

# Expert Opinion

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## Potential therapies for coronaviruses

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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 aurointricarboxylic 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, aurointricarboxylic acid, canine coronavirus, chloroquine, SARS-CoV, small interfering RNAs

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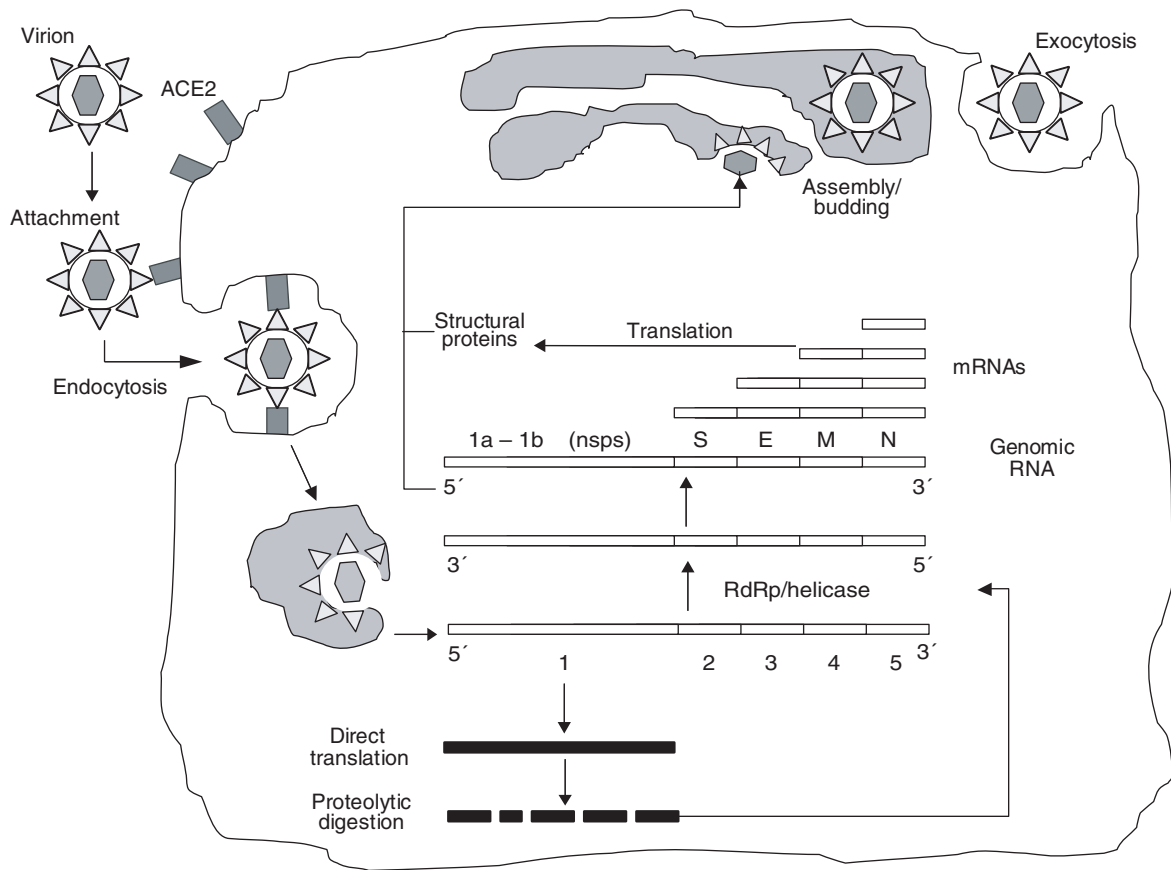
### 1. Introduction

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,



**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 replicase 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 gemination 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.

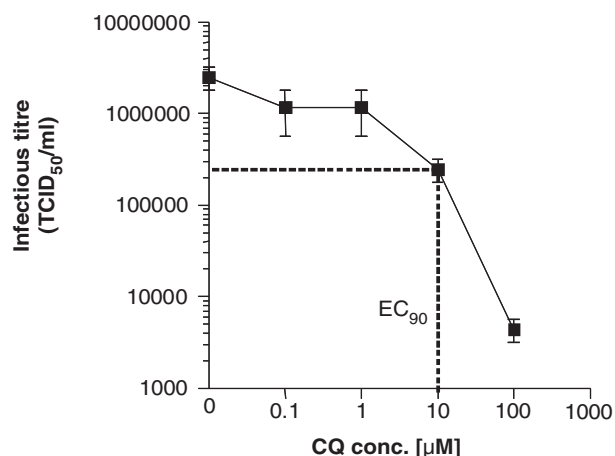
Another strategy to elicit neutralising antibodies is the use of specific vaccines [104]. However, the description of vaccination strategies would go beyond the scope of this review, which deals with therapeutic approaches.

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 [3]). 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  $8.8 \pm 1.2$   $\mu$ M, within the range of blood concentrations achievable during antimalarial treatment [16]. The dose-inducing 50% cytostatic activity was much higher, that is,  $261.3 \pm 14.5$   $\mu$ M. 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.

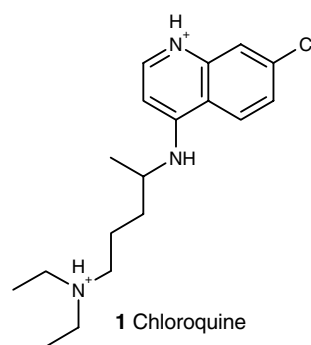
The use of quinoline compounds, including chloroquine, in combination with a multivitamin preparation has been claimed to treat different viral infections including those caused by coronaviruses [106]. Patent [107] discloses an invention consisting of an adhesive patch containing an essential oil and 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 anticoronavirus effect of chloroquine.



**Figure 2. Effects of chloroquine (CQ) on replication of CCoV in canine fibrosarcoma A72 cells.** A72 cells were loaded for 1 h with CQ and then infected with CCoV. Supernatants were collected at peak and the infectious titre was assayed in A72 cells. Means  $\pm$  S.D. Data are representative of three experiments. CCoV: Canine coronavirus; CQ: 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 expectedly 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 coronavirus 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 chymotrypsin-like folding with a root mean square deviation from bovine  $\alpha$ -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  $\alpha$ -helical and plays a critical role in enzyme dimerisation [26]. The chymotrypsin-like folding of 3CLpro is intriguing because the overall sequence identity between the 3CLpro and  $\alpha$ -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 anticoronaviral effect of HIV-1 PIs, although robust data providing a causal link between 3CLpro inhibition and impairment of SARS-CoV replication are still lacking.

