

Therapies for coronaviruses. Part I of II – viral entry inhibitors

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Expert Opinion

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Therapies for coronaviruses. Part I of II – viral entry inhibitors

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Background: Severe acute respiratory syndrome (SARS) coronavirus emerged fleetingly in the winter of 2002 and again in the winter of 2003, resulting in the infection of ~ 8000 people and the death of ~ 800. The identification of the putative natural reservoir suggests that a re-emergence is possible. The functions of many coronaviral proteins have now been elucidated, resulting in many novel approaches to therapy. **Objective:** To review anti-coronaviral therapies based on inhibition of viral entry into the host cell and to cast light on promising approaches and future developments. **Method:** The published literature, in particular patent publications, is searched for relevant documents. The information is organized and critiqued. **Results/conclusion:** The approaches to combating coronaviral infections are built on the foundation of antivirals against other viruses and the fundamental insights gained by dissection of the coronaviral lifecycle. These approaches include the prevention of viral entry, reviewed here, and interference with the intracellular lifecycle of the virus in the infected cell, reviewed next. Of the viral-entry inhibitors, monoclonal antibodies have demonstrated efficacy, clinical application in other viral infections, and the potential to impact a future epidemic. Moreover, combinations of monoclonal antibodies have been shown to have a broader spectrum of antiviral activity.

Keywords: ACE2, cathepsin L, coronavirus, heptad repeat, monoclonal antibody, SARS, SARS-CoV, severe acute respiratory syndrome

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1. Introduction

The coronaviruses are enveloped, positive-sense, single-stranded RNA viruses (Baltimore group IV). They and the other genus of arteriviruses belong to the order Nidovirales, sharing the same transcriptional strategy of creating a nested set of subgenomic mRNAs [1]. However, at around 30 kb, the coronaviruses have larger genome, in fact, the largest among the RNA viruses. Coronaviruses are animal viruses and circulate in humans, other mammals, and birds. The human coronaviruses (H-CoV), OC43, 229E, NL63, HKU1, and SARS-CoV are found in groups 1 and 2, along with various mammalian coronaviruses; the avian coronaviruses are found in an exclusive group 3. The recently discovered SARS-CoV is a zoonosis and jumped to humans from bats [2-5], and so is H-CoV-OC43, which probably arose from bovine CoV [6], a possible consequence of the domestication of the cattle.

SARS-CoV is the etiologic agent of SARS [7], a viral pneumonia with 10% fatality rate [8-12]. The disease emerged in late 2002 in southern China, spread to 29 countries within a few weeks, and infected ~ 8000 people, resulting in close to 800 deaths. Critical respiratory impairment, subclinical immune dysfunction, and diarrhea are the primary manifestations of illness. A pandemic was averted with some luck and a lot of hard work [13]. In the several years since the epidemic, the molecular evolution [14] of the virus has been worked

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out; the natural reservoir identified; the viral lifecycle, genome and proteome dissected; animal disease models established; structural information on viral enzymes obtained; and HCoV-229E- and SARS-CoV-based non-infectious replicon cell lines established, paving the way for *in-silico* and high-throughput screening of chemical compound libraries for drug leads [15,16].

The coronavirus lifecycle begins with attachment to specific receptors expressed on a permissive cell of the native or a closely related animal species. H-CoV 229E, transmissible gastroenteritis virus, and feline infectious peritonitis virus use the zinc metalloprotease aminopeptidase N (APN, CD13) as receptors [17]. The mouse hepatitis virus (MHV) employs the immunoglobulin superfamily of cell-surface proteins as receptors.

The cellular receptor for SARS-CoV [18] and HCoV-NL63 [19] spike protein is angiotensin I converting enzyme 2 (ACE2). ACE2 is expressed in various tissues and organs of the body – including the respiratory tract, gastrointestinal tract, liver, and kidneys – resulting in infection of these organs by SARS-CoV. Following viral attachment to ACE2, fusion of the viral lipid envelope with that of the cell membrane takes place after the formation of a fusion peptide as a result of conformational change of the S2 region of the spike protein. This change involves heptad repeats (HR₁ and HR₂), oligomerization of S, and extracellular proteolytic cleavage or cathepsin L (CTSL)-mediated cleavage in endocytic vesicles [20].

