outbreak of the severe acute respiratory syndrome (SARS) during 2002/2003 that extensive efforts went into the research for specific anticoronavirus therapies. The emergence of the SARS coronavirus (SARS-CoV), capable of killing ~900 people in a few months, clearly indicated that more resources had to be put into coronavirus research. In the 3 years after 2003, more potential anticoronaviral compounds have been patented than in the previous decades of coronavirus research. Here, the authors review the published patents/patent applications on the most relevant compounds/approaches for potential anticoronaviral therapies, focusing on those approaches targeting one specific step of the coronavirus life cycle as they are more likely to be used as lead compounds for further drug development. In case the reader needs information on an anticoronavirus drug that has been left ‘orphan’ of a molecular target, they are directed to excellent reviews in [1,2]. Finally, the authors highlight those compounds that are likely to have an immediate clinical application in case SARS re-emerges.

2. Principal drug targets and specific inhibitors

2.1 The spike glycoprotein/receptor interaction

Coronaviruses display a rather complex life cycle (schematised in Figure 1). To enter target cells, these viruses exploit a wide variety of cellular receptors. Transmissible gastroenteritis virus (TGEV), human coronavirus (hCoV)-229E and canine coronavirus (CCoV) use a cell membrane-bound metalloprotease, aminopeptidase N, also referred to as CD13. CD13 is widely distributed in cells in many tissues, including respiratory, enteric epithelial, neuronal and glial cells [3]. SARS-CoV and another human coronavirus, hCoV-NL63, both use a cell-surface zinc peptidase,
Potential therapies for coronaviruses

Figure 1. The life cycle of SARS-CoV. The life cycle of coronaviruses consists of: i) attachment of the S glycoprotein to the cellular receptors; ii) endocytosis of the viral particle/receptor complex; iii) uncoating of the virus and entry of the viral positive-sense RNA; iv) direct translation of the non-structural proteins (nsps) from the replicate gene 1; v) cleavage of the 1a and 1b polyproteins by means of a main protease (3CLpro) and one or two papain-like proteases (PLpro) embedded in the polyproteins; vi) synthesis of an RNA filament complementary to the genomic RNA; vii) synthesis of new genomic RNAs or mRNAs for the structural proteins; and viii) assembly and gemmation of the new virions.

angiotensin-converting enzyme 2 (ACE2) as their receptor [4,5]. Interesting insights on this viral/cellular protein interaction have been uncovered by crystallographic studies. The crystal structure of the ACE2 ectodomain [6,7] shows a claw-like N-terminal peptidase domain, with the active site at the base of a deep groove, and a C-terminal ‘collectrin’ domain. The spike (S) glycoprotein of SARS-CoV is responsible for binding ACE2. A fragment of the S glycoprotein’s S1 region, residues 318 – 510, is sufficient for tight binding to the peptidase domain of ACE2 [8]. This fragment, the receptor-binding domain (RBD), is the critical determinant of virus–receptor interaction and, thus, of viral host range and tropism [9]. The crystal structure at 2.9 Å resolution of the RBD bound with the peptidase domain of human ACE2 was recently resolved by Li et al. (Protein Data Bank/PDB accession: 2AJF) and shows that the RBD presents a concave surface, which cradles the N-terminal lobe of the peptidase (the ACE2 peptidase domain has two lobes that close toward each other after substrate engagement) [6]. The SARS-CoV S glycoprotein contacts the tip of one lobe of ACE2. It does not contact the other lobe, nor does it occlude the peptidase active site. Binding of the S protein to ACE2 is not altered by the addition of a specific ACE2 inhibitor [9]. These crystallographic data suggest that specific SARS-CoV inhibitors could be developed to block the ACE2/S protein interaction and these SARS-CoV inhibitors should be different from ACE2 inhibitors blocking the peptidase activity (inhibitors of the ACE2 enzymatic activity have been patented by Millennium Pharmaceuticals, Inc. [101]). The atomic details at the interface between the two proteins described by Li et al. [9] give important information for virtual screening of antiviral compounds. Alanine-scanning mutagenesis analysis indicated that, in ACE2, charged amino acids between residues 22 – 57 were important, K26 and D30, in particular [10].

A soluble form of ACE2 has been described for potential SARS treatment [101]. Although it may be applied as a decoy for neutralising circulating viruses, this approach could hardly find a clinical application, as suggested by lessons
learnt from AIDS research. A similar approach, that is, the use of soluble CD4, the main receptor for HIV-1, was postulated for the treatment of HIV-1/AIDS. Despite the in vitro data being encouraging [11], soluble CD4 has not found a clinical application in ~20 years.

Specific inhibitors of the S glycoprotein/ACE2 interaction have been produced using isolated portions of the S glycoprotein [102]. Using a system in which a lentivirus expressing green fluorescent protein and pseudotyped with the SARS-CoV S glycoprotein infects 293T cells stably expressing ACE2, the inventors detected potent antiviral activity of a peptide comprising amino acids 318–510 of the S glycoprotein (IC$_{50}$ < 10 nM). Approaches using peptides in order to prevent viral entry are encouraged by results from AIDS research. Indeed, proof-of-concept has been provided that systemic administration of a peptide from a highly conserved region of the HIV-1 viral envelope glycoprotein gp41, significantly impacts on viral load [12]. An easy strategy to prevent the attachment of a virus to a cell is the use of neutralising antibodies. Monoclonal antibodies directed to the S glycoprotein of SARS-CoV have been produced [103]. These antibodies inhibit the interaction of the virus with ACE2. This strategy may be applied in passive immunotherapy or immunoprophylaxis after exposition to SARS-CoV, and, based on the affinity of these antibodies with the S1 region responsible for binding ACE2, this invention might be exploited in screening assays for anti-SARS-CoV compounds. Compounds binding the S glycoprotein at the site for interaction with ACE2 should also prevent the interaction with the antibodies.