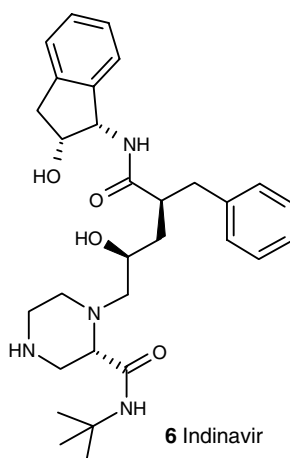
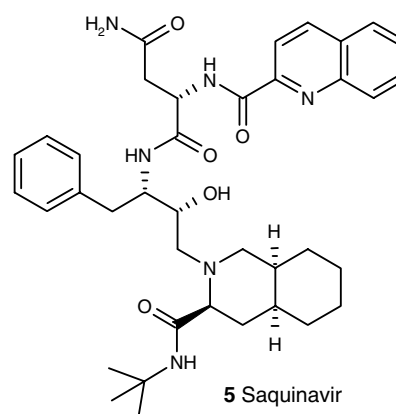
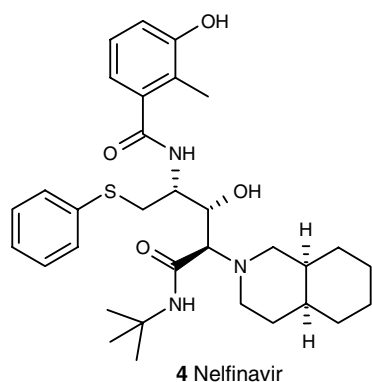
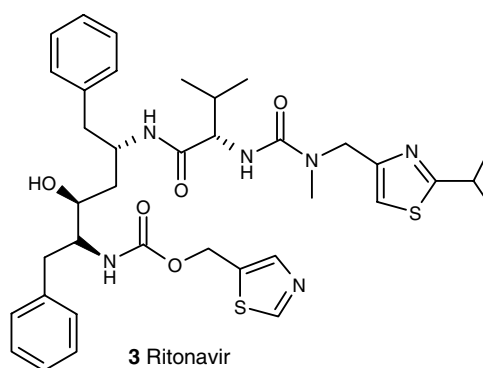
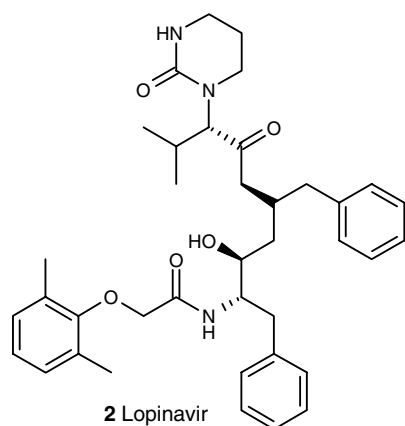
The story of HIV-1 PIs as a potential SARS treatment began during the 2003 SARS outbreak with the empirical administration to affected individuals of the antiretroviral preparation Kaletra (Abbott), containing lopinavir (2) and ritonavir (3) at a ratio of 4:1 (w/w) [28]. A retrospective matched cohort study of Chan *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  $\mu\text{M}$ ), 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  $\mu\text{M}$ ) 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 to 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  $\text{EC}_{50}$  value of lopinavir was in the range of 1.6–6.4, and 6.4–12.8  $\mu\text{M}$  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  $\text{EC}_{50}$  value of lopinavir against the prototype SARS-CoV strain 39849 was in the range of 3.2–6.4  $\mu\text{M}$  in fRHK-4 cells, and 6.4–12.8  $\mu\text{M}$  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 ( $K_i = 5.6 \times 10^{-25}$  M). These authors attribute to the other Kaletra component, lopinavir, a  $K_i$  value of  $8.7 \times 10^{-20}$  M [34]. Instead, Enwitesuk *et al.* [35] attribute a  $K_i$  value of  $10^{-7}$  M to ritonavir. More recent calculations attribute a  $K_i$  value of  $10^{-5}$  M to ritonavir [34]. This  $K_i$  value is more realistic than those previously reported given that HIV-1 PIs, at the highest concentrations tested (i.e., 10  $\mu\text{M}$ ), 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.

