Expert Opinion

Therapies for coronaviruses. Part 2: inhibitors of intracellular life cycle

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Background: Severe acute respiratory syndrome (SARS) coronavirus emerged from an animal reservoir in 2002 and has the potential to reemerge, as shown by the occurrence of non-laboratory-associated new cases in the winter of 2003. In the absence of a vaccine, broad spectrum anticoronaviral medications are needed. *Objective*: Anticoronavirals targeting viral entry were reviewed in part I. Here we review anticoronaviral therapies directed against the intracellular life cycle, with an emphasis on allowed patents and pending patents. *Method*: The published literature, in particular, patent publications is searched for relevant documents. The information is organized and critiqued. *Results/conclusion*: Many promising anticoronaviral strategies are identified. Monoclonal antibodies, protease inhibitors, interferon-based drugs and nucleic-acid based antivirals are most advanced, each having its own advantages and disadvantages. A multi-pronged approach, keeping all venues open, is advocated.

Keywords: 3CLpro, coronavirus, interferon, polymerase, protease, RNAi, SARS-CoV, severe acute respiratory syndrome, viroporin

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1. Introduction

The coronaviruses and the arteriviruses belong to the order Nidovirales. They are enveloped positive-sense single-stranded RNA viruses (Baltimore group IV) [1,2]. The coronavirus life cycle begins with the entry into its host cell after attachment to its species-specific cellular receptor. Following entry of viral nucleocapsid into the cytosol and uncoating of the viral genome, the 5′-capped and methylated and 3′-polyadenylated viral genome is recognized by the translational machinery of host cells and begins translating polyproteins 1a and 1ab, the latter regulated by a hairpin-pseudoknot secondary structure and achieved with a -1 ribosomal frameshift just 5′ of the termination codon of ORF1a [3]. The polyprotein is co- and post-translationally processed into a multisubunit viral 'replication/transcription complex' (RTC) by two enzymes encoded in ORF1a: a papain-like (accessory) cysteine protease (PLpro) function of nsp3 and mainly by a 3C-like (main) protease (termed 3CLpro or Mpro) function of nsp5 [3-6]. PLpro has two domains, PL1pro and PL2pro, in most coronaviruses [severe acute respiratory syndrome (SARS)-CoV does not have the PL1pro domain]. Pp1a and pp1ab are processed into nsp1 to nsp11, and nsp1 to nsp10 and nsp12 to nsp16, respectively [7]. The next phase involves transcription of a nested set of functionally monocistronic subgenomic mRNA (defining viruses of the order Nidovirales). These are transcribed through a unique discontinuous mechanism resulting in the acquisition of a common 5′-terminal leader sequence from the 5′ end of the genome (see **Figure 3** in [8]). The synthesis of mRNA, the negative-sense RNA template and production

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of progeny genomic RNA take place within endoplasmic reticulum or autophagy vacuole-derived membrane-associated replication/transcription complexes [9,10]. The viral intracellular life cycle is complete with the assembly of virus particles in membrane-bound endoplasmic reticulum/Golgi intermediate compartment, post-translation modification (glycosylation, acylation and cleavage) of structural proteins using host Golgi apparatus/endoplasmic reticulum and trafficking of the virions to the surface for release, again using host intracellular highways.

The comparatively largest genome (~ 30 Kb) among RNA viruses is replicated by a complex series of time-consuming steps (generation time of 17 – 19 h in FRhK cells [11]) and is reflected in the complexity of its proteome, which contains enzymatic activities not found in other RNA viruses. An example is 3′-to-5′ exonuclease activity (ExoN) possibly involved in RNA proofreading and repair, activities unknown among RNA viruses [8].

In defending itself from viral infections, host cells use RNA interference (RNAi) [12], the interferon system [13], various intracellular molecules that retard viral processes (MxA protein; 2′,5′-oligoadenylate synthetase [14]; RNA-specific adenosine deaminase, $ADAR_1$ [15]; PKR (p68 kinase); RNaseL; APOBEC [16,20] etc.), components of the innate immune system (mannose-binding lectin [17,18]) and the adaptive immune system to prevent viral dissemination. The importance of the later is underscored by the efficacy of engineered antibodies in certain viral infections [19,20], including SARS-CoV, reviewed in part I. Many antiviral strategies against SARS-CoV may find application in other coronaviral infections, such as the newly discovered HCoV-NL63 [21].

Here we focus on recent patents and patent applications against the intracellular life cycle of coronaviruses, especially those with a promise of broad spectrum activity across the genus.

2. SARS-CoV proteases as promising drug targets

SARS-CoV 3CLpro, the main viral protease responsible for catalytic cleavage of as many as 11 sites in polyproteins 1a (pp1a) and 1ab (pp1ab), also releases the key replicative functions, such as RNA-dependent RNA polymerase (RdRp) and the helicase [8]. Accordingly, 3CLpro is the focus of much effort for finding a broad spectrum inhibitor [4,22-27]. This effort is relevant to the recent discovery of novel coronaviruses in animal reservoirs [28,29]. The immense interest in this viral enzyme has spawned a variety of different 3CLpro expression constructs and kinetic assays, which need to be standardized in order that inhibitors being developed can be compared [30].

2.1 Catalytic site of 3CLpro

SARS-CoV 3CLpro is a 33-kDa cysteine protease using the nucleophilic thiol group of cysteine at the catalytic dyad of Cys145 and His41 situated in a chymotrypsin-like fold formed from the β-barrel domains I and II for enzymatic cleavage. A further residue Asp187 may be involved, with enhanced catalytic efficiency, after a molecule of water trapped by Asp187 and His41 is displaced by the binding of substrate (see Section 2.2 later) [31]. Because coronavirus 3CLpro molecules are dimeric owing to association between the third unique α-helical domains, which were proven critical for its function, domain III was proposed as a new target for design of specific inhibitors [32]. The main protease is essential for the formation of the membrane-bound viral replication complex and shows remarkable conservation of the cleavage sites within the genus. Comparison of the previously known coronavirus cleavage sites with those identified in the SARS-CoV main protease suggests conservation of the P4 (Ser, Thr, Val, Pro, Ala [STVPA]), P2 (Leu, Ile, Val, Phe, Met [LIVFM]), P1 (Gln[Q]) and P1′ (Ser, Ala, Gly, Asn, Cys [SAGNC]) residues. This was experimentally confirmed by *trans*-cleavage assays using expressed SARS-CoV 3CLpro and *in-vitro*-translated substrates or synthetic peptides derived from group 1 (TGEV) and group 2 (MHV) N-terminal 3CLpro autoprocessing sites. Expected cleavage products were detected and cleavage was abrogated by targeted mutation in the Cys-to-Ala active-site mutant [3]. A drug with Gln ↓ (Ser, Ala, Gly) specificity (↓ denotes cleavage site) against SARS 3CLpro may also have activity against the other members [33].

Recently, the highly conserved crystal structures of several groups II and III coronaviruses, including HCoV-HKU1, and inhibitory and toxicity data of Michael acceptor inhibitors (N3, N27 and H16) derived therefrom, have been added to the literature [34,35].

2.2 Catalytic triad in 3CLpro active site

Because proteins, including enzymes, are not rigid, attempts to screen for inhibitors based on crystal structure may be complemented by terascale computing to model flexible regions of proteins [31,36,37]. Instead of a catalytic dyad of Cys145 and His41, Pang *et al.* demonstrated a more efficient catalytic triad of Asp187, His41 and Cys145 as shown by 420 different molecular dynamics simulations with experimental substrate $ATVRLQ^{P1}A^{P1'}$ bound to 3CLpro [31]. Using this information from molecular modeling studies, small molecule inhibitors were designed with a docking program (EUDOC). This direct approach from genome to drug lead resulted in the discovery in 2 months of a small molecule inhibitor of SARS-CoV 3CLpro, which eluded screening against two crystal structures (CS11; see Section 2.3.1 later). The drug discovery process can be shortened to 1 month by the available 3.4 teraflops computing resource and further shortened by petascale computing in the near future. This approach also broadens the scope of virtual screening of pharmacophore and can potentially accelerate the development of therapeutics to meet the challenges of emerging infectious diseases.