Another strategy to elicit neutralising antibodies is the use of specific vaccines [104]. However, the description of vaccination strategies would go beyond the scope of this review, which deals with therapeutic approaches.

Another group of strategies against coronaviruses is aimed at targeting the cellular receptor rather than the virus itself. Receptor down-modulation is postulated to be a valuable approach to limit the entry of coronaviruses inside target cells [105]. An invention uses double- and single-stranded oligomeric antisense compounds, particularly single- or double-stranded oligonucleotides (RNA or RNA-like), and single-stranded oligonucleotides (DNA or DNA-like) for use in modulating expression of aminopeptidase N [105]. Multiple mechanisms exist by which short synthetic oligonucleotides can be used to modulate gene expression in mammalian cells. These include degradation by RNase H or RNA interference (RNAi; an excellent review on these mechanisms can be found in [9]). An advantage of this approach would be the extreme selectivity due to the oligonucleotide match requirement, while a disadvantage would be the extreme complexity in the administration of these molecules. Furthermore, it is not yet known what would be the effect of ACE2 down-modulation in humans.

The most promising approach to limit the entry of a coronavirus into target cells is probably represented by an old antimalarial drug, chloroquine (1), which has the advantage of being a small molecule and, therefore, presenting no difficulties in administration. Past studies have supported the idea that chloroquine might inhibit coronavirus replication. Cells infected with hCoV-229E and treated with nocodazole (a microtubule depolymerising agent that blocks transport from early to late endosomes) produced decreased amounts of hCoV-229E antigens [13]. This result indicated that endosomal transport is needed for hCoV-229E infection and cells treated with chloroquine expressed decreased amounts of hCoV-229E antigens [13]. Meanwhile, unpublished data obtained from the authors’ group showed that chloroquine potently inhibited replication of CCoV at therapeutically achievable concentrations (Figure 2). As: i) chloroquine is also endowed with anti-inflammatory properties (reviewed in [14]); and ii) clinical worsening of individuals with SARS in week 2 is apparently related to immunopathological damage [15], some of the authors suggested that chloroquine be considered in the treatment of SARS and tested against SARS-CoV [14]. The authors’ hypothesis was confirmed in two independent in vitro studies. Researchers at the Belgian Catholic University of Leuven found that chloroquine inhibited SARS-CoV replication with an EC$_{50}$ value of 8.8 ± 1.2 μM, within the range of blood concentrations achievable during antimalarial treatment [16]. The dose-inducing 50% cytostatic activity was much higher, that is, 261.3 ± 14.5 μM. Time-of-addition experiments indicated that chloroquine affected an early stage of SARS-CoV replication [16]. Researchers at the Centers for Disease Control and Prevention (Atlanta, GA, USA) reported potent anti-SARS-CoV effects of chloroquine in vitro, attributable to a deficit in the glycosylation of the SARS-CoV receptor ACE2 [17]. Again, the antiviral drug concentrations were not cytotoxic. These results await confirmation in animal models. As discussed by Van Ranst et al., chloroquine is given prophylactically to people travelling to malaria-endemic areas. If SARS re-emerges, chloroquine can be of great importance as prophylactic medication for people living in and travelling to the affected area. Chloroquine is ubiquitously available, of low cost and easy to administer. The drug is generally well-tolerated and may also be administered to pregnant women. However, one disadvantage could be the possibility of retinopathy in cases of chronic exposure to the drug, such as those of prolonged prophylactic use. Nevertheless, this side effect is reversible at the early stages and, thus, can be prevented by regular examination of the fundus.

The use of quinoline compounds, including chloroquine, in combination with a multivitamin preparation has been claimed to treat different viral infections including those caused by coronaviruses [106]. Patent [107] discloses an invention consisting of an adhesive patch containing an essential oil and an antimicrobial compound, such as chloroquine, in order to filter and kill pathogens such as coronaviruses entering the respiratory tract. Given the mechanism of action of chloroquine (acting on cells rather than on the coronavirus itself), this invention will not result in the anticonvirus effect of chloroquine.
2.2 Viral RNA
The genomic RNA of coronaviruses contains a capped, polyadenylated, single-stranded, positive-sense genomic RNA that is 27 – 32 kb in length and is the largest known RNA virus genome. Coronavirus-infected cells contain a characteristic 3′ coterminal and nested mRNA. The mRNA has a capped leader sequence consisting of < 70 nucleotides that is derived from the 5′ end of the genome. A non-translated region (UTR) of 200 – 400 nucleotides follows this leader sequence. Another UTR at the 3′ end of the RNA genome is followed by a polyA tail of variable length. Both the 3′- and 5′-UTRs are important in RNA replication and transcription, as is the transcription-regulatory sequence, which is a typical feature of coronaviruses. This short motif is usually near the beginning of each open reading frame (ORF) and the 3′ end of the leader sequence. Moreover, the consensus sequence, 5′-CUAAAC-3′, is found immediately in front of the S protein, and membrane (M) protein genes and ORF 10 [3]. Gene organisation in most coronaviruses follows the same pattern of genes coding for polyproteins 1a and 1b, S, M, envelope protein and nucleocapsid protein (Figure 1). Some group II coronaviruses possess a haemagglutinin esterase. Coronavirus genomes also contain a variety of additional ORFs that encode 2 – 4 nonstructural proteins (nsps) with no known function; these genes are not conserved among coronaviruses. Whereas the genes encoding the functional proteins are directly translated from genomic RNA, production of the structural proteins requires the synthesis of specific mRNAs by the functional proteins (Figure 1) [3]. Both the genomic and the mRNAs can be targeted by the RNA interference technique described in the previous section [3]. The use of small interfering RNAs (siRNAs) has been claimed for the treatment of SARS in humans [108]. An advantage of this approach would be the expected low toxicity due to the extreme selectivity of coronavirus inhibition. Again, a disadvantage could be the complexity in the administration of these molecules. In this context, encouraging results are derived from the SARS-CoV-infected *Rhesus* macaque model. Intranasal administration of chemically synthesised siRNA duplexes resulted in the reduction of: i) SARS-CoV infection-induced fever; ii) SARS-CoV viral levels; and iii) acute diffuse alveoli damage. Macaques did not show any toxic effects [18].