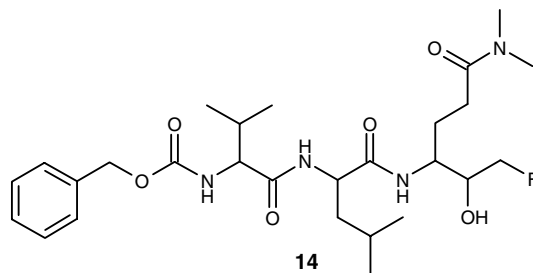
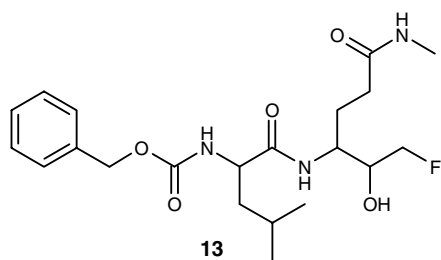
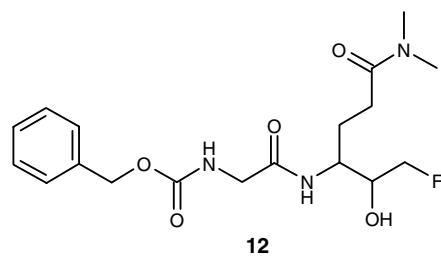
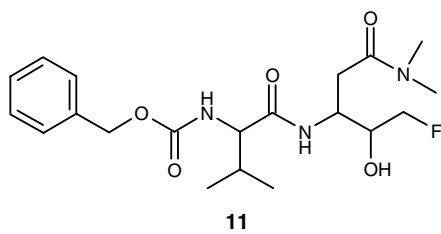
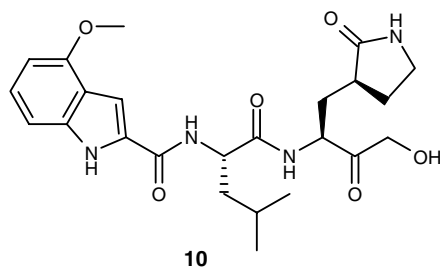
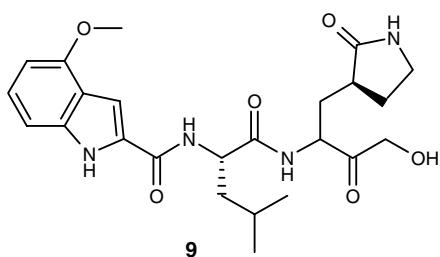
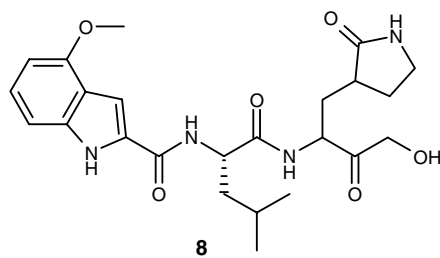
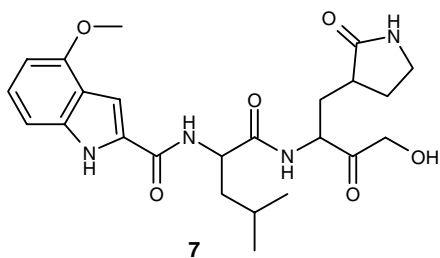
There is no patent specifically devoted to claiming the use of HIV-1 PIs as SARS-CoV inhibitors. However, one of the previously quoted patents [107] claims the use by inhalation of different compounds, including all principal HIV-1 PIs,



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  $\mu\text{g/ml}$  and  $IC_{50}$  value of > 50  $\mu\text{g/ml}$ , which gave a selectivity index (SI) of > 24. Compound of Example 24 (12) was found to have an  $EC_{50}$  value of 0.02  $\mu\text{g/ml}$  and  $IC_{50}$  value of > 10  $\mu\text{g/ml}$ , suggesting a potentially high therapeutic index. The same compound inhibited 100% of the cytopathic effect of SARS-CoV at 1  $\mu\text{M}$ , 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  $\mu\text{M}$ , 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-acrylate 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 R<sub>1-4</sub> 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  $\beta$ -lactamases (proteases and  $\beta$ -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

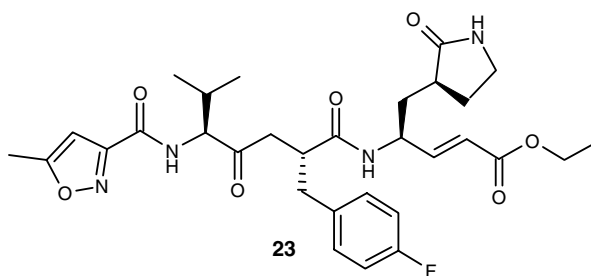
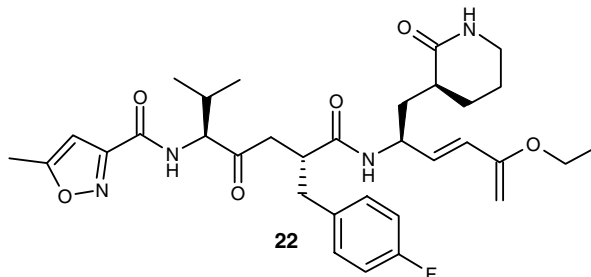
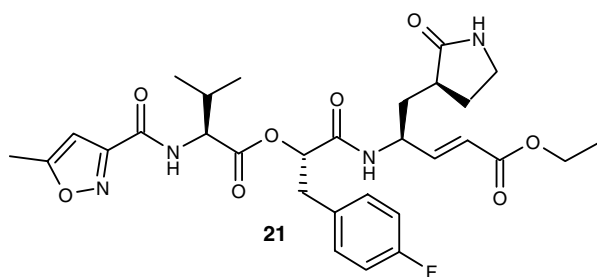
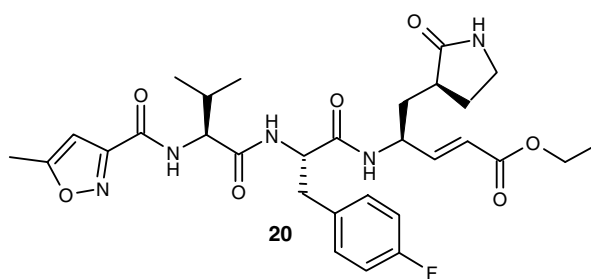
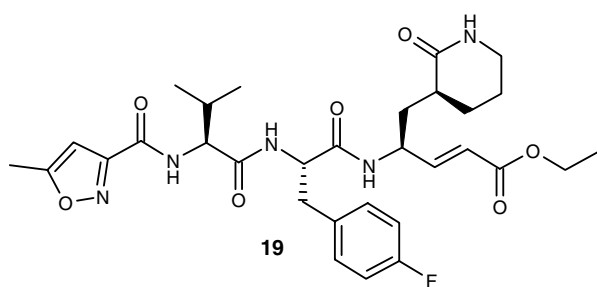
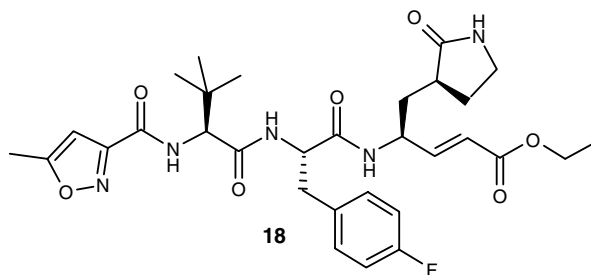
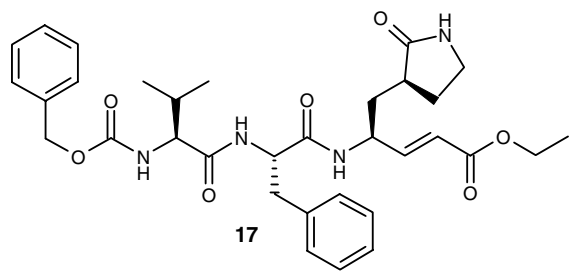
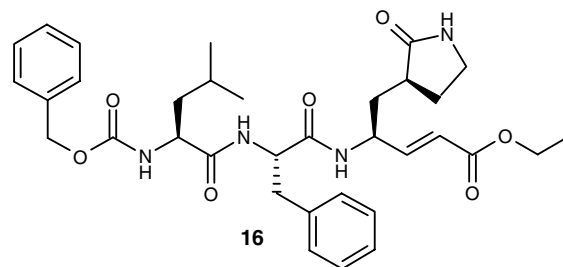
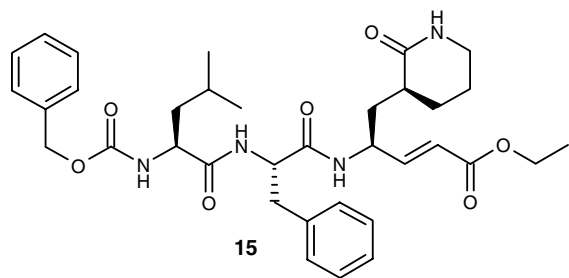
largest of the nsps [21] and they are defined by homology to the papain-like fold [20] and constitute the peptidase family C16 [38]. Although mutational analyses supported the presence of a Cys-His catalytic dyad [21,39], it is now clear that these enzymes also have a catalytic triad Cys-His-Asp [40]. 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 down- and upstream 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 permuted) 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 deubiquitylating 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 I 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 PLpro 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 IC<sub>50</sub> value of 1.3  $\mu$ M [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  $\mu$ M (data not shown). Therefore, PLpro inhibitors patentable in the future are likely to contain zinc.