Table 1. Coronaviral proteases and other viral protein inhibitors.

Target/technology	Exemplary drugs	IC_{50} and other measures of efficacy	Ref.
3CLpro (SARS-CoV)	CS11	EC_{50} = 23 μM; CC_{50} = 76 μM; SI = 3.3	[38]
3CLpro (SARS-CoV)	Cinanserin and derivatives	IC_{50} = 19 – 34 μM	[39,42]
3CLpro (SARS-CoV)	Compound 2	EC_{50} = 3 μM; no cytotoxicity at 12 mM	[44,45]
3CLpro (SARS-CoV)	Compound 1	Low EC_{50} (values not provided)	[46]
3CLpro (SARS-CoV)	Formula II	Reduces viral titer by 4.7 log at 5 μM (SARS-CoV)	[6,47]
3CLpro (SARS-CoV) subsite with conserved Ser139, Ser144, Ser147	Organic boron-containing compounds, e.g., FL-166	Ki^{app} (apparent inhibition constant) = 22 nM	[49]
3CLpro (SARS-CoV)	Nelfinavir	EC50 = 0.0484 μM; CC_{50} = 14.6; SI = 301.6	[67]
RdRp	Nucleoside analogue, e.g., 4-amino-7-(2-C-methyl-β-D-ribofuranosyl)-7H-pyrrolo[2,3-d]pyrimidine	–	[80]
CoV E protein	Cinnamoylguanidine	Reduces SARS-CoV viral titer by 76% at 10 μM	[85]

CC_{50}: Cytotoxic concentration that reduced cell viability to 50%; EC_{50}: Plasma concentration required for obtaining 50% of the maximal effect *in vivo*; IC_{50}: Concentration of a drug that is required for 50% inhibition of viral replication *in vitro*; SI (selectivity index): CC_{50}/EC_{50}.

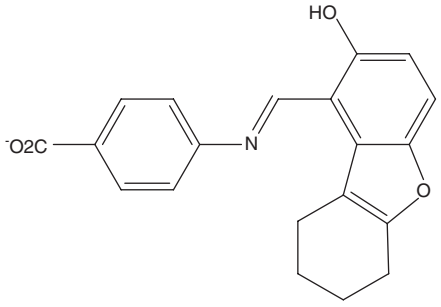

Figure 1. CS11. This molecule is structurally similar to Tamiflu™. It inhibits SARS-CoV 3CLpro with an EC_{50} of 23 μM (CC_{50} = 76 μM; SI = 3.3).

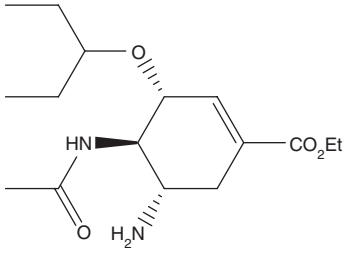

Figure 2. Tamiflu (Oseltamivir). This compound inhibits the neuraminidase of influenza A and B.

2.3 Patent pending small molecules with 3CLpro inhibitory activity

Table 1 lists the properties of protease inhibitors and other small molecular inhibitors of the intracellular life cycle of coronaviruses.

2.3.1 CS11

In US patent publication US2007/0149487 [38], Pang *et al.* disclosed CS11 (**Figure 1**), structurally similar to Tamiflu™ (**Figure 2**), with EC_{50} of 23 μM on SARS-CoV (Tor 2 strain) cultured in Vero E6 cells (CC_{50} = 76 μM; SI = 3.3). Four other compounds (CS 08, 09, 10 and 12) showed 13 – 17% inhibition at a drug concentration of 32 μM (66% for CS11). EUDOC-generated CS11-bound complex with the main protease suggested that simple chemical modifications of CS11 would improve the potency, for example, by replacement of a 4-aminobenzoic acid moiety with 4-amino-3-methylbenzoic acid.

2.3.2 Cinanserin and analogues

Pending patent US 2006/0142383 disclosed three-dimensional structure of SARS-CoV 3CLpro and cinanserin (**Figure 3**) and its analogues as specific inhibitors [39]. Following virtual screening of 8,000 existing drugs by a docking approach against a homology model of SARS-CoV 3CLpro and the crystallographic structure of the binding pocket, cinanserin (SQ 10,463), a serotonin antagonist that has undergone clinical testing in humans in the 1960s [40] but abandoned because of hepatoma in mice with prolonged high dosage [41], was chosen for further experimental evaluation [42]. Binding of both cinanserin and its hydrochloride with 3CLpro of both SARS-CoV and HCoV-229E was demonstrated with surface plasma resonance. Further testing with a fluorogenic substrate revealed an IC_{50} of 5 μM against both enzymes. HCoV-229E replicon-based tissue culture assay [43] and quantitative test assay with infectious SARS-CoV and HCoV-229E all demonstrated strong inhibition of viral replication (up to 4 logs; IC_{50} = 19 – 34 μM) at nontoxic drug concentrations. These experiments conclusively demonstrated the anti-3CLpro activity of cinanserin against both SARS-CoV and HCoV-229E. By contrast, no binding or inhibitory activity was demonstrable with human rhinovirus (HRV)-14 3Cpro, indicating the specificity of these compounds for coronavirus 3CLpro.

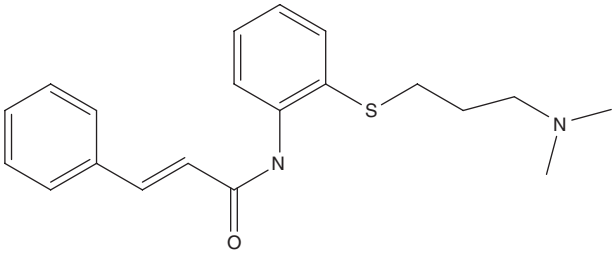

Figure 3. Cinanserin [2′-(3-dimethylaminopropylthio) cinnamanilide]. This serotonin antagonist inhibits both SARS-CoV and HCoV-229E 3CLpro.

2.3.3 *US patent application 2006/0160866*

Wong *et al.* provided 35 exemplary compounds with inhibitory activity against SARS-CoV [44]. Vero E6 cells preincubated with 10 µl of the test compounds were inoculated with 100 $TCID_{50}$/well of SARS-CoV (H.K.). All compounds had EC_{50} between 0.85 and 100 µM by inhibition of cytopathic effect (CPE), with no cytotoxicity at EC_{50} concentration. The inhibitory activities were confirmed by immunofluorescence ELISA, immunofluorescence assay (IFA), western blot, flow cytometry and 3CLpro inhibition assay. Compound **2** (**Figure 4**), developed as a transition-state analogue inhibitor of HIV -1 protease, had EC_{50} of 3 µM against SARS-CoV 3CLpro. It is non-cytotoxic to Vero E6 at > 12 µM. Docking simulation shows that it is folded into a ring-like structure in the active site of 3CLpro. It is extended when bound to HIV-1 and feline immunodeficiency virus proteases [45].

2.3.4 *US patent application 2006/0019967*

Wu *et al.* disclosed 25 exemplary dicyclic or multi-cyclic compounds with inhibitory activity against SARS-CoV main protease [46]. The compounds were added at serial dilutions to a mixture of 50 nM SARS-CoV protease and 6 µM of fluorogenic peptide substrate and fluorescence change was measured. The initial velocities of the reactions were used to derive the IC_{50} values. Compounds **1** (**Figure 5**) – **4** had very low IC_{50} values (details not provided).