2.3 3C-like protease
Following cell infection, the viral replicase gene (fundamental for genomic RNA replication and structural protein production) is translated directly from the viral genome [19]. Autocatalytic processing by two types of proteases, which are part of the replicase polyprotein, releases nsps [20-22]. These form a membrane-bound RNA replication complex [23,24]. One of the two types of coronaviral proteases, that is, the 3C-like protease (3CLpro), resides in nsp5 and, after autocleavage, releases the downstream replicase subunits [21]. 3CLpro is responsible for cleavages at most polyprotein processing sites and, thus, is termed the ‘main’ protease, whereas coronaviral PLpro(s) cleave only at the three N-terminal polyprotein processing sites and, thus, are termed ‘accessory’ protease(s). 3CLpro is a cysteine protease. In the active site of SARS-CoV 3CLpro, Cys145 and a His41 form a catalytic dyad [25]. Cysteine proteases usually display a catalytic triad consisting of serine, histidine and aspartate; however, the 3D structure of SARS-CoV 3CLpro shows no aspartate residue in the vicinity of Cys145 and His41. SARS-CoV 3CLpro is a homodimer with each monomer comprising three domains [25]. The first two domains of 3CLpro show a cysteine protease-like fold with a root mean square deviation from bovine α-chymotrypsin of only 2.6 Å [22]. The folding of the first two domains is responsible for the catalytic reaction, whereas the third domain is α-helical and plays a critical role in enzyme dimerisation [26]. The cysteine protease-like folding of 3CLpro is intriguing because the overall sequence identity between the 3CLpro and α-chymotrypsin is quite
low (11%). Chymotrypsin-folding of 3CLpro raised the hypothesis that 3CLpro was originally a chymotrypsin-like serine protease that later evolved into a cysteine protease [27]. The potential usefulness of 3CLpro as a drug target is supported by: i) its fundamental role in coronavirus replication; ii) its well defined 3D structure; and iii) preliminary clinical observation indicating that drugs cross-targeting this enzyme, that is, the HIV-1 protease inhibitors (HIV-1 PIs; 2 – 6) produced some clinical benefits in patients treated with IFNs and ribavirin.

The most thoroughly studied group of drugs targeting 3CLpro are represented by HIV-1 PIs. Clinical, virological, biochemical and bioinformatic data concur in suggesting 3CLpro as the main target for the anticovalviral effect of HIV-1 PIs, although robust data providing a causal link between 3CLpro inhibition and impairment of SARS-CoV replication are still lacking.

The story of HIV-1 PIs as a potential SARS treatment began during the 2003 SARS outbreak with the empirical administration to affected individuals of the antiretroviral preparation Kaletra (Abbott), containing lopinavir (2) and ritonavir (3) at a ratio of 4:1 (w/w) [28]. A retrospective matched cohort study of Chen et al. [29] found that the addition of lopinavir/ritonavir to standard initial treatment protocols was associated with a reduction in the overall death rate (2.3%) and intubation rate (0%) when compared with a matched cohort who received standard treatment (15.6 and 11.0%, respectively, p < 0.05), and with a lower necessity of methylprednisolone at high doses. In another study [30], Chu et al. evaluated the effects of Kaletra in association with ribavirin, a drug that causes mutations in the RNAs of many viruses. The adverse clinical outcome (acute respiratory distress syndrome or death) was significantly lower in the treatment group than in the historical controls treated with ribavirin alone (2.4 versus 28.8%, p < 0.001) at day 21 after the onset of symptoms.

The clinical effects of antiretroviral drugs on SARS were initially attributed to non-specific anti-inflammatory effects, but in vitro studies later on showed that some members of the HIV-1 PI class could indeed exert direct antiviral effects against SARS-CoV [31]. The HIV-1 PI nelfinavir (4; 10 µM), but not ritonavir (3), significantly and efficiently inhibited viral antigen expression (measured by immunofluorescence), the production of virions (measured by real-time reverse transcriptase-polymerase chain reaction [RT-PCR]) and the cytopathic effect (measured by the methyl tetrazolium assay) in Vero E6 cells infected with SARS-CoV at a multiplicity of infection of 0.01. Moreover, time-of-addition experiments showed that nelfinavir inhibited SARS-CoV replication at a postentry step and that lopinavir (3; 6.4 µM) inhibited the cytopathic effect of SARS-CoV (measured by plaque-reduction assay) in fetal rhesus kidney-4 (fRHK-4) cells [30,31]. Of note, the effects of lopinavir were synergistic to those of ribavirin, a drug shown to be otherwise ineffective in the treatment of SARS [30]. Therefore, Chu et al. attribute this antiviral synergism the clinical benefits observed in individuals with SARS and treated with ribavirin plus Kaletra [30]. Chen et al. [32] screened the effects of different antiviral compounds against ten clinical isolates of SARS-CoV. These compounds included the nucleosidic reverse transcriptase inhibitors (NRTIs) zidovudine and stavudine, the non-nucleosidic reverse transcriptase inhibitor (NNRTI) nevirapine, and the PIs ritonavir and lopinavir. Again, only lopinavir resulted in exerting detectable effects against SARS-CoV. The EC50 value of lopinavir was in the range of 1.6 – 6.4, and 6.4 – 12.8 µM at 48 and 72 h postinfection respectively, in the fRHK-4 cell line [32]. However, they warn that the antiviral effects are variable and cell line-dependent. For example, the EC50 value of lopinavir against the prototype SARS-CoV strain 39849 was in the range of 3.2 – 6.4 µM in fRHK-4 cells, and 6.4 – 12.8 µM in vero cells [32]. Therefore, it is likely that the antiviral effects of some anti-SARS compounds reported in the literature have been exaggerated or underestimated depending on the assay and cell line used by the different groups. In this regard, the example of ribavirin is interesting. Some report that it is an inhibitor of SARS-CoV replication, whereas others report that it exerts its antiviral effects only at concentrations too high to be reached in vivo [30,32,33]. The latter view is also supported by clinical data.