The virus lifecycle, the host cell, and the organism as a whole represent many opportunities for therapy [21-23]. Previous efforts focus mostly on the virus. However, the host cell also presents opportunities because the virus does not encode all the proteins it needs for its lifecycle, usurping host proteins for its purpose. Finally, the innate and adaptive immune responses of the organism present further opportunities to prevent and treat coronaviral and other viral infections. Part I of this review of patent literature on therapies for coronaviruses, focuses on viral-entry inhibitors.

2. Viral-entry inhibitors

2.1 Coronaviral spike (S) protein and ACE2 receptor interaction as antiviral target

Denying entry of an intracellular pathogen as an antiviral strategy is conceptually most appealing. This serves not only to protect the unexposed host (prophylaxis) but also to prevent the infection of more host cells in the exposed/infected host (treatment). Normally non-permissive cells have been rendered suitable for the propagation of SARS-CoV by expressing the cellular receptor, ACE2 [18], by genetic engineering [24] [202], thus attesting to the importance of this phase of the viral lifecycle. Various strategies to prevent entry of coronavirus into its host cell are list in Table 1 and discussed below.

2.1.1 Inhibitors derived from ACE2 receptor

Pending patent US2005/0282154 by Farzan *et al.* teaches methods of stably expressing ACE2 in cell lines to impart permissivity for SARS-CoV, and methods of assaying inhibitory activity of agents that disrupt binding between S-protein and ACE2. Inhibitors claimed include soluble ACE2, antibodies against ACE2, and small molecule inhibitor (Figure 1) of ACE2 catalytic activity [24].

Soluble decoy receptors that saturate viral receptor-binding proteins, such as SARS-CoV S protein, could be used to prevent viral binding to cells. Lessons learned from HIV1 suggest that unmodified decoy receptors are not sufficiently potent [25-27] and require modification, such as fusion with another protein, of which CD4-IgG is an example [28-30]. This chimeric molecule has four binding regions for HIV1 gp41, D₁D₂ domains of human CD4 engineered into the IgG₂ molecule, replacing the variable domains. It has been tested in human subjects (ClinicalTrials.gov, identifier NCT00000876) [31,32]. The chimera overcomes the problems of soluble CD4, such as enhancement of viral infection, low neutralizing activity, and short half-life *in vivo*. CD4-IgG₂ (PRO 542) [33] was evaluated in HIV-infected adults in a Phase I study that found reductions in plasma HIV RNA and plasma viremia with no dose-limiting toxicities [31]. Another Phase I/II clinical trial in children with HIV1 infection also demonstrated reduction in viral burden [32]. In a similar fashion, engineered multivalent soluble ACE2 (sACE2)-immunoglobulin might also be efficacious in neutralizing SARS-CoV [34]. Tailoring sACE2 to SARS-CoV can conceivably be improved by employing residues 90 – 93 of civet ACE2 [35].

2.1.2 ACE2 inhibitor

As surmised, inhibition of ACE2 catalytic activity is accompanied by anti-SARS-CoV activity. One lead compound (*N*-(2-aminoethyl)-1 aziridine-ethanamine [NAAE]) (Figure 2) was identified among ~ 140,000 small molecules by *in silico* molecular docking. It inhibited both ACE2 catalytic activity and S-protein-induced cell–cell fusion by virtue of shifting of spike-binding residues upon occupation of the enzymatic site by NAAE [36]. Others did not expect dual inhibitory effect on ACE2 catalytic activity and SARS-CoV binding because the catalytic site of ACE2 is distinct from the S-protein-binding domain [18,34]. Nevertheless, NAAE did show antiviral activity, with IC₅₀ measured in the micromolar range. The control heptad repeat peptide inhibitor used in the experiments was orders of magnitude more potent. Cytotoxicity data are not available.

Because ACE2 and the angiotensin II type 1a receptor have recently been found to be protective of lung injury in SARS-CoV infection, as well as acute respiratory distress syndrome from a variety of causes, inhibiting ACE2 as an antiviral strategy appears to be physiologically unsound and needs further consideration [37,38].