2.4 Peptide 3CL-inhibitor

TaiGen Biotechnology developed a peptide-like 3CL protease inhibitor (TG-0205221) with K_i of 53 nM and remarkable activity against SARS-CoV and HCoV-229E, reducing viral titer by 4.7 log at 5 µM for SARS-CoV and even more potently for HCoV-229E (5.2 log at 1.25 µM). The crystal structure at 1.93 Å resolution reveals a unique binding mode comprising a covalent bond, hydrogen bonds and numerous hydrophobic interactions [6]. Patent publication US2005/0143320 disclosed a peptide-like compound with viral protease-inhibiting activity based on formula II (**Figure 6**) [47].

2.5 Subsite adjacent to active site of 3CLpro as drug target

Analysis of the active site of SARS-CoV reveals adjacent subsites such as a cluster of serine residues (Ser139, Ser144 and Ser 147), especially Ser147, that when replaced with alanine, inhibited dimerization and resulting in loss of enzymatic activity [48]. This subsite is also susceptible to inhibition by compounds containing boronic acid, in particular bifunctional aryl boronic acid compounds, as expected of the hydroxyl group in serine residues. This novel molecular scaffold is highly conserved in similar proteases from 20 coronaviruses and could be further optimized as a drug candidate [22].

US2005/0267071 provides organic boron-containing compounds for inhibiting coronavirus protease(s) at the conserved Ser139, Ser144 and Ser147 subsite [49]. This subsite is adjacent to the catalytic site; Ser 147 is located 9 Å away from the dimer interface. Drugs targeting this novel site do not need to compete with the attractive forces between subunits to be effective. Moreover, the conservation across coronaviruses and crippling mutations (replacing Ser147 with alanine prevents dimerization and function of 3CLpro) suggest that broad spectrum inhibitors are feasible and are at low risk of viral escape. FL-166 (**Figure 7**), derived from another biphenyl boronic acid, has apparent inhibition constant (Ki^{app}) of 22 +/- 10 nM. This compound has an α of 1.8 ($\alpha = \infty$ for purely competitive inhibitor and ranges between 1 and ∞ for a noncompetitive inhibitor).

2.6 Papain-like cysteine protease inhibitors

The papain-like protease of coronaviruses also shows conservation of both position and sequences of polyprotein cleavage sites. As predicted, the only SARS-CoV papain-like cysteine protease (PLpro), a Zn-ribbon-containing protease, cleaves the N-proximal polyprotein regions at three sites. SARS-CoV, like infectious bronchitis virus (IBV), does not have a paralogous PL1pro that is present in other family members [8]. Also like IBV, SARS-CoV PLpro, in the absence of a second PLpro with overlapping activities, probably resulted in greater conservation of cleavage sites and narrower substrate specificities. This feature of SARS-CoV and IBV could be exploited for the development of selective inhibitors [8].

The catalytic triad used by this PLpro recognizes and cleaves substrates with the consensus sequence LXGG [50], including the C-terminal LRGG of ubiquitin and C-terminal LRLRGG of ISG15 [51]. Its deubiquitinating (DUB) and de-ISGylating activities are thus similar to adenoviral protease and influenza NS1, respectively [51-53]. Conjugation of cellular proteins with the 15-kD protein encoded by interferon (IFN)-stimulated gene (ISG15) is speculated to be part of the innate antiviral response.

In addition, SARS-CoV PLpro interacts with IRF-3, a latent type I IFN transcription factor essential in innate antiviral immunity. It does so by preventing phosphorylation and nuclear translocation of IRF-3, thereby intercepting the signal for activation of type I IFN responses through both Toll-like receptor 3 and retinoic acid-inducible gene I (RIG-I)/melanoma differentiation-associated gene 5 (MDA5) pathways [54,55].

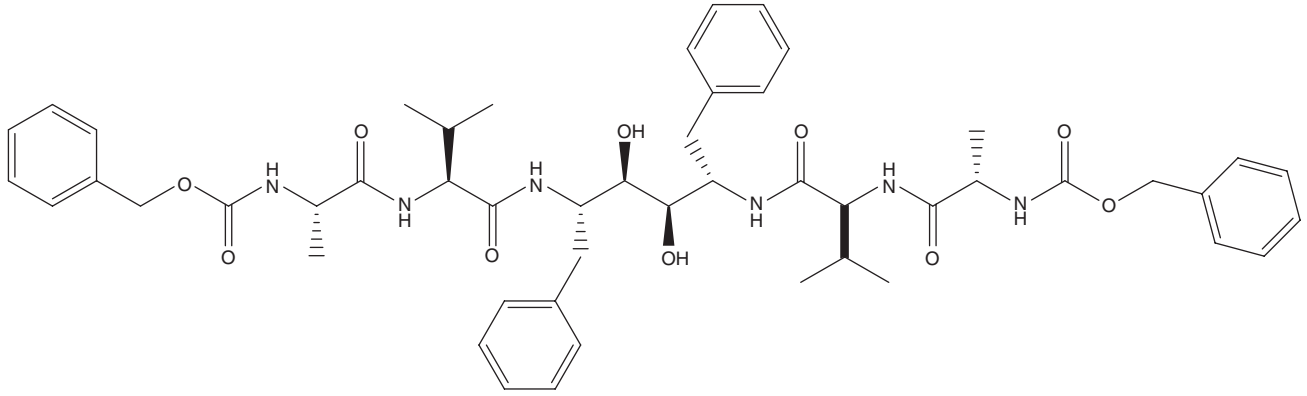

Figure 4. Compound (2) of US2006/0160866. This compound was developed as a transition-state HIV-1 protease inhibitor and turned out to potently inhibit SARS-CoV 3CLpro with EC_{50} of 3 μM.

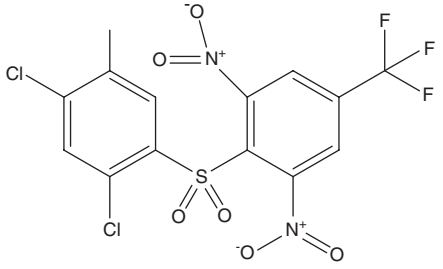

Figure 5. Compound (1) of US2006/0019967. This molecule had low IC_{50} inhibitory activity against SARS-CoV 3CLpro.

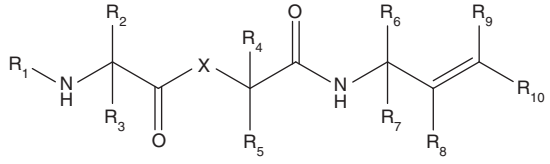

Figure 6. Formula II of US2005/0143320. A peptide coronaviral 3CLpro inhibitor.

A drug such as GRL0617 that inhibits this enzyme is, therefore, likely to serve to interfere with the life cycle and attenuate the virulence of SARS-CoV, as proven in principle by Ratia *et al.* [56].

2.7 Clinical and laboratory experience with protease inhibitors

It was noted during the 2002 – 3 SARS epidemic that HIV-positive patients on HAART seem to be protected against SARS [57,58]. Researchers in Hong Kong investigated the utility of lopinavir–ritonavir in a multi-center retrospective matched cohort study as initial and rescue therapy for SARS [59,60]. Patients who received this therapy as initial treatment had better outcome (reduced death and intubation rate) compared with an uncontrolled group, and also had lower rate of use of methylprednisolone at a lower mean dose. These clinical results are in agreement with structural studies that predicted the utility of lopinavir (**Figure 8**), ritonavir, niclosamide and promazine against 3CLpro [61], with lopinavir and nelfinavir also showing *in vitro* activity [60,62,63]. However, these results are not entirely duplicated by other studies, which found an IC_{50} of 50 μM (K_i = 14 μM) for lopinavir alone and no activity for ritonavir and saquinavir at 50 μM [45]. Several other lopinavir-like structures were found to have IC_{50} of ~ 25 μM. Real-time fluorimetric assay showed that niclosamide has no inhibitory activity against SARS-CoV 3CLpro [64]. In addition, nelfinavir was found to have only 10% of the activity of hexachlorophene, which has a K_i of 4 μM by a FRET-based assay [65]. Moreover, nelfinavir failed to protect an animal model (BALB/c mice) from SARS-CoV challenge [66]. Conflicting experience with nelfinavir (**Figure 9**) is disclosed by Fujii *et al.*, who report EC_{50} of 0.0484 μM (cytopathic effect) and CC_{50} = 14.6 μM (SI = 301.6) [67].