The limited experimental evidence available for an inhibitory effect of PIs on 3CLpro is supported by computational simulations revealing that the catalytic site of 3CLpro allows the docking of several HIV-1 PIs. Clinically used anti-HIV-1 PIs only partially fill the binding cavity of 3CLpro, but a recent bioinformatic study [34] indicates stable ligand–protein interactions. Among three well-known HIV-1 PIs, that is, indinavir (5), saquinavir (6) and ritonavir (3), only ritonavir was found to display significant hydrogen bonding with 3CLpro. These observations are in line with previous calculations of other groups using 3CLpros of SARS-CoV and TGEV [35,36]. Among the various compounds tested, Zhang et al. [36] indicated ritonavir as the compound with the highest binding affinity (Kd = 5.6 x 10-25 M). These authors attribute to the other Kaletra component, lopinavir, a Ki value of 8.7 x 10-20 M [34]. Instead, Enwiwheesuk et al. [35] attribute a Ki value of 10-7 M to ritonavir. More recent calculations attribute a Ki value of 10-9 M to ritonavir [34]. This Ki value is more realistic than those previously reported given that HIV-1 PIs, at the highest concentrations tested (i.e., 10 µM), have been determined to inhibit the 3C-like protease only partially [31]. One advantage of HIV-1 PIs is that these are among the very few drugs for which clinical evidence for potential usefulness in the treatment of SARS is provided, although it is still very limited. A randomised trial would be needed to validate these results if SARS were to return. A disadvantage of Kaletra might be found in the adverse effects on lipid metabolism and by the high costs of these molecules.

There is no patent specifically devoted to claiming the use of HIV-1 PIs as SARS-CoV inhibitors. However, one of the previously quoted patents [107] claims the use by inhalation of different compounds, including all principal HIV-1 PIs.
Potential therapies for coronaviruses

against different respiratory infections, such as those caused by coronaviruses. Administration of these compounds by inhalation should allow the achievement of local drug concentrations sufficient to inhibit viral replication and to avoid systemic side effects.

Other peptidic inhibitors of 3CLpro are being developed. Different peptidic inhibitors of 3CLpro, such as compounds 7–10, have been described together with procedures for their synthesis [109]. Published application [110] describes methods for the synthesis of other SARS-CoV 3CLpro inhibitors. Biological data on the activity of some compounds against SARS-CoV are also supplied. Compound 11 was found to have an EC$_{50}$ value of 2.1 µg/ml and IC$_{50}$ value of > 50 µg/ml, which gave a selectivity index (SI) of > 24. Compound of Example 24 (12) was found to have an EC$_{50}$ value of 0.02 µg/ml and IC$_{50}$ value of > 10 µg/ml, suggesting a potentially high therapeutic index. The same compound inhibited 100% of the cytopathic effect of SARS-CoV at 1 µM, with no detectable toxicity in cell cultures. For two other compounds, only the data on inhibition of the viral cytopathic effect are provided. At 1 µM, compound 13 inhibited 100% of the cytopathic effect of
SARS-CoV, whereas compound 14 inhibited it by 94.5%. Not enough data are available on this group of drugs to draw further conclusions; however, the in vitro data seem to be encouraging.

Based on the structural similarity between the 3C protease of rhinoviruses and coronaviral 3CLpro, the use of the rhinovirus inhibitors, such as compounds 15–23, against SARS-CoV has been proposed by Pfizer and Agouron Pharmaceuticals [111,112]. These compounds contain the ethyl-acylate ester moiety as terminal warhead and, thus, act as Michael acceptors. According to docking studies conducted by the inventors, the nucleophilic carbons in position 2,3 should covalently bind to the active site of SARS-CoV 3CLpro, thus inactivating it irreversibly. Other Michael acceptor inhibitors of 3CLpro (24–44) are described in Pfizer’s patent application [111] and similar compounds, such as 45 and 46, have been claimed by Agouron Pharma [113]. Although the inventors describe the procedures for testing these compounds in infected cell cultures, only molecular docking data are provided. Other inhibitors presenting an acrylate group have been described and include a natural substance, namely, cinanserin (47) [114].

In general, nucleophilic protease inhibitors, due to their theoretical capacity to covalently inactivate 3CLpro, are expected to potently inhibit SARS-CoV in vivo. However, the safety of these molecules should be carefully tested in cell cultures and animal models, given the potential toxicity of nucleophilic molecules.