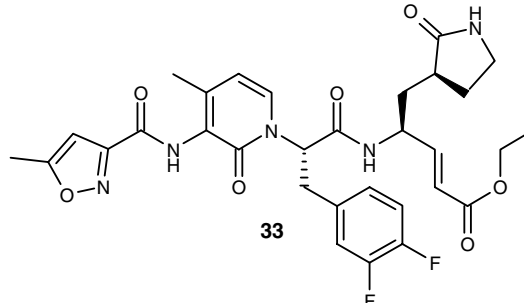
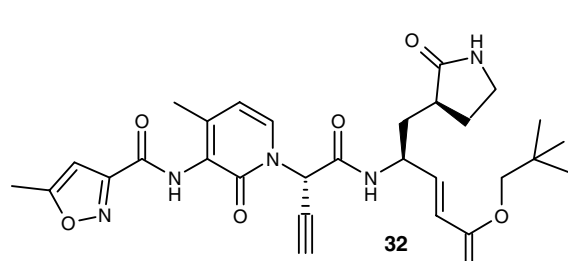
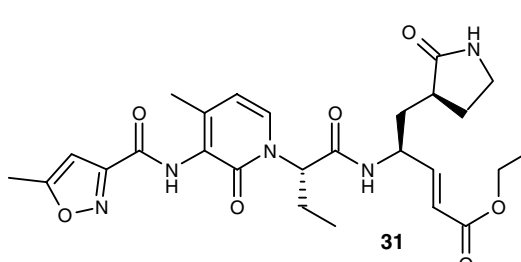
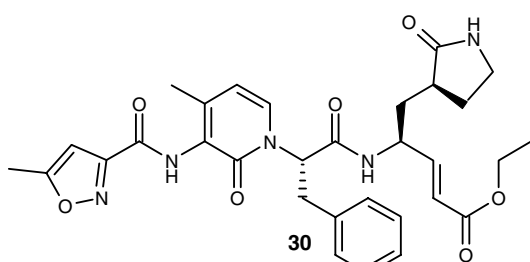
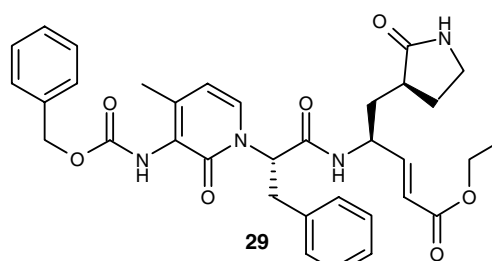
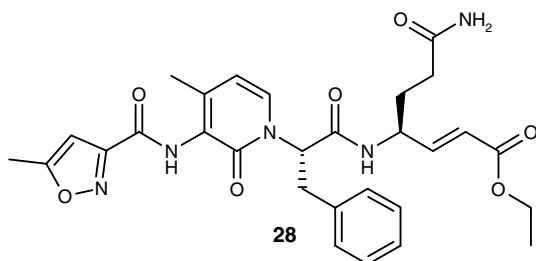
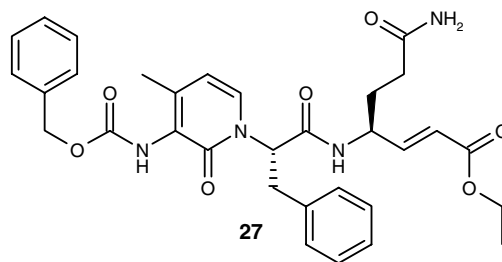
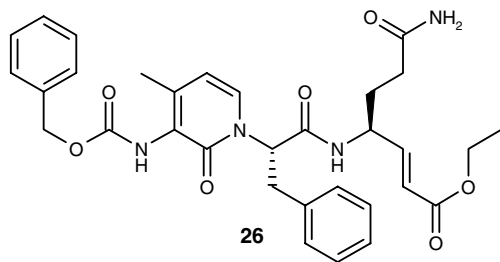
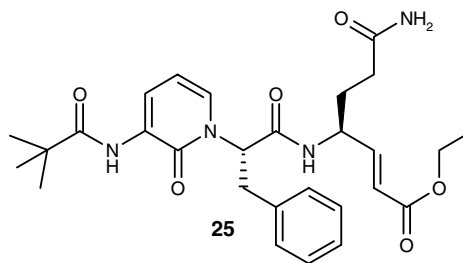
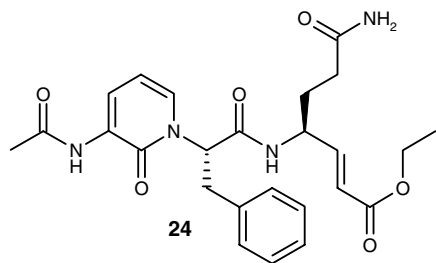
### 2.5 RNA-dependent RNA polymerase