Table 1. Coronaviral entry inhibitors.

Target/technology	Exemplary drugs	IC ₅₀ and other measures of efficacy	Ref.
ACE2	Anti-ACE2 antibody, soluble ACE2 and ACE2-inhibitor	–	[24]
S protein/MAb	S3.1	~ 5 ng/ml (100% viral neutralization)	[48]
S protein/MAb	MAbs 11A and 256, binding to S not containing Asp at 480 position	–	[55]
S protein/MAb	CR3022 in combination with CR3014 for SARS	Neutralizing titer (66% protection of wells) CR3014 = 128 – 256 (2.4 – 4.9 µg/ml) CR3022 = 32 – 64 (9.8 – 19.5 µg/ml)	[55,56]
S protein/MAb	Clone 7-508-201	IC ₅₀ = 0.7 nM (50% viral neutralization <i>in vitro</i>) ED ₅₀ = 6 nM (full length S1255 binding) See Table 3 of ref. [61]	[60,61]
S protein/MAb	30F9 (Conf IV) and 33G4 (Conf V), including different chimeric constructs and humanized constructs	50% neutralizing dose (ND ₅₀) Conf IV = 0.005 µg/ml Conf V = 0.009 µg/ml	[63,64]
S2 (heptad repeats)	Peptide heptad repeat mimetic	–	[80]
S2 (loop region)	Peptides analogous to S2 loop region	IC ₅₀ = 2 – 4 µM > 80% plaque reduction at 15 – 30 µM	[82]
S peplomers	Peptides (P2, P6, P8, and P10) inhibiting oligomerization of S protein	IC ₉₀ (P8) = 24.9 ± 6.2 µg/ml	[84]
Cathepsin L	Val-Phe-containing compounds (MDL 28170/Z-Val-Phe-CHO/PN-001) and various formulas for SARS and Ebola	IC ₅₀ = 1 nM (fluorogenic substrate) EC ₅₀ = 100 nM (entry of pseudotype virus into 293T cells in a luciferase assay)	[95]

IC₅₀: Concentration of a drug that is required for 50% inhibition of viral replication *in vitro*; C₅₀: Plasma concentration required for obtaining 50% of the maximal effect *in vivo*; CC₅₀: Cytotoxic concentration that reduced cell viability to 50%; SI (selectivity index) = CC₅₀/EC₅₀.

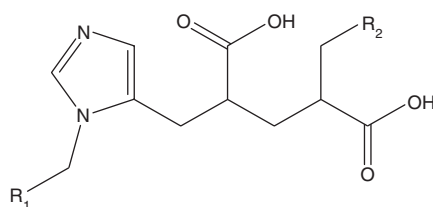


Figure 1. Small molecule inhibitor of ACE2. Formula 1 is illustrated. R1 is selected from the group consisting of: phenyl optionally substituted with a group selected from NO₂, Cl, a C₁–C₃ alkyl, and CF₃O–; an alkylcyclohexyl, wherein said alkyl is a C₁–C₃ alkyl; and cyclohexyl optionally substituted with a C₁–C₃ alkyl; and R₂ is selected from the group consisting of phenyl and a straight or branched C₁–C₃ alkyl.

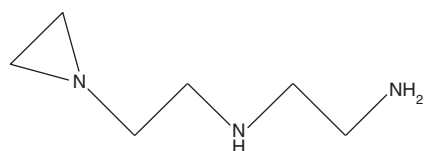


Figure 2. N-(2-aminoethyl)-1 aziridine-ethanamine (NAAE). Another small molecule inhibitor of ACE2.

2.1.3 Engineered monoclonal antibodies against receptor-binding domains

Antibodies have long been used to combat infections. They have been in and out of favor because of the development of small molecular anti-infectives and the accumulation of immunocompromised hosts and drug-resistant microbes [39]. The subject was reviewed recently [40,41]. Competing for space on the shelf are small-molecule drugs and vaccines. Like most vaccines, therapeutic antibodies need to be refrigerated (2 – 8°C) during distribution and storage. With recent advances in the rapid development of fully humanized monoclonal antibodies (MAb), thus limiting the side effects, our therapeutic arsenal promises to be as unlimited as the diversity of antibodies [41].