Other miscellaneous inhibitors of 3CLpro have been described, such as dicyclic or multicyclic compounds 48–72 [115], and molecules presenting the backbone (73) [116]. These chemically diverse molecules are in too early a phase of testing to allow any conclusions to be drawn. Invention [117] describes boron-containing 3CLpro inhibitors. These inhibitors are possible protease inhibitors and may have the backbones (74–88), wherein the R1,4 moieties can be H or different substituents, including aromatic cyclic compounds or heterocycles; X, Y and U are various linker groups, and Q is represented by different moieties, including ketones, alkanes, alkenes or cyclic compounds. The inventors chose to use boron-containing compounds to inhibit coronavirus 3CLpro based on the use of boric acid and various boronic acids as inhibitors of β-lactamases (proteases and β-lactamases have in common the capacity of hydrolysing an amide bond). These compounds are effective in inhibiting 3CLpro in enzymatic assays, but no biological data from infected cells are provided. Again, it is too early to draw any conclusions about these particular molecules, but the use of boron-containing compounds to inhibit viral proteases seems to be interesting in light of recent crystallographic data using the HIV-1 protease [37].

### 2.4 Papain-like protease(s)

The processing of the amino-proximal nsps is carried out by one or two paralogous protease domains within nsp3, the largest of the nsps [21] and they are defined by homology to the papain-like fold [20] and constitute the peptidase family C16 [38]. Although mutational analyses supported the presence of a Cys-His catalytic dyad [21,39], it is now clear that these enzymes also have a catalytic triad Cys-His-Asp [40]. Most coronaviruses harbor two such papain-like protease sequences (PLpro), PL1pro and PL2pro, whereas SCoV and the avian infectious bronchitis coronavirus (IBV) utilise only one, which is equivalent to PL2pro [41]. PL2pro may cleave downstream of nsp3 [38,41], but only upstream cleavages were associated with PL1pro [21,42,43]. In actual fact, coronaviral PLpros, although built on the common papain-like scaffold, look structurally more like ubiquitin-specific proteases (USPs, MEROPS family C19) than like papain (MEROPS family C1) [38,40]. Coronaviral PLpros have a two-domain organisation with a (circularly permutated) Zn-finger domain nested in the middle of the papain-like fold, precisely as in the case of USPs [20,40]. PLpros have been recently shown to possess deubiquitinating activity [42]. This might be a means by which coronaviral proteins may escape the proteasome degradation pathway, thus limiting their antigenic presentation on HLA class 1 molecules and avoiding the immune responses. These recent observations strengthen the hypothesis that PLpro(s) may be an important drug target for therapeutic interventions. Although substances inhibiting SARS-CoV PLPpro have been described [43], no patents or patent applications have been published yet on the search engines consulted. It is hoped that the recent resolution of its structure [42] will help in the development of new effective inhibitors of coronavirus replication.

Interestingly, zinc ions inhibited the protease activity potently with an IC₅₀ value of 1.3 µM [43]. The inhibition is specific because other divalent metals, such as Mg, Mn, Ca, Ni and Co, had no effect on the activity of SARS-CoV PLP2 at 10 µM (data not shown). Therefore, PLpro inhibitors patentable in the future are likely to contain zinc.

### 2.5 RNA-dependent RNA polymerase

One of the proteins released by the proteolytic activity of the 3CLpro main protease is nsp12, acting as a RNA-dependent RNA polymerase (RdRp; nsp12). Coronaviral RdRp catalyses the production of new full-length genomic RNAs to be packaged into virions as well as a nested set of monocistronic mRNAs that encode all structural proteins. These activities require the formation of intermediate negative-sense RNA (Figure 1). Due to its pivotal role in viral RNA synthesis and, consequently, in protein synthesis and genome duplication, the 106 kDa RdRp represents an attractive target for anti-SARS therapy [44]. However, there is a lack of structural and biochemical information on any coronavirus RdRp, and structural predictions are complicated by the fact that the coronavirus RdRps are significantly diverged from cellular and viral RNA polymerases. Recently, a structure model was built for the catalytic domain of the SARS-CoV RdRp [45]. The model provides first insights into the active site of the
Potential therapies for coronaviruses

Chemical structures of potential coronaviral inhibitors:

- Structure 24
- Structure 25
- Structure 26
- Structure 27
- Structure 28
- Structure 29
- Structure 30
- Structure 31
- Structure 32
- Structure 33
Potential therapies for coronaviruses
Potential therapies for coronaviruses

![Chemical structures](image)

---

*Expert Opin. Ther. Patents (2006) 16(9)*
Potential therapies for coronaviruses

Aim at preventing degradation by RNAses include reversi-
molecules through the plasma membrane. Modifications
The last of these properties facilitates the transition of these
RNAs, and increase hybridisation properties or lipophilicity.

A patent application claims the use of aurintricarboxylic
acid (ATA; 89) as an inhibitor of SARS-CoV RdRp [118].
According to the inventors, ATA inhibits SARS-CoV replica-
tion in Vero cells by ≥ 100-fold at 0.4 mg/ml and by
> 1000-fold at 0.8 mg/ml. A significant inhibition of S glyco-
protein production was observed in SARS-CoV-infected cells
treated with ATA as a result of inhibition of viral protein syn-
thesis. No detectable toxicity was observed in uninfected Vero
cells treated with similar concentrations of ATA. However,
ATA is quite a non-specific inhibitor of different DNA/RNA
polymerases. Thus, the potential usefulness of ATA per se
should be considered with caution, given the past experience
in testing this compound as a HIV-1 integrase inhibitor.
Despite the initial encouraging results, this compound was
abandoned due to insufficient specificity for HIV-1 integrase
inhibition. In the case of coronaviruses, research on this com-
pound should not be abandoned, given the paucity of
reported RdRp inhibitors. For example, ATA could serve as a
valuable lead for the development of specific inhibitors of
coronaviral RdRp.