The disagreement between molecular modeling by computation and *in vitro* and *in vivo* data are to be expected. In addition, the observed effect of the compound *in vivo* may not necessarily be due to the enzyme inhibitor activity.

3. SARS-CoV polymerase as drug target

SARS-CoV RdRp is the only coronaviral RdRp characterized structurally and functionally [68]. It resides on nsp12, with a second RdRp (nsp8) functioning as a primase [69], and working together with nsp7 in a cylindrical macromolecular complex that confers processivity to nsp12 [70].

RdRps are not encoded in mammalian cells but are found in all RNA viruses except retroviruses. The crystal structures of several RNA viruses are available and used as starting point for the construction of the structural model of SARS-CoV RdRp [68]. Xu *et al.* used a novel method of first identifying conserved sequence motifs, followed by manual alignments and prediction of the secondary structure using the program PHD (profile network from HeiDelberg). Such a model led to a series of predictions on the properties

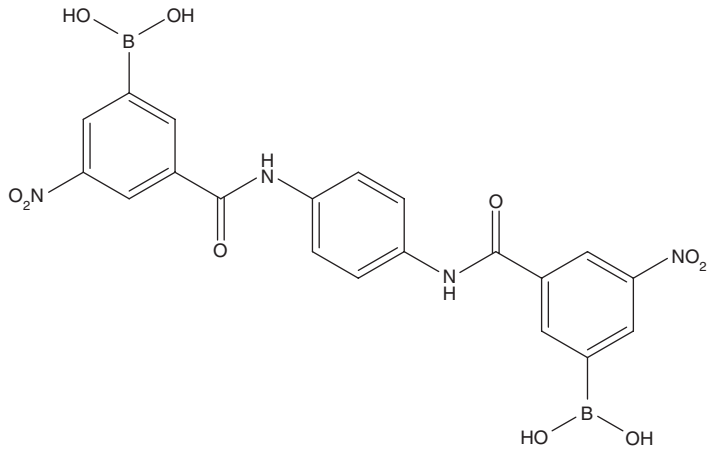

Figure 7. FL-166. This 3CLpro inhibitor derived from biphenyl boronic acid had apparent inhibition constant (Ki^{app}) of 22 +/- 10 nM.

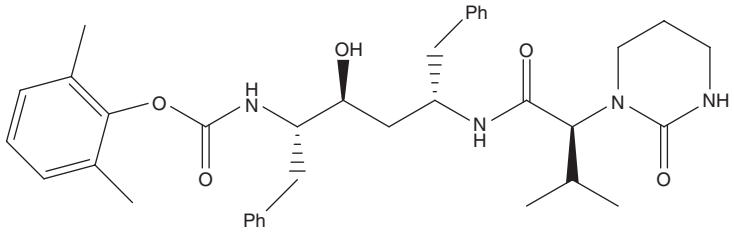

Figure 8. Lopinavir. This HIV-1 protease inhibitor was found in one study to inhibit SARS-CoV with an IC_{50} of 50 μM (K_i = 14 μM).

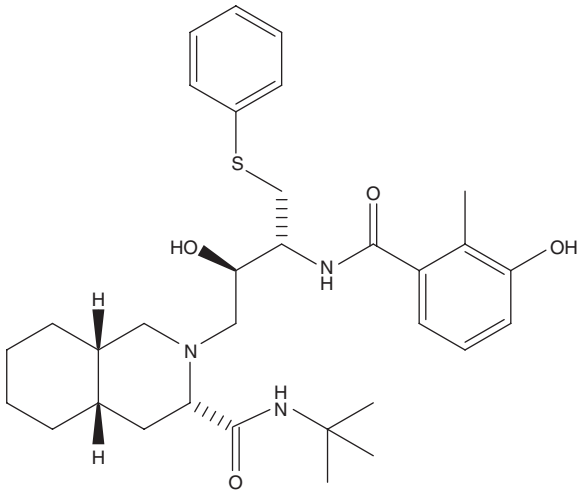

Figure 9. Nelfinavir. This HIV-1 protease inhibitor inhibits SARS-CoV 3CLpro with different efficacies by different assays.

of potential nucleoside analogue inhibitors, including having groups at the 2′ and 3′ positions that can hydrogen bond with the neighboring Asp 623, and Asn691, and having the C3′ *endo* sugar puckering conformation to maintain its stability for making a hydrogen bond at the 3′ position and to avoid steric conflicts at the 2′ position. Thus, it was suggested that 2′-*C*-methyladenosine and 2′-*O*-methylcytidine might be potential inhibitors of SARS-CoV RdRp. The structural model also shows that SARS-CoV RdRp lacks a hydrophobic non-nucleoside inhibitor-binding pocket as in the case of HIV-1 RT or HCV RdRp, making it unlikely to be inhibited by such inhibitors.

These predictions are supported by experiments showing that a 2(-*O*-methylcytidine derivative, N^4-Benzoyl-5′-*O*-(dimethoxytrityl)-5-methyl-2′-*O*-methylcytidine and β-D-N^4-hydroxycytidine (**Figure 10**) showed some inhibitory activity against SARS-CoV [71]. Several non-nucleoside HIV-1 RT inhibitors (α-APA, R90384 and HBY 097) were indeed found to be inactive against SARS-CoV RdRp [69].

3.1 Ribavirin

Unlike influenza virus, hepatitis C virus (HCV: a Flavivirus) and many agents of viral hemorrhagic fevers, SARS-CoV shows primary resistance towards ribavirin [1-(β-D-Ribofuranosyl)-1H-1,2,4-triazole-3-carboxamide]. Ribavirin (**Figure 11**) is a long half-life purine nucleoside analogue prodrug derived from D-ribose. It is activated by cellular kinases into the pharmacologically active 5′-triphosphate nucleotide. Thus, ribavirin is incorporated by RdRp into viral RNA as base analogues of either adenine or guanine, resulting in lethal 'error catastrophe'. It also inhibits certain viral RdRps, as well as having other activities, such as depletion of intracellular

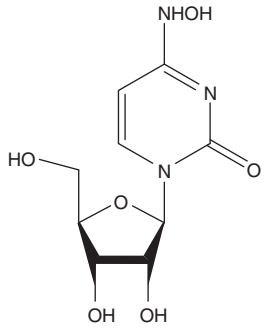

Figure 10. β-D-*N*^{4}-hydroxycytidine. This nucleoside analog was shown to have selective activity against SARS-CoV RdRp.

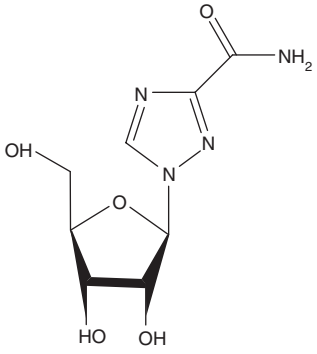

Figure 11. Ribavirin. Ribavirin had low selectivity index (< 1) against SARS-CoV.

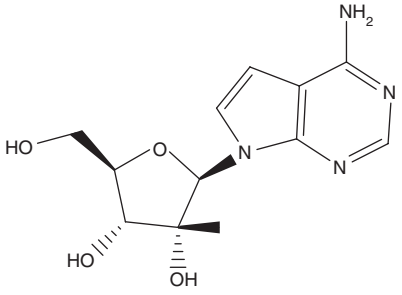

Figure 12. Compound A – 4-amino-7-(2-*C*-methyl-β-D-ribofuranosyl)-7*H*-pyrrolo[2,3-d]pyrimidine. This nucleoside analog by Merck was disclosed as combination therapy against coronaviruses.