FDA approval of passive immunoprophylaxis of neonatal respiratory syncytial virus (RSV) infections prompted an evolution from polyclonal hyperimmune globulin (RespiGam®) to a humanized MAb palivizumab (MEDI-493, Synagis®) [42,43]. More potent and longer-lasting second- and third-generation MAbs against RSV are currently undergoing Phase III clinical trials (motavizumab) and development (Numax-YTE) [43]. These developments are familiarizing industry, regulatory agencies, clinicians, and the public to the new era of MAb-based anti-infectives.

With regard to SARS, it is encouraging that neutralizing convalescent or engineered antibodies has shown therapeutic

potential [44]. During the SARS epidemic, convalescent serum was used in SARS patients (and in mice) without ill effect [45,46]. Experimental studies employing HIV(S), an engineered HIV1-expressing SARS-CoV spike protein on the surface and encoding luc as a reporter system, have demonstrated the necessity and specificity of the interaction of S with the then unknown cellular receptor ACE2, as well as the ability of convalescent sera to inhibit the process [47].

2.1.3.1 MAb from human memory B cells of convalescent patients

When developing MAb against SARS-CoV, Lanzavecchia's group interrogated the B-cell memory repertoire of an immune SARS patient and rapidly and efficiently isolated 35 neutralizing MAbs. These were identified as IgG1 antibodies, without other IgG isotypes or IgA or IgM antibodies.

IgG+ memory B cells were isolated by a combination of magnetic and fluorescence-activated cell sorting (FACS). These cells were directly immortalized by EBV, potentiated by a polyclonal B-cell activator, CpG 2006 (a CpG oligonucleotide that activates toll-like receptor 4), in the presence of irradiated allogeneic mononuclear cells. The antibodies neutralize SARS-CoV (Frankfurt strain) at concentrations of ~ 5 ng/ml [48].

One of these MAbs, S3.1 – an IgG1-kappa neutralizing antibody – prevented SARS-CoV replication in murine lungs when given prophylactically [49]. Mice given the control IgG and S3.1 intraperitoneally at doses of 50, 400, and 800 µg were subsequently challenged with 10^4 TCID₅₀ of SARS-CoV (Urbani strain). Two days later, the nasal turbinates and lungs were examined for viral titers. The lungs were better protected than the turbinates; no virus was detected in the lungs of animals given more than 200 µg of the antibody ($\leq 1.5 \log_{10}$ TCID₅₀/g tissue).

Also starting from SARS convalescent patients, Duan *et al.* constructed an immune antibody phage-display library, from which B1, a human single-chain variable region fragments (scFv) recognizing an epitope on S2 (residues 1023 – 1189), was identified [50]. Peripheral blood lymphocytes from four SARS convalescent patients were isolated. RNA was isolated and cDNA was synthesized, from which a library of primary scFv was created after amplification of the variable regions of the immunoglobulin heavy and light chains. B1 is deduced from DNA sequence to consist of V_{H1} and V_{K3}. It has high affinity for SARS-CoV virions (equilibrium dissociation constant, $K_d = 105$ nM) and shows dose-dependent neutralization of SARS-CoV pseudovirus infection of Vero E6 cells (50% neutralizing dose ≈ 4.25 µg/ml).

2.1.3.2 MAb from non-immune human antibody libraries

Without resorting to the immune patients, early validation of MAb against SARS-CoV was demonstrated by the Marasco group, which employed naïve human antibody libraries. Eight recombinant human scFvs against the receptor-binding domains (RBD) of S protein were identified from a vast

library. 80R IgG₁, a monoclonal antibody engineered from one such fragment, possesses potent neutralizing activity *in vitro* and was demonstrated to confer resistance in a mouse model when given prophylactically [51,52]. The authors further demonstrated that not all strains of SARS-CoV (e.g., GD03) they have tested are sensitive to the 80R MAb, making it strategically important to monitor the viral genotype for prediction of efficacy [51]. The related patent application [53] claims an isolated MAb that binds to SARS-CoV S protein not containing an aspartic acid residue at a specified location. However, the International Searching Authority is of the opinion that the claims are anticipated by van den Brink (see next paragraph) [54-56].