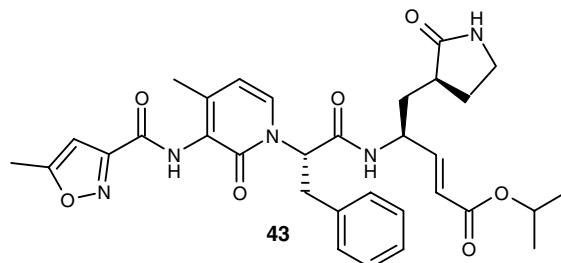
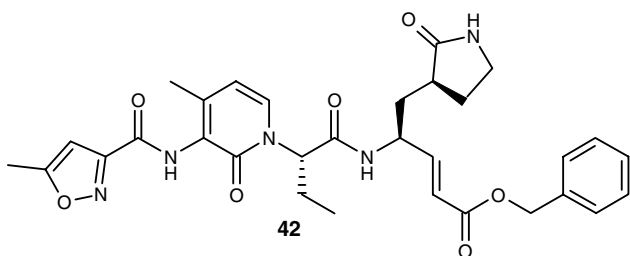
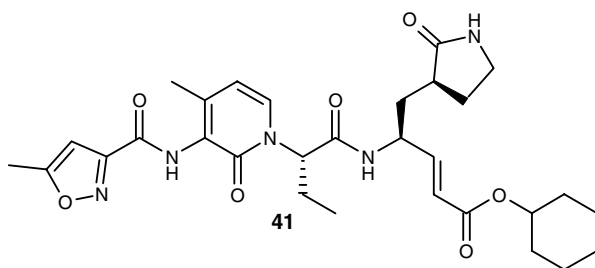
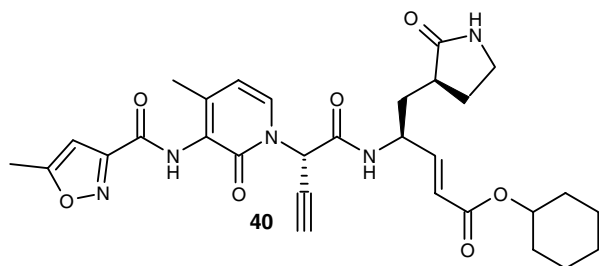
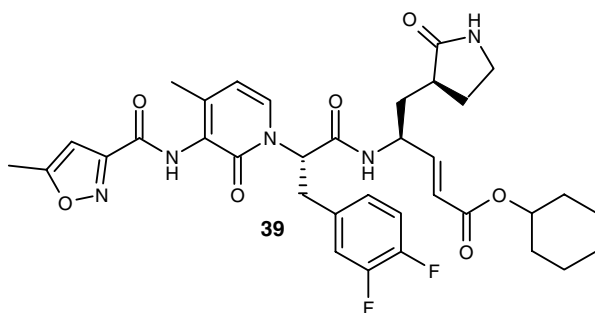
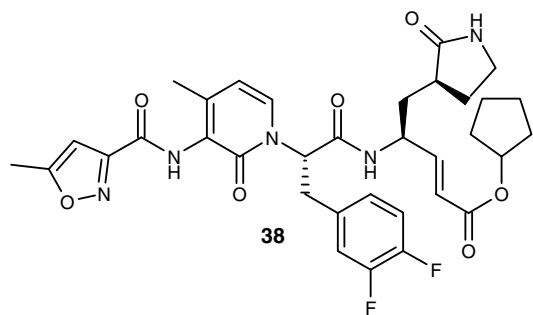
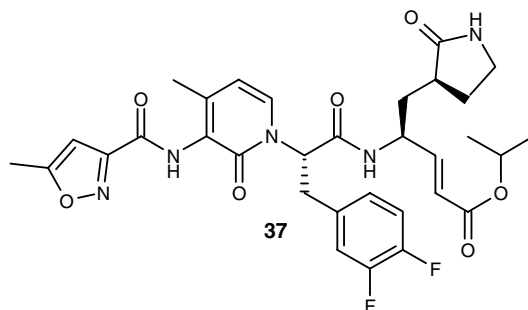
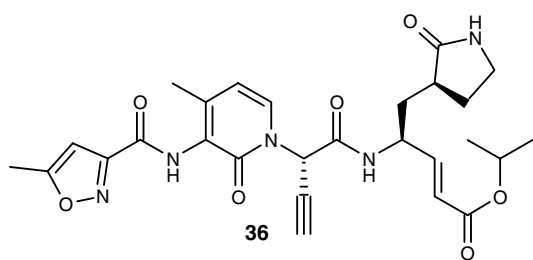
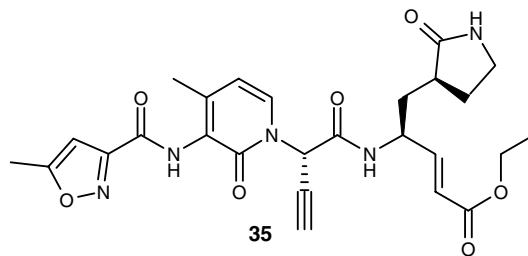
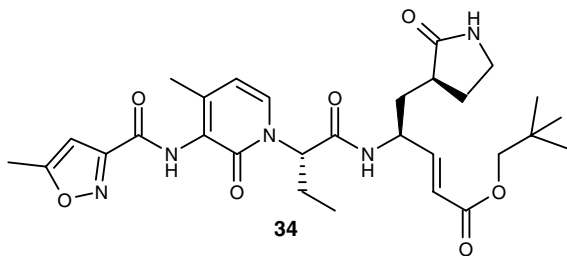
One of the proteins released by the proteolytic activity of the 3CLpro main protease is nsp12, acting as a RNA-dependent RNA polymerase (RdRp; nsp12). Coronaviral RdRp catalyses the production of new full-length genomic RNAs to be packaged into virions as well as a nested set of monocistronic mRNAs that 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