SARS-CoV RdRp could also be inhibited by oligonucle-
otides and mimics thereof [119]. The appropriate sequence of
bases (indicated as Bx in the following structural formulae)
may be created by regular oligonucleotides or oligonucleo-
tide-like molecules. These oligonucleotide-like molecules
might have multiple applications. Modified oligonucleotides
and their mimics have been designed to prevent degradation
by RNAses before they come into contact with the viral
RNAs, and increase hybridisation properties or lipophilicity.
The last of these properties facilitates the transition of these
molecules through the plasma membrane. Modifications
aimed at preventing degradation by RNAses include reversi-
bly protected oxygens or substituents in position 2′ (90),
and dicyclic substituents of the ribose (91). To increase the bind-
ing properties to a target RNA, the use of tricyclic heterocyclic
moieties, such as compound 92, has been taken into account.
Peptide nucleic acids (93) using the uncharged amide bond
instead of the phosphate diester linking adjacent riboses could
not only increase hybridisation properties, but also facilitates
the passage of the molecule through the cellular membrane.
Morpholino-based oligomeric compounds (94) are non-ionic
mimics of oligonucleotides more likely to pass through the
cellular membrane and less likely to form complexes with pro-
teins as compared with their oligonucleotide counterparts.
The same invention takes into account the use of cyclohexen-
yl nucleic acids (CeNA). These molecules have the general
formula of compound 95 where the furanose ring is replaced
with a cyclohexenyl ring. In general, the incorporation of
CeNA monomers into a DNA chain increases the stability of
a DNA/RNA hybrid. Apart from their potential for RNAi
strategies, these molecules might be used for forming com-
plexes with the viral positive-sense RNA, thus hindering its
transcription/duplication. The inventors are aware of the
potential difficulties with the systemic administration of these
molecules, and consider the inhalation route as a possible way
of administering the molecules to humans.

2.6 NTPase/helicase

Another protein released by the proteolytic activity of
3CLpro is nsp13, a NTPase–helicase (Hel) that specifically
unwinds polynucleotide duplexes with a 5′ to 3′ polarity.
Hels from hCoV-229E and SARS-CoV have very similar
properties: a 5′ to 3′ polarity of unwinding; and a stimulation
of ATPase activity by single-stranded polynucleotides. The
ability of Hel to hydrolyse a wide variety of ribonucleoside-
diphosphates (NTPs) and deoxyribonucleosidetriphosphates
dNTPs) is consistent with the triphosphate moiety forming
the basis of the recognition factor for binding and hydrolysis.
It is possible that this lack of selectivity in NTP hydrolysis
also enables the helicase to act as a broad RNA 5′-triphos-
phate in viral 5′ capping. The basal ATPase activity of coro-
aviral helicase is stimulated by most polynucleotides 15- to
25-fold, with the exception of poly(G) and poly(dG). A sig-
nificant variation in strength of homopolynucleotide binding
was reported, suggesting that the SARS-CoV helicase may
possess elements of secondary structure/sequence recogni-
tion capability. It has been hypothesised that this specificity
may play a role in the localisation of a putative RdRp–helicase
complex to selected genomic locations during subgenomic
RNA synthesis, augmenting proposed base-pairing factors.
The 5′ to 3′ direction of unwinding suggests that the helicase
may aid the replicative process by disrupting secondary struc-
ture or displacing bound proteins and annealed RNA frag-
ments, putative roles that will require experimental
validation. Coronaviral Hel possesses a cystein-rich putative
metal binding domain at the N-terminal portion that forms
mutational analysis in the related equine anaemia virus and
has been implicated in subgenomic mRNA synthesis, genome
replication and virion biogenesis [46]. Hel is thought to be
essential for nidovirus viability and, therefore, is a potential
target for the development of anti-SARS drugs. Interestingly,
Hel was reported to be inhibited by the adamantine/pyridoxal
conjugates, bananins (95 – 98) [47]. Unfortunately, the authors
found no published patents or patent applications regarding
these compounds in the search engines consulted. In case no
specific patents exist, bananins may serve as valuable lead
compounds for patentable Hel inhibitors.

3. Expert opinion

Many compounds with possible anticonoravirus activity
have been researched, especially after the major SARS
outbreak in 2003. Some of these compounds deserve particular attention because they are drugs that are already registered for other uses in humans. These drugs include the antimalarial chloroquine and HIV-1 PIs. The anthelmintic compound, niclosamide (96) is one such drug; however, its molecular target has not been defined yet [48]. Drugs with well-known and long-studied toxicity profiles represent interesting compounds that might be immediately tested in clinical trials in case SARS re-emerges. The claims covered by the patents contain novel and interesting applications of existing drugs. These include drug administration by inhalation. This route of administration could of course reduce the systemic side effects of the drugs and allow an increase in local concentrations. Increased local concentrations could be particularly important in an anticoronavirus use of drugs designed for other disease conditions. The existence of such patents will probably allow the industrial development of new ways of administering old drugs.

On the other hand, the patents/applications on the use of very promising compounds, such as chloroquine or HIV-1 PIs, in coronavirus infections do not cover all of their possible applications. For the uses of chloroquine and HIV-1 PIs not covered by the existing patents/applications, these drugs might be sold at a lower price, which could be of particular interest for resource-poor countries. As for chloroquine, its use as a prophylactic weapon for residents in, or travellers to, SARS-affected areas (encouraged by the several-decade long use of this drug in antimalarial prophylaxis) is, to the authors’ knowledge, not covered by any patents. The same goes for the use of HIV-1 PIs as a systemic treatment for
potential therapies for coronaviruses. of note, the potential of chloroquine for providing prolonged prophylaxis is unique among the different investigational drugs.

in addition, many patents cover the use of brand new agents inhibiting validated drug targets within the coronavirus life cycle. these compounds will possibly represent new therapeutic weapons if they prove to have satisfactory toxicity profiles and sufficient anticoronavirus activity in animal models. alternatively, they may represent interesting drug leads. of note, no patents or patent applications have been published yet on potential inhibitors of PLP(pro(s)) or helicase. as these enzymes seem to be novel and extremely interesting drug targets due to their multiple functions, it will be necessary in the near future to fill this gap by developing more specific inhibitors.

finally, lessons for the choice of the best antiviral strategies should be learnt from the successes and failures of a quarter-century of research against another viral infection, such as HIV-1/AIDS.

acknowledgements

in part granted by the italian ministry of health-iss-rome under a specific SARS-project.

bibliography

papers of special note have been highlighted as either of interest (*) or of considerable interest (**) to readers.