GTP by inhibition of cellular inosine monophosphate (IMP) dehydrogenase [72] and enhancing antiviral Th1 function in the treatment of HCV infection [73].

For SARS-CoV, ribavirin has a low selectivity index of < 1 [74]. It also lacks demonstrable clinical benefit [75,76]. The mechanism of resistance to ribavirin has been attributed to structural characteristics of the RdRp [68] and the 3′-to-5′ exonuclease (ExoN) activity of nsp14, which is speculated to perform viral RNA proofreading, repair and/or recombination [8]. Recently, ribavirin and other IMP dehydrogenase inhibitors were found to prolong and enhance SARS-CoV replication in the lungs of BALB/c mice [77,78], suggesting that ribavirin and IMP dehydrogenase inhibitors be avoided in SARS-CoV infection in humans [76,79].

3.2 Other nucleoside analogues

US2004/0259934 disclosed Compound **A**, 4-amino-7-(2-*C*-methyl-β-D-ribofuranosyl)-7H-pyrrolo[2,3-d]pyrimidine (**Figure 12**) for coronaviral infection and prophylaxis administered in combination with another active agent, such as interferon, 2′-*C*-methylcytidine or inosine monophosphate dehydrogenase inhibitors (ribavirin, levovirin, viramidine) [80]. 2′-*C*-methylcytidine (**Figure 13**) and 2′-*C*-methyladenosine (**Figure 14**) showed EC_{50} of < 100 μM against HCoV-OC43 by cytopathic effect inhibition assay and ≤ 10 μM with neutral red (NR) uptake assay. Cytotoxicity screening was done against cultured hepatoma cells (HuH-7). CC_{50} data were not provided.

4. Coronavirus envelope (E) protein

Many viruses encode selective ion channels. Examples are HIV-1 Vpu protein, HCV p7 and Dengue virus M protein. SARS-CoV envelope (E) protein as well as E proteins from coronaviruses of all three taxonomic groups (HCoV-229E, MHV, IBV) were recently shown to form cation-selective ion channels in planar lipid bilayers, although the functional role in the intact virion or virally infected cell is not entirely clear [81]. The envelope (E) protein of coronaviruses also mediates viral assembly and morphogenesis. Virus ion channels are the latest antiviral targets known as 'viroporins'. Pharmacological blockage inhibits viral budding and replication. Hexamethylene amiloride was recently shown to inhibit HCoV-229E and MHV replication by blocking E protein [82], as well as HIV-1 budding (by blocking Vpu) and the ion-channel activity of HCV p7 [83,84].

Pending patent US2007/0099968 disclosed several ion channel inhibitors against coronaviruses and other viruses [85]. A bacterial bio-assay was developed for screening potential SARS-CoV E protein ion channel blocking drugs. The temperature-inducible SARS-CoV E protein was expressed in *Escherichia coli*, the growth inhibition of which on exposure to drugs signifies dissipation of the normal Na^+ gradient. One viroporin inhibitor is an acylguanidine, cinnamoylguanidine (**Figure 15**), which reduced SARS-CoV (Hong Kong strain) and MHV viral titer by 76 and 88% at a concentration of 10 μM, respectively. At 2.5 μM, it showed 47 and 57% inhibition of HCoV-229E and HCoV-OC43, respectively. Test data on porcine respiratory coronavirus and bovine coronavirus, as well as several other viruses, were also presented.

5. RNAi and nucleic acid antiviral strategies

RNA interference specifically inhibits mRNA synthesis or translation as guided by a short strand of RNA, typically

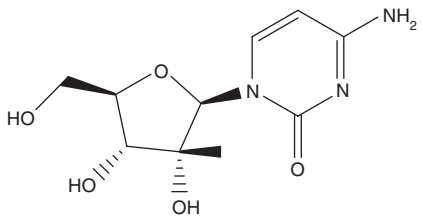

Figure 13. 2′-*C*-methylcytidine. This nucleoside analog showed inhibitory activity against HCoV-OC43.

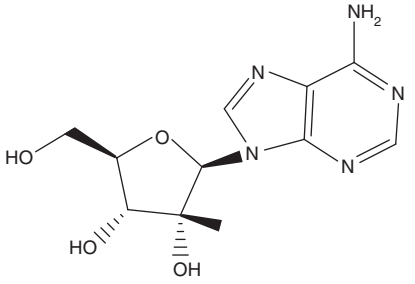

Figure 14. 2′-*C*-methyladenosine. This nucleoside analog showed inhibitory activity against HCoV-OC43.

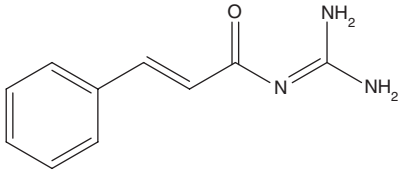

Figure 15. Cinnamoylguanidine. This "viroporin" inhibitor was found to inhibit coronavirus E protein and replication of SARS-CoV, MHV, HCoV-229E and HCoV-OC43 at low micromolar concentrations.

21 – 23 nt in size. It, therefore, does not result in global shutdown of protein synthesis found in the interferon-induced state. The phenomenon is also thought to have mechanisms for control and amplification [86]. Double-stranded viral RNA (an intermediate during viral replication) or precursor miRNA (pre-miRNA) are substrates of the enzyme Dicer (a class III ribonuclease that cleaves dsRNA), which produces siRNA and miRNA, respectively. When the anti-sense strand of the 21 – 23 nt double-stranded siRNA (or miRNA) is bound to an enzyme complex comprising both members of the argonaute family, eIF2C1 and/or eIF2C2, and a yet to be identified endonuclease, a cytosolic effector complex, RNA-induced silencing complex (RISC), is formed. This RISC (a 5′ phosphomonoester-producing RNA endonuclease [12]) directs post-transcriptional degradation of mRNA. At the transcriptional level, siRNAs targeted to CpG islands within promoters induce DNA methylation and inhibition of transcription. The intranuclear effector, RITS (RNA-induced initiation of transcriptional gene silencing) complex, includes DNA methyltransferase (DNMT) 1 and 3B. RITS also methylates histone H3 on Lys9 resulting in inhibition of gene transcription.

Experience with RNAi against human viruses *in vitro* have been reported for SARS-CoV [87-91], HIV-1 [92] and HCV [93]. For prevention of respiratory syncytial virus (RSV) infection, ALN-RSV01, an siRNA directed against the mRNA of RSV N-protein, not only showed specific *in vitro* and *in vivo* anti-RSV activity, it was also shown to be well tolerated and apparently safe when administered intranasally in 101 healthy adult volunteers (ClinicalTrials.gov Identifier: NCT00496821) [94].

The subject of RNAi against viruses and viral counter-strike (HIV-1 Tat protein and adenovirus virus-associated RNAs I and II, among others, show RNAi-suppression activity) was recently reviewed by Haasnoot [12]. The intellectual property aspects of siRNA as a new drug is reviewed by Schiffelers *et al.* [95].

Table 2 summarizes the properties of various nucleic-acid-based anticoronaviral strategies.

5.1 SiRNA targeting SARS-CoV ORF1a

He *et al.* designed six 21-mer siRNAs targeting various regions of the SARS-CoV replicase gene (ORF1a). Monkey kidney cells (FRhk-4 cells) were transfected with the siRNA 8 h before infection with SARS-CoV (GZ50 strain). Cytopathic effect (CPE) was determined with phase-contrast microscopy, followed by fixation and immunostaining with SARS-CoV-specific antibody isolated from acute convalescent sera of confirmed SARS patients. Various combinations of the siRNA were also evaluated, as well as their effects on other strains of SARS-CoV (GZ34, HKR_1 and HKR_2). SARSi-4, SARSi-2 and SARSi-3 showed consistent and marked reduction in viral replication *in vitro*, as confirmed by quantitative real-time polymerase chain reaction (RT-PCR: 85.8 – 92.5% reduction) [96]. These siRNAs were equally efficacious against the various strains of SARS-CoV tested. However, no synergism was observed. Mock transfected cells showed no morphological change. These *in vitro* results are encouraging, although clinical usefulness remains to be determined.