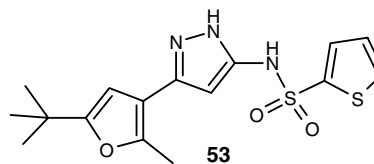
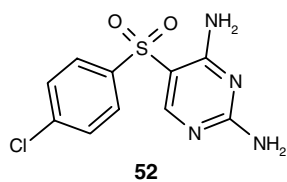
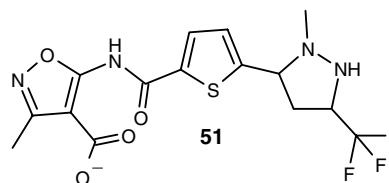
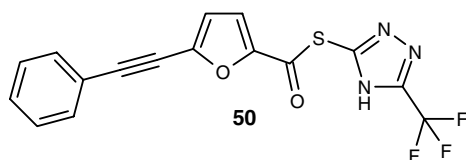
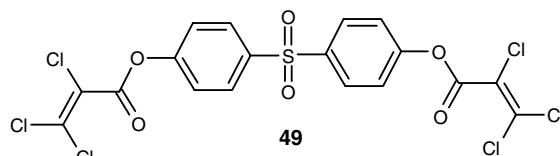
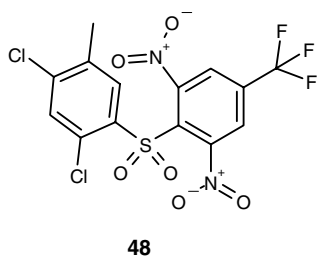
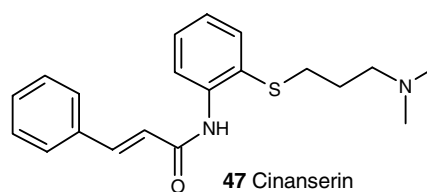
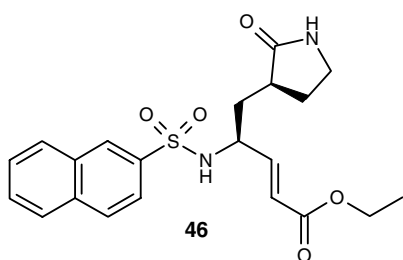
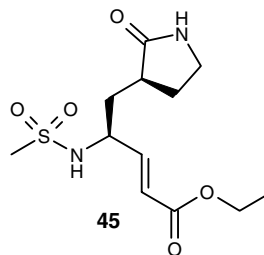
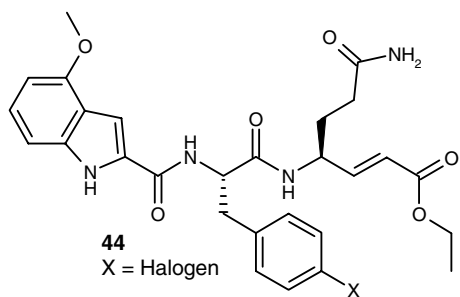


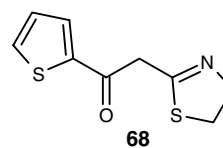
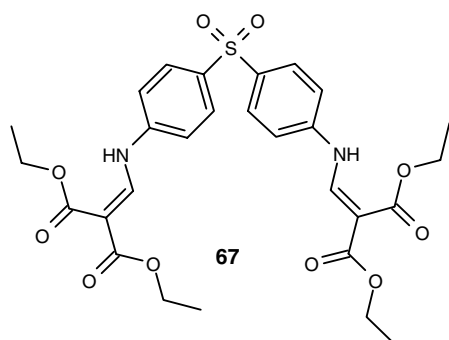
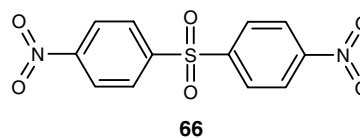
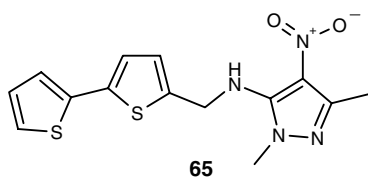
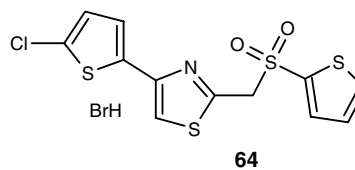
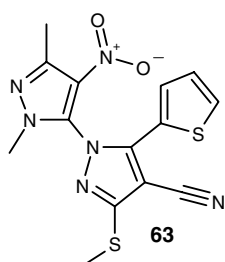
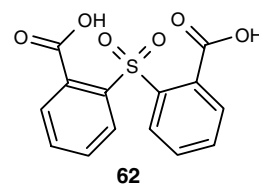
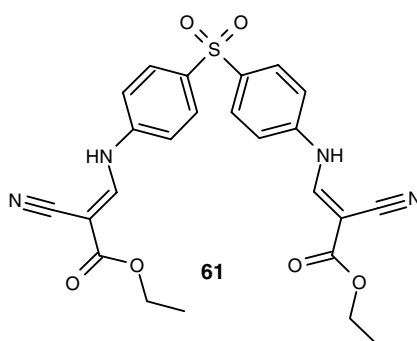
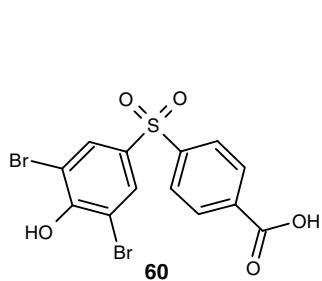
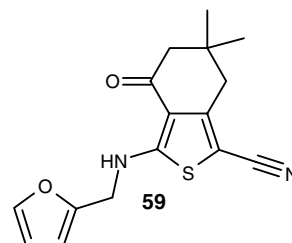
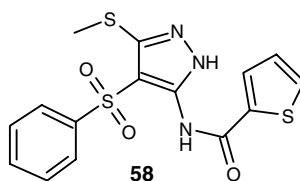
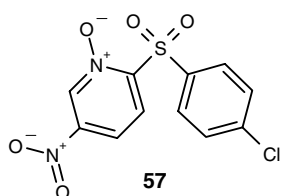
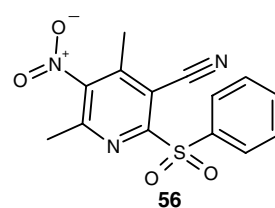
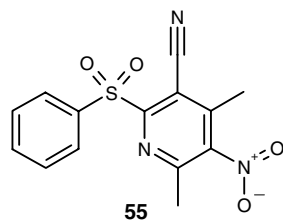
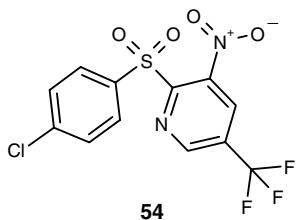