1. WU Y-S, LIN W-H, HSU J, HSIEH HP: Antiviral drug discovery against SARS-CoV. Curr. Med. Chem. (2006) (In Press).
** A comprehensive review on SARS-CoV inhibitors.
2. DE CLERCQ E: Potential antivirals and antiviral strategies against SARS coronavirus infections. Expert Rev. Anti-Infect. Ther. (2006) 4(2):291-302.
** A comprehensive review on SARS-CoV inhibitors.
3. WU CJ, CHAN YL: Antiviral applications of RNAi for coronavirus. Expert Opin. Investig. Drugs (2006) 15(2):89-97.
4. LI W, MOORE MJ, VASILIEVA N et al.: Angiotensin-converting enzyme 2 is a functional receptor for the SARS coronavirus. Nature (2003) 426(6965):450-454.
5. HOFMANN H, PYRC K, VAN DER HOEK L, GEIER M, BERKHOUT B, PÖHLMANN S: Human coronavirus NL63 employs the severe acute respiratory syndrome coronavirus receptor for cellular entry. Proc. Natl. Acad. Sci. USA (2005) 102(22):7988-7993.
This paper provides interesting structural insights on the SARS-CoV receptor.

7. BARTLAM M, YANG H, RAO Z: Structural insights into SARS coronavirus proteins. *Curr. Opin. Struct. Biol.* (2005) 15(6):664-672.

8. XIAO X, CHAKRABORTI S, DIMITROV AS, GRAMATIKOFF K, DIMITROV DS: The SARS-CoV S glycoprotein: expression and functional characterization. *Biochem. Biophys. Res. Commun.* (2003) 312(4):1159-1164.

9. LI W, ZHANG C, SUI J et al.: Receptor and viral determinants of SARS-coronavirus adaptation to human ACE2. *EMBO J.* (2005) 4(0):1634-1643.

10. HAN DP, PENN-NICHOLSON A, CHO MW: Identification of critical determinants on ACE2 for SARS-CoV entry and development of a potent entry inhibitor. *Virology* (2006) 350(1):15-25.

11. SMITH DH, BYRN RA, MARSTERS SA et al.: Blocking of HIV-1 infectivity by a soluble, secreted form of the CD4 antigen. *Science* (1987) 238(4834):1704-1707.

12. KILBY JM, HOPKINS S, VENNETTA TM et al.: Potent suppression of HIV-1 replication in humans by T-20, a peptide inhibitor of gp41-mediated virus entry. *Nat. Med.* (1998) 4(11):1302-1307.

13. BLAU DM, HOLMES K: Human Coronavirus hCoV-229E enters susceptible cells via the endocytic pathway. *Adv. Exp. Med. Biol.* (2001) 494:193-198.

14. SAVARINO A, BOEULAERT JR, CASSONE A et al.: Effects of chloroquine on viral infections: an old drug against today’s diseases? *Lancet Infect. Dis.* (2003) 3(11):722-727.

15. PEIRIS JS, CHU CM, CHENG VC: Clinical progression and viral load in a community outbreak of coronavirus-associated SARS pneumonia: a prospective study. *Lancet* (2003) 361(9371):1767-1772.

16. KEYAERTSE, VJIGEN L, MAES P: *In vitro* inhibition of severe acute respiratory syndrome coronavirus by chloroquine. *Biochem. Biophys. Res. Commun.* (2004) 323(1):264-268.

**This article describes the first experiments showing an anti-SARS-CoV effect of chloroquine.**

17. VINCENT MJ, BERGERON E, BENJAMINET S: Chloroquine is a potent inhibitor of SARS coronavirus infection and spread. *Virology* (2005) 2(69).

**This research article provides the mechanisms of SARS-CoV inhibition by chloroquine.**

18. LI BJ, TANG Q, CHENG D et al.: Using sRNA in prophylactic and therapeutic regimens against SARS coronavirus in Rhesus macaque. *Nat. Med.* (2005) 11(9):944-951.

**An article furnishing proof-of-concept of the potential benefits of sRNA in vitro.**

19. ZIEBUHR J: The coronavirus replicase. *Curr. Top. Microbiol. Immunol.* (2005) 287:57-94.

20. SULEA T, LINDNER HA, PURISIMA EO, MENARD R: Binding site-based classification of coronaviral papain-like proteases. *Proteins* (2006) 62(3):760-775.

21. ZIEBUHR J, SNIJDER EJ, GORBALENYA AE: Virus-encoded proteases and proteolytic processing in the Nidovirales. *J. Gen. Virol.* (2000) 81(4):853-879.

22. BROCKWAY SM, LU XT, PETERS TR, DERMDY TS, DENISON MR: Intracellular localization and protein interactions of the gene 1 protein p28 during mouse hepatitis virus replication. *J. Virol.* (2004) 78(21):11551-11562.

23. PRENTICE E, MCAULIFFE J, LU X, SUBBARAO K, DENISON MR: Identification and characterization of severe acute respiratory syndrome coronavirus replicase proteins. *J. Virol.* (2004) 78(18):9777-9896.

24. SALONEN A, AHOLA T, KAARAINEN L: Viral RNA replication in association with cellular membranes. *Curr. Top. Microbiol. Immunol.* (2005) 285:139-173.