The corresponding US patent 7,129,223 disclosed SARSi-2, 3, 4, 7, 8, 9, 10 and 11, targeting SARS-CoV replicase 1a, S, E, M and N proteins as having inhibitory activity against SARS-CoV infection and replication *in vitro* [97]. SARSi-4, targeting replicase 1a, showed broad spectrum antiviral activity against three different strains of SARS-CoV (GZ43, HKR_1 and HKR_2). In addition, a synergistic effect is now observed with various combinations targeting different genes rather than the replicase 1a gene alone [96]. Thus, viral titers were reduced 18-fold by the combination of SARSi-4/7 and SARSi-7/8 and between 6- and 12-fold by SARSi-4/8, 4/9 and 7/9. The success of combination RNAi strategy permits lower dosage and greater clinical potential.

US patent application 2007/0203082, filed by Intradigm Corporation, disclosed several siRNAs targeting the replicase

Table 2. RNA interference and other nucleic acid antiviral strategies.

Target/technology	Exemplary drugs	IC_{50} and other measures of efficacy	Ref.
RNAi-replicase or combinations	SARSi-4; SEQ ID NO:4; nucleotide sequence 1194 – 1213	Viral titers were reduced 18-fold by the combination of SARSi-4/7 and SARSi-7/8	[97,98]
RNAi	siSC2 (target nsp12), siSC5 (target RdRp)	In macaques, provided relief from pyrexia, reduction in viral replication and reduced diffuse alveolar damage	[99]
RNAi conserved regions, such as RdRp and 3′UTR and/or shared leader sequence of SARS-CoV	Chemically modified synthetic siNA with increased resistance to nuclease degradation *in vivo* and/or improved cellular uptake	–	[100]
Frameshift/Antisense	Oligonucleotide (329729, 339289)	IC_{50} = 0.2/0.15 μM	[102]
Disruption of base-pairing between 5′ leader and body TRS	Oligonucleotide compound and method for treating nidovirus infections	–	[103,104]

IC_{50}: Concentration of a drug that is required for 50% inhibition of viral replication *in vitro*; RNAi: RNA interference; TRS: Transcriptional regulatory sequences.

gene (ORF1b) and a structural gene (Spike) of the SARS-CoV genome [98]. SC2 and SC5, targeting the S gene and nsp12 (RdRp), respectively, demonstrated significantly prolonged (72 h) reduction of CPE (> 80%) and viral titer in culture supernatant by quantitative RT-PCR, when administered before infection of cultured FRhk-4 cells. Further exhaustive search for siRNA showed that 2 extra siRNAs from among 40 showed similar potency in reducing CPE (SC14 targeting nsp13 and SC15 targeting nsp16). Given after inoculation, the therapeutic effects of siRNA were much weaker, with SC15 being the only siRNA having significant therapeutic effect. Control siCONa-b had no antiviral activity, essentially ruling out nonspecific interferon response. The data also suggest that the antiviral mechanism seemed to be disruption of the viral genome rather than the effect of targeting specific genes. In a unique experiment so far, siSC2 and siSC5, targeting S and ORF1b (nsp12, RdRp), respectively, were tested in a primate (Rhesus macaque) model of SARS-CoV by nasal delivery and demonstrated relief from pyrexia, reduction in viral replication and reduced diffuse alveolar damage. The 10 – 40 mg/kg accumulated dosages did not result in any sign of siRNA-induced toxicity [99]. Although the results are promising, it is not clear why only three of four macaques in each treatment group showed response to the therapy.

5.2 SiRNA with modified structures

Sirna Therapeutics disclosed short interfering nucleic acid (siNA) specific for conserved regions of SARS-CoV, such as the 3′-untranslated end and/or leader sequence common to the genomic RNA and nested mRNA transcripts or the viral polymerase RdRp [100]. The siNA may be chemically modified for increased resistance to nuclease degradation *in vivo* and/or improved cellular uptake. Examples of modification include phosphorothioate, phosphonoacetate and/or thiophosphonoacetate internucleotide linkages [101], 2′-deoxyribonucleotides, 2′-*O*-methyl ribonucleotides, 2′-deoxy-2-fluororibonucleotides, 'universal base' nucleotides, 'acyclic' nucleotides, 5-*C*-methyl nucleotides and terminal glyceryl and/or inverted deoxy abasic residue incorporation.

5.3 Oligonucleotides targeting coronavirus programmed frameshift

US patent 7,339,051 disclosed oligonucleotides 329722, 329724, 329727, 329550 and 329731 as effective inhibitors of the -1 frameshift of SARS-CoV [102]. Ribosomal frameshift is widespread, being observed in viruses, bacteria, protozoa and mammals. Frameshifting in viruses regulates the relative quantity of two encoded polypeptides. Coronaviruses are among some viruses that use frameshift to regulate gene transcription. Importantly, coronaviruses do not carry in the virion the requisite RdRp for replication. It is instead produced from proteolytic autocleavage from pp1ab, the larger of the two products of ORF1. Thus, interruption of frameshifting and the abrogation of RdRp is an attractive anticoronaviral strategy. The disclosed siRNA were effective at concentrations ranging from 5 to 25 μM, where IC_{50} values were estimated to be in the 5 – 10 μM range. Oligonucleotides 329729 and 339289 were even more effective, having an IC_{50} of 0.2 and 0.15 μM, respectively. One oligonucleotide (329721) increased the efficiency of the frameshift; indicating that both reduced and enhanced frameshifting efficiency are possible with oligonucleotides. The group had previously used RNAi to successfully target HCoV-NL63 S [21]. SiRNA1 and siRNA2 had IC_{50}, CC_{50} and SI of 5 nM, > 200 nM, > 40; and 3 nM, > 200 nM, > 66, respectively.

5.4 Nucleic acids targeting coronavirus subgenomic mRNA synthesis

A genomic expression strategy that characterizes Nidoviruses (including coronaviruses) is the synthesis of a nested set of subgenomic mRNAs before translation of genes encoding the structural proteins. Critical for this phase of the coronavirus

life cycle is the base pairing between the body transcriptional regulatory sequences (TRS) at the 3′ end of the nascent minus-strand mRNA and the complementary leader TRS at the 5′ end of the positive-strand genomic RNA (see **Figure 3** in [8]). This step lends itself to antiviral compounds disclosed by AVI BioPharma's patent application [103].

The patent application disclosed oligonucleotide compounds that disrupted the synthesis of nested subgenomic mRNA. It is 4 – 25 nucleotide bases in size and is characterized by a nuclease-resistant backbone and has a sequence that is complementary to ≥ 8 bases contained in either the 5′ leader sequence that includes an internal leader TRS or a negative-strand 3′ subgenomic intermediate that includes an internal body TRS. The oligonucleotide forms heteroduplex with genomic RNA or negative-strand subgenomic intermediate by specific base pairing, thus disrupting base pairing between the TRSs and the synthesis of nested subgenomic mRNA. Sequences determined to be inhibitory to various nidoviruses include SEQ ID NOS: 26, 27 and 36 – 43 for SARS-CoV, 22 and 23 for HCoV-229E, 24 and 25 for HCoV-OC43, 20 and 21 for feline coronavirus and 44 and 45 for simian hemorrhagic fever virus. The findings were replicated by a recent study that showed siRNA targeting the leader TRS were more potent than siRNA targeting SARS-CoV S gene [104].