Potential therapies for coronaviruses

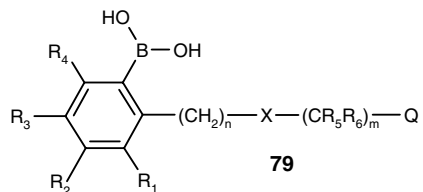
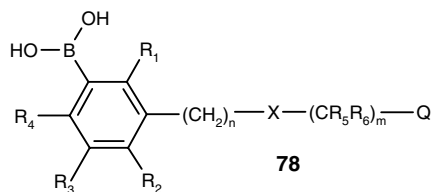
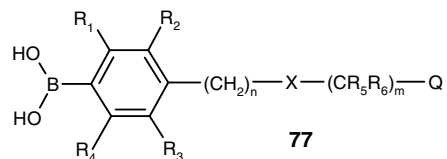
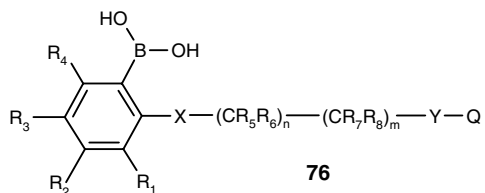
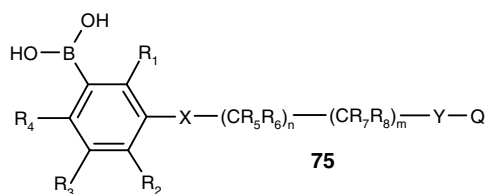
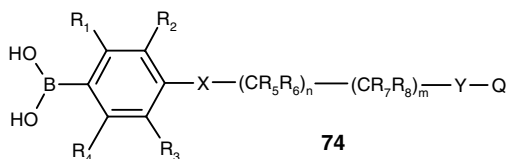
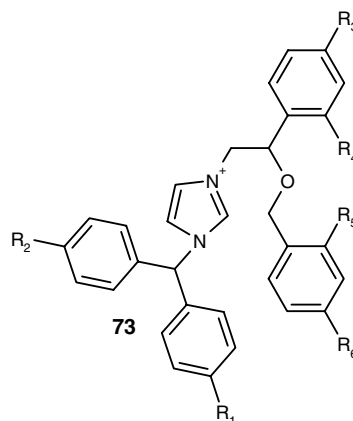
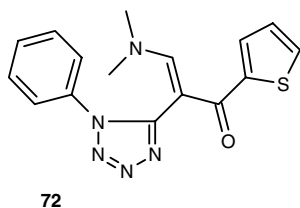
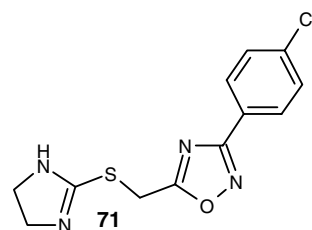
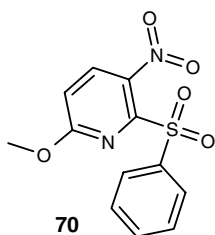
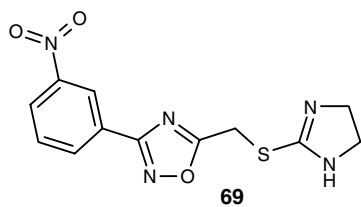


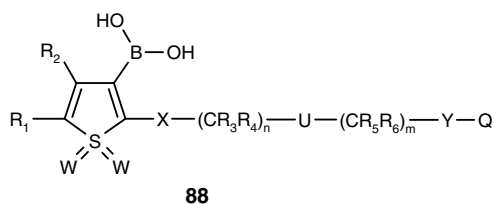
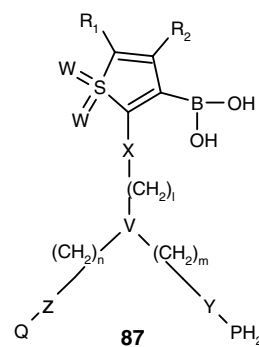
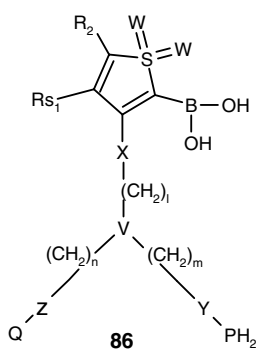
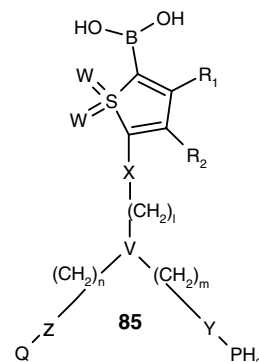
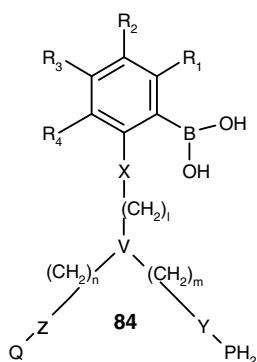
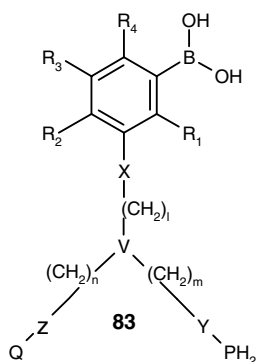
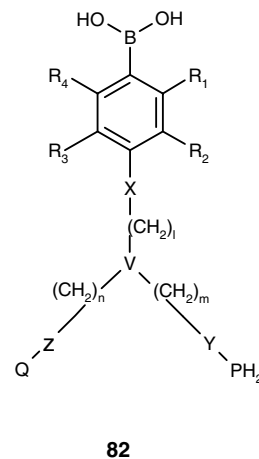
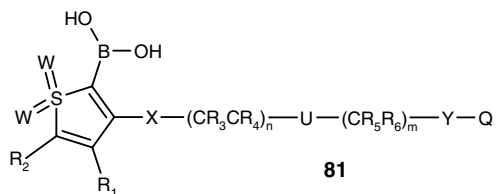
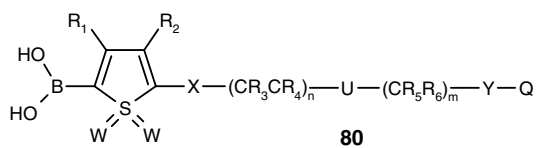