25. ANAND K, ZIEBUHR J, WADHWANI P, PRENTICE E, MCAULIFFE J, LU X: Coronavirus main proteinase (3CLpro) structure: basis for design of anti-SARS drugs. *Science* (2003) 300(5626):1763-1767.

26. SHI J, WEI Z, SONG J: Dissection study on the severe acute respiratory syndrome 3C-like protease reveals the critical role of the extra domain in dimerization of the enzyme: defining the extra domain as a new target for design of highly specific protease inhibitors. *J. Biol. Chem.* (2004) 279(23):24765-24773.

27. SHAN YF, LI SF, XIU GJ: A novel auto-cleavage assay for studying mutational effects of the active site of severe acute respiratory syndrome coronavirus 3C-like protease. *Biochem. Biophys. Res. Commun.* (2004) 324(2):579-583.

28. CHENG VC, TONG BS, WU AK, CHU CM, YUEN KY: Medical treatment of viral pneumonia including SARS in immunocompetent adult. *J. Infect.* (2004) 49(4):262-273.

29. CHAN KS, LAI ST, CHU CM: Treatment of severe acute respiratory syndrome with lopinavir/ritonavir: a multicentre retrospective matched cohort study. *Med. J.* (2003) 9(6):399-406.

30. CHU CM, CHENG V, HUNG IF et al.: Role of lopinavir/ritonavir in the treatment of SARS: initial virological and clinical findings. *Thorax* (2004) 59(3):252-256.

31. YAMAMOTO N, YANG R, YOSHINAKA Y, AMARI S, NAKANO T, CINATL J: HIV protease inhibitor nelfinavir inhibits replication of SARS-associated coronavirus. *Biochem. biophys. res. commun.* (2004) 318(3):719-725.

32. CHEN F, CHAN K H, JIANG Y, KAO R, LU H, FAN K: *In vitro* susceptibility of 10 clinical isolates of SARS coronavirus to selected antiviral compounds. *J. Clin. Virol.* (2004) 31(1):69-75.

33. STROHER SU, DICARO A, LI Y, STRONG J, AOKI F, PLUMMER F: Severe acute respiratory syndrome-related coronavirus is inhibited by IFN-α. *J. Infect. Dis.* (2004) 189(7):1164-1167.

34. SAVARINO A: Expanding the frontiers of existing antiviral drugs: possible effects of HIV-1 protease inhibitors against SARS and avian influenza. *J. Clin. Virol.* (2005) 34(3):170-178.

**This paper reviews the information available on the use of HIV-1 protease inhibitors against SARS-CoV and provides the results of a molecular docking study.**

35. JENWITHEESUK E, SAMUDRALA R: Identifying inhibitors of the SARS coronavirus protease. *Bioorg. Med. Chem. Lett.* (2003) 13(22):3989-3992.
Potential therapies for coronaviruses

This bioinformatic analysis of drug/enzyme docking provides interesting insights on the use of HIV-1 protease inhibitors as lead compounds for development of 3Clpro inhibitors.

An interesting bioinformatic approach evaluating the fitness for the 3Clpro active site of compounds already approved for use in humans.

From nonpeptide toward noncarbon proteinase with HIV, psychotic and parasite proteinase. Biochem. Pharmacol. (2006)

The papain-like protease(s) (PLpro) – Papain-like protease (PLpro) – Papain-like protease 2 (PLP2) from severe acute respiratory syndrome coronavirus (SARS-CoV): expression, purification, characterization, and inhibition. Biochemistry. (2005) 44:10349-10359.

Patents

101. MILLENNIUM PHARM., INC.: US6194556 (1997).
102. THE BRIGHAM AND WOMEN'S HOSPITAL, INC.: US20050113298 (2004).
103. DANA FARBER CANCER INST., INC.: WO2005060520 (2004).
104. CHIRON CORP.: WO2004092360 (2004).
105. COZEN O'CONNOR PC: US20050073607 (2005).
106. PHOENIX BIOSCIENCES: US20050245502 (2005).
107. SCHWEGMAN, LUNDBERG, WOESSNER, KLUTH: US20040071575 (2003).
108. DICKSTEIN SHAPIRO MORIN, OSHINSKYLLP: WO20040071575 (2003).
109. PFIZER, HOFFMAN: WO2004093860 (2004).
110. AGOURON PHARMA.: US2004235952 (2004).
111. AGOURON PHARMACEUTICALS, INC.: US20060014821 (2006).
112. SHANGHAI INSTITUTE OF PLASMA PHYSICS: WO2004108914 (2004).
113. FISH & RICHARDSON PC: US20060019967 (2005).
114. UNIVER BEIJING: CN1569841 (2005).
115. FULLCRUM PHARMACEUTICALS INC.: WO2005041904 (2005).
116. CANADA NATURAL RESOURCES: WO2005123965 (2005).
117. ISI PHARMACEUTICALS INC.: WO2005020885 (2005).

Affiliation
Andrea Savarino†1 MD,
Canio Buonavoglia2 DVM,
Sandro Norelli3 MS, Livia Di Trani5 PhD &
Antonio Cassone4 MD
†Author for correspondence
1Department of Infectious, Parasitic and Immune-mediated Diseases, Istituto Superiore di Sanità, Viale Regina Elena, 299 00161 Rome, Italy
Tel: 0039 0649902305; Fax: 0039 0649387065; E-mail: asavarino@medscape.com
2Department of Animal Health and Well-being, Faculty of Veterinary Medicine, University of Bari, Strada per Casammallia Km 3, 70010 Valenzano, Bari, Italy
Fax: 0039 0804679843; E-mail: c.buonavoglia@vet.uniba.it
3Department of Food and Animal Health, Istituto Superiore di Sanità, Viale Regina Elena, 29900161 Rome, Italy
Tel: 0039 0649902453; Fax: 0039 0649387077; E-mail: ditrani@iss.it