6. Interferon and related intracellular antiviral defense

The interferon system is an antiviral shield that is targeted by numerous viruses [13,105]. They are cytokines elaborated by virally infected cells and innate immune cells and function to mount an antiviral innate immune system in the host organism. It is coupled with pathogen pattern recognition receptors (toll-like receptors, PKR, RNA helicases such as retinoic acid inducible gene-I (RIG-I) and the homologous melanoma differentiation-associated gene 5 (mda-5) [106]) and antiviral effectors. These include RNA-dependent protein kinase (PKR), 2′,5′-oligoadenylate synthetase (2 – 5 AS), RNase L, RNA-specific adenosine deaminase ($ADAR_1$), protein Mx GTPase and inducible nitric oxide synthase, among others.

SARS-CoV uses various strategies against the innate immune response and interferon system. IFN-regulatory factor 3 (IRF-3), a latent transcription factor downstream of the pathogen pattern recognition receptors, is prevented from phosphorylation and nuclear translocation by SARS-CoV PLpro [54]. Other SARS-CoV proteins, including ORF3b, ORF6 and N also prevent nuclear import of activated IRF3 [107] and hence activation of IFN-β gene transcription [55]. In a similar manner, SARS-CoV ORF6 antagonizes STAT1, and hence interferon function, by sequestering nuclear import factors on the rough endoplasmic reticulum/Golgi membrane [108]. Thus, signals of viral infection are prevented from entering the nucleus. This is one viral mechanism to prevent activation of interferon-stimulated response element (ISRE) and gamma-activated sequence (GAS) elements in interferon-responsive genes [108]. Also, nsp1 reduces STAT1 phosphorylation [109] and promotes host mRNA degradation [110]. By degrading host mRNA, crucial antiviral proteins are not translated even if the genes are transcribed. The result is abrogation of the antiviral cellular proteins induced by interferon.

Anticoronaviral strategies using intracellular innate defense are listed in **Table 3**.

6.1 Increasing intracellular 2–5-oligoadenylate synthetase as anticoronaviral strategy

One of the innate intracellular antiviral effectors, 2–5 AS, comprises several isoforms encoded by three duplicated genes on chromosome 12, catalytically converting ATP into various oligoadenylates of the general structure ppp(A2′p)*n*A, which dimerizes and activates RNase L, an IFN-inducible, constitutively expressed, latent endoribonuclease that breaks down viral and cellular RNAs, including rRNA.

US Patent 7,354,908 claims a method of inhibiting an RNA virus infection within a patient by increasing the degree of 2–5 AS activity within the cell [14]. By delivery of a genetic cargo encoding murine 2–5 AS or at least one catalytically active fragment thereof, such as the p40, p69 or p100 subunits, the inventors provided evidence of reduction in epithelial cell damage, in peribronchiolar and perivascular inflammation, in thickening of the alveolar septae, of viral titer in the lungs, and of the levels of chemokines such as MIP1-α using RSV (strain A2) and $STAT4^{-/-}$ BALB/c mice in the experiments. Whether the result can be extrapolated to humans and other RNA viruses, including the coronaviruses, remains to be proven.

6.2 Interferon as anticoronaviral medicine

Being signaling molecules, interferons are best used prophylactically before viral infection. They have also shown clinical efficacy in blocking viral dissemination from cell to cell in the organism as a whole. Several uncontrolled clinical studies during the SARS epidemic have suggested clinical efficacy of interferons against SARS-CoV [74, 111-115]. *In vitro* and animal studies, including in primate, have further demonstrated the efficacy of interferon therapy [62,111,116-121].

US patent publication 2006/0035859 disclosed specific anti-SARS-CoV activity of Alferon N® (IFN-α-n3) in Vero 76 cell culture: EC_{50} = 5,696 +/- 1,703 (SEM) IU/ml (visual cytopathic effect) and 10,740 +/- 5,161 (SEM) IU/ml (neutral red assay) [122]. Viral yield reduction assay indicated an EC_{90} (viral load reduction by 1 $\log_{10}$) of 78,000 +/- 22,000 (SEM) IU/ml. The divisional application US2007/0141080 included various combinations of natural interferon and dsRNA (Ampligen™) [123]. Tan *et al.* also showed an IC_{50} of 0.8 IU/ml (IC_{95} = 200 IU/ml) for Alferon™ on plaque reduction assay [124]. Using BALB/c

Table 3. Interferon and related intracellular antiviral defense.

Target/technology	Exemplary drugs	IC_{50} and other measures of efficacy	Ref.
Host innate antiviral mechanism	2′-5′-oligoadenylate synthetase or coding sequence	–	[18]
IFN	Natural IFN (Alferon N)	EC_{50} = 5.7 +/- 1.7 IU/µl (CPE) and 10.7 +/- 5 IU/µl (NR assay)	[122]
IFN and IFN inducer	Combinations of natural IFN (Alferon N®) and dsRNA (Ampligen™)	Alferon N: EC_{50} = 5.7 +/- 1.7 IU/µl (CPE) and 10.7 +/- 5 IU/µl (NR assay)	[123]
IFN	IFN alpha-n1, n3, human leukocyte IFN or –IFN beta-1b	–	[124]
IFN	Combination of type I or III IFN receptor agonist and/or type II IFN receptor agonist against coronaviruses	–	[129]
IFN inducer	Artificial CpG single-stranded oligodeoxynucleotide	–	[135]

CPE: ; EC_{50}: Plasma concentration required for obtaining 50% of the maximal effect *in vivo*; IC_{50}: Concentration of a drug that is required for 50% inhibition of viral replication *in vitro*; IFN: Interferon; NR: Neutral red.

mice as an animal model of SARS infection, Barnard *et al.* showed that IFN-α B/D, a hybrid interferon consisting of residues 1 – 60 from HuIFN-αB and residues 61 – 166 from HuIFN-αD, with broad cross-species activity, and Ampligen™, a mismatched double-stranded RNA interferon inducer, were effective in reducing viral titers in the lungs as assessed by CPE titration assay [66]. As expected from the species specificity of interferon molecules, intraperitoneally administered human natural interferon, Alferon™, failed to demonstrate protective effect on BALB/c mice from SARS-CoV challenge [66]. In the only primate model studied, pegylated IFN-α given before infection protected macaque type 1 pneumocytes against infection by SARS-CoV [125]. Type II interferon (INF-γ) is not effective when used alone. However, it enhances type I interferons when administered together [126-128].

Pending patent US2006/0153803 further provides experimental data on the SARS-CoV inhibitory effects of several interferons, IFN-β1b (Betaferon™), IFN-αn3 (Alferon™) and IFN-α (Multiferon™), as determined by plaque reduction assay on Vero E6 cells infected with SARS-CoV (strain 2003VA2774), with IC_{50} of 0.2 – 2 IU/ml [124]. These interferons showed no cytotoxicity at or near inhibitory concentrations.

Blatt disclosed monotherapy and combination therapy (types I and III) with IFN-s [129]. The role of interferon in the treatment of SARS is recently reviewed [130-132].

6.3 Interferon inducers

CpG dinucleotides are rare in the mammalian genome, and unmethylated CpG even rarer [133,134]. Unmethylated CpG is considered by the eukaryotic cell as a signal of intracellular pathogen invasion and is recognized by the homodimeric TLR-9, a surface and endosomal membrane-bound pathogen recognition receptor (PRR) expressed by plasmacytoid dendritic cells (pDC, previously known as IFN-α–producing cell), B lymphocytes and NK cells.

6.3.1 CpG single-stranded oligodeoxynucleotide

US patent publication 2007/0155683 disclosed novel artificial CpG single-stranded oligodeoxynucleotide (ODN) DVAX-1 that induces IFN production by pDC and peripheral blood mononuclear cells (PBMN) [135]. The diluted supernatant of cultured PBMN treated with DVAX-1 protected Vero cells from infection with influenzavirus and SARS-CoV Sino-5 strain. BW001 (another CpG ODN), with B-type CpG ODN structural feature at the 5′ end and A-type CpG ODN structural feature at the 3′ end, protected Vero cells from SARS-CoV infection [136].