Potential therapies for coronaviruses





protein and also enables conclusions to be drawn about the properties of potential nucleoside analogue-inhibitors of coronavirus RdRps. Thus, it was proposed that potential nucleoside analogue-inhibitors should contain groups at their 2' and 3' positions that are capable of making hydrogen bonding interactions with RdRp residues 623 and 691. Clearly, direct structural information is highly desirable for the development of effective inhibitors of this key enzyme.

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 replication in Vero cells by  $\geq 100$ -fold at 0.4 mg/ml and by  $> 1000$ -fold at 0.8 mg/ml. A significant inhibition of S glycoprotein production was observed in SARS-CoV-infected cells treated with ATA as a result of inhibition of viral protein synthesis. 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 compound 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 oligonucleotides and mimics thereof [119]. The appropriate sequence of bases (indicated as Bx in the following structural formulae) may be created by regular oligonucleotides or oligonucleotide-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 reversibly protected oxygens or substituents in position 2' (90), and dicyclic substituents of the ribose (91). To increase the binding 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 proteins as compared with their oligonucleotide counterparts. The same invention takes into account the use of cyclohexenyl 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 complexes 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.

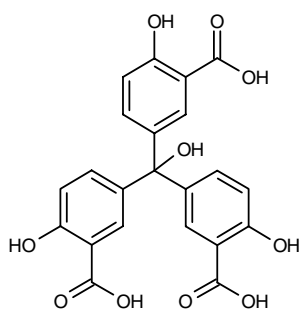
### 2.6 NTPase/helicase

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. Hel 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-triphosphates (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'-triphosphatase in viral 5' capping. The basal ATPase activity of coronaviral helicase is stimulated by most polynucleotides 15- to 25-fold, with the exception of poly(G) and poly(dG). A significant variation in strength of homopolynucleotide binding was reported, suggesting that the SARS-CoV helicase may possess elements of secondary structure/sequence recognition 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 structure or displacing bound proteins and annealed RNA fragments, putative roles that will require experimental validation. Coronaviral Hel possesses a cysteine-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 adamantane/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.

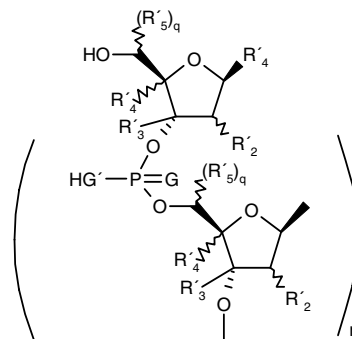
## 3. Expert opinion

Many compounds with possible anticoronavirus activity have been researched, especially after the major SARS

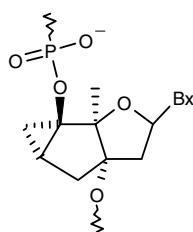




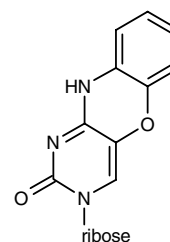
89 ATA



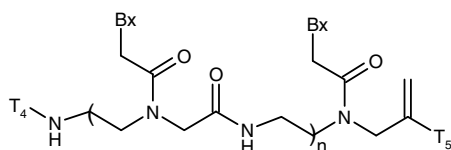
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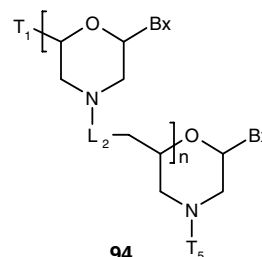
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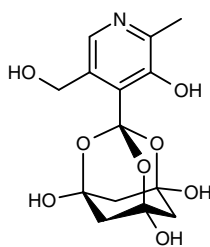
94

T<sub>1</sub> = Hydroxyl or a protected hydroxyl  
 T<sub>5</sub> = H or a phosphate or phosphate derivative  
 L<sub>2</sub> = Linking group  
 n = 2 – 50

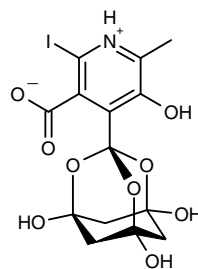
outbreak in 2003. Some of these compounds deserve particular attention because they are drugs that are already registered for other uses in humans. These drugs include the antimalarial chloroquine and HIV-1 PIs. The antihelminthic compound, niclosamide (96) is one such drug; however, its molecular target 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.

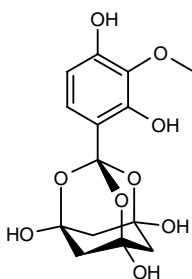
On the other hand, the patents/applications on the use of very promising compounds, such as chloroquine or HIV-1 PIs, in coronavirus infections do not cover all of their possible applications. For the uses of chloroquine and HIV-1 PIs not covered by the existing patents/applications, these drugs might 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



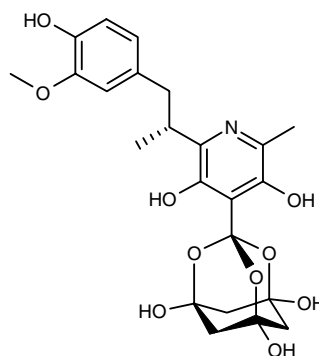
95 Bananin



96 Iodobananin



97 Vanillinbananin



98 Eubananin

coronavirus infections. 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.

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