6.3.2 Mismatched double-stranded RNA: Polyl:polyC12U (ampligen)

Similar to CpG ODN, Ampligen, a synthetic dsRNA recognized by lysosomal/endosomal TLR-3 in DC, T lymphocytes and NK cells, is disclosed in US2006/0035859 and a divisional application US2007/0141080 as being effective in acute and severe viral infections and SARS [122,123]. Ampligen, $rI_n.r(C_{12}U)_n$, Poly A.Poly U or $rI_n.r(C_{29},G)_n$, in which r is ribose, is a mismatched derivative of double-stranded RNAs and was recently reviewed by De Clercq [137]. Ampligen alone protected mice from coxsackie B3 virus-induced myocarditis [138] and flavivirus-induced encephalitis [139] but offered only limited protection against lethal pichinde virus challenge [140]. When given with interferon, Ampligen amplifies its effects. It also has synergistic activities with most antiretrovirals [141] and is well tolerated by HIV positive patients in a multi-center, randomized, double-blind study [142]. Recently, Barnard *et al.* evaluated two IFN-inducers, Ampligen and poly IC:LC, in a mouse model of SARS [78]. Without prophylactic treatment, 70% of the BALB/c mice exposed to SARS-CoV lost significant body weight (> 30%) followed by death 3 – 5 days later. With prophylaxis, virus lung scores were reduced, although lung viral titers were not. However, mice given interferon

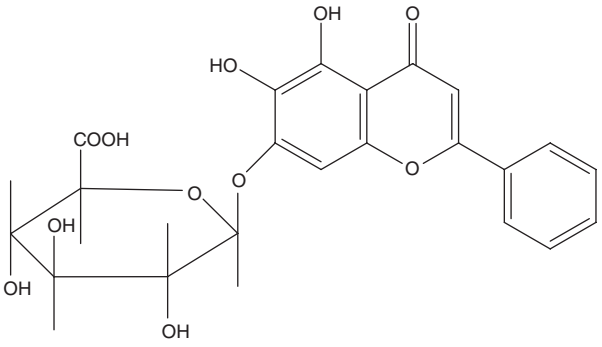

Figure 16. Baicalin. This Chinese medicinal herb inhibits SARS-CoV by an unknown mechanism.

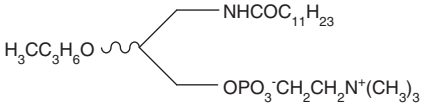

Figure 17. Phospholipid compound with Anti-SARS-CoV activity.

stimulators were significantly protected from weight loss and fatal infection.

7. Other patent applications with unknown targets

7.1 Baicalin

US patent publication 2005/0215494 by Yuen *et al.* disclosed the anti-SARS-CoV activity of Chinese medicinal herbal compound Baicalin (**Figure 16**), which had EC_{50} of 12.5 – 25 μg/ml at 48 h and 25 – 50 μg/ml at 72 h, CC_{50} of > 100 μg/ml and SI of > 8 [143]. The mechanism of action is unknown [62].

7.2 Phospholipids

US 2005/0187192 disclosed a phospholipid compound (**Figure 17**) with inhibitory activity against SARS-CoV (Urbani strain) in Vero cells, as determined by neutral red assay [144]. The EC_{50}, CC_{50} and SI were 3 μg/ml, 40 μg/ml and 13, respectively.

Table 4 compares the properties of these agents with unknown mechanism of anticoronaviral activity.

8. Conclusion

The coronaviruses are animal viruses that are well adapted in humans, other mammals and birds. The cross-species transmission of an animal coronavirus in 2002 – 2003 resulted in the pandemic spread of a serious illness, SARS. There is compelling reason to continue the search for a vaccine and to develop antiviral therapies for the coronaviruses as a global strategy against emerging diseases. The continued engagement of the best minds in this quest is necessary because we are also threatened by other emerging diseases and serious pathogens, all of which demand a similar set of knowledge and skills. In developing therapeutic agents for coronaviruses, many different approaches are being taken. We have reviewed strategies preventing viral entry into host cells in part I and small molecules that are more readily taken into the cytosol to interfere with the intracellular viral life cycle in this review. It is fair to conclude that all of the approaches are necessary and elegant, and complementary rather than exclusive.

The fact that viruses are so successful reinforces the widespread notion that viral escape from antivirals is virtually certain. Antiviral strategies evolved by the host cell and organism as a whole, are all known to be subject to viral counterattacks. RNA interference has met with anti-Dicer (such as HIV-1 tat protein), the interferon system is routinely under attack (SARS-CoV PLpro interferes by binding IRF-3) and a point mutation can potentially enable viral escape from monoclonal antibodies, small molecule inhibitors of viral enzymes and nucleic acid-based therapies such as antisense oligonucleotide and siRNA. It is, therefore, imperative to keep all antiviral venues open and use a multi-pronged strategy.

9. Expert opinion

Among the antiviral agents reviewed in this series, monoclonal antibody technology promises to deliver a safe and potent agent(s) within a few weeks of an epidemic. The success of placental animals probably owe in some part to the passive immunity conferred on their offspring. Owing to the fear of vaccine reaction and doubt of efficacy as the main reasons for a 75% acceptance rate for influenza vaccine [145], it is conceivable that MAb be derived from vaccinated individuals for the prophylaxis of those who refuse vaccination. It is, however, expensive and of limited impact on the psychological need of a population in panic because of the time to develop them and the limited capacity to produce them at present.

The issue of capacity also pertains to natural interferons, although recombinant interferons can be readily produced in greater quantities. Because interferons have been shown to be efficacious and are relatively safe, they and the interferon inducers should be available to those who do not desire MAb or other newer medications.

Despite viral counterattack on RNAi, as therapeutics, this technology seems to be very promising. A shortcoming is, as with vaccination, an element of uncertainty as to who will be successfully protected. The short development time and the largely innocuous nature of well-designed agents that do not accidentally knockout host proteins, are some important advantages of this approach. These agents can also be very cheap to produce. Issues to consider are route of administration and innovative measures to target specific cellular populations [146].

Table 4. Other patent applications with unknown targets.

Target/technology	Exemplary drugs	IC_{50} and other measures of efficacy	Ref.
	Baicalin	IC_{50} = 12.5 – 25 μg/ml (48 h); CC_{50} > 100 μg/ml; SI > 8	[62,143]
	Phospholipid	EC_{50} = 3 μg/ml; CC_{50} = 40 μg/ml; SI = 13	[144]

CC_{50}: Cytotoxic concentration that reduced cell viability to 50%; EC_{50}: Plasma concentration required for obtaining 50% of the maximal effect *in vivo*; IC_{50}: Concentration of a drug that is required for 50% inhibition of viral replication *in vitro*; SI (selectivity index): CC_{50}/EC_{50}.

Small molecule inhibitor of coronavirus main protease is an attractive approach that appeals because of the potential for broad spectrum activity and a higher certainty of a clinical response. If the drugs were known to be safe, it would appeal to the popular psyche and be real ammunition. However, most studies have not produced data beyond feasibility and preliminary toxicology, with one providing *in vivo* toxicity results on chicken embryos [34]. Agents with a narrow toxicity window may be viable drug leads if potent but have no utility in the clinical setting. Stockpiling small molecule drugs and distributing them should not be a serious logistical issue. Several competing compounds can be produced and stockpiled as contingencies.

The development of the new class of antiviral, viroporin inhibitors, should be watched. Finally, existing drugs and natural products that have been shown to be effective and having a good safety profile should not be forgotten [131,147].

Declaration of interest

The author states no conflict of interest and has received no payment in preparation of this manuscript.

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