2.1.3.3 Combination of monoclonal antibodies

Also starting with a naïve antibody library, antibody phage display technology was used to identify a human IgG₁ antibody, CR3014 [54,57]. This exhibited potent *in-vitro* viral neutralization and *in-vivo* protection of ferrets from macroscopic lung pathology [57]. CR3014 has a neutralizing titer (defined as protection of 66% of cells from CPE) against wild-type SARS-CoV (Frankfurt 1 strain) on Vero cells of 42 nM (see example 7, in Table 7, ref. 56). It apparently recognizes a conformational epitope within the S1 domain that cannot be resolved by PEPSCAN analysis and is probably different from that recognized by 80R [54]. Another MAb, CR3006, is susceptible to viral escape following loss of its binding affinity with S by the introduction of naturally occurring amino-acid substitutions of residues Y442 or F360, L472, D480, and T487, which are present in two different SARS-CoV isolates. Because viral escape from neutralizing antibodies occurs, and viral enhancement might be the consequence of subneutralization, the Ter Meulen group further provided combination of monoclonal antibodies. This group showed that CR3022, a newly identified neutralizing antibody against CR3014 escape mutants, is not prone to new escape variants and is synergistic to CR3014, allowing a lower dose of either antibody for passive immunoprophylaxis of SARS-CoV infection [58].

2.1.3.4 Monoclonal antibodies from immunized transgenic mice with human immunoglobulin genes

The Ambrosino group employed transgenic mice with human immunoglobulin genes and vaccinated them with recombinant SARS-CoV S protein [59-61]. They then used immunoprecipitation analysis to define the neutralizing epitopes of two monoclonal antibodies identified in the process. The fully human MAb 201 binds within the receptor-binding domain of S at residues 490 – 510, whereas the chimeric MAb 68 binds externally at residues 130 – 150. MAb 201 and MAb 68 provided effective immunoprophylaxis of non-immunized mice. Postexposure therapy with MAb 201 of Golden Syrian hamster, a model of SARS-associated pulmonary pathology, resulted in reduction of the viral burden and alleviation of pulmonary

pathology [62]. MAb 201 is planned for clinical trial should SARS return [59]. However, the International Searching Authority is of the opinion that the claims are lacking in inventive steps.

2.1.3.5 Monoclonal antibody from immunized non-transgenic mice

Jiang *et al.* disclosed the method for producing 23 potent neutralizing MAbs against several distinct conformational epitopes of S-protein RBD [63,64]. BALB/c mice were immunized with protein-A sepharose-purified RBD-Fc fusion protein. Splenocytes of immunized mice were fused with SP2/0 myeloma cells to generate hybridoma colonies in a standard fashion. MAbs recognizing conformation-dependent epitopes (Conf) IV and V were shown to be efficient at blocking SARS pseudovirus entry of ACE2-transfected 293T cells. The 50% neutralizing dose (ND₅₀) was 0.009 µg/ml for MAb 30F9 (Conf IV) and 0.005 µg/ml for MAb 33G4 (Conf V). MAb recognizing Conf I (26E1) and II (31H12) did not significantly inhibit RBD binding with ACE2 but were also potent neutralizing antibodies (ND₅₀ = 0.354 µg/ml and 0.139 µg/ml, respectively). MAbs 4D5 and 17H9, recognizing linear epitopes, had ND₅₀ values of > 100 µg/ml. Humanization of these murine antibodies are required to prevent human antimouse antibody responses if used for immunoprophylaxis or therapy of SARS.

2.1.4 Discussion

2.1.4.1 Techniques for generating monoclonal antibodies

As noted earlier, human MAbs have been generated from transgenic mice expressing human immunoglobulins with neutralizing activity against SARS-CoV S protein [59,65]. Although this process is more laborious and hence has a slower turn-around than other methods, because transgenic mice are immunologically less robust and require more immunizations and antibody screenings, a successful immunization can yield a multiplicity of MAbs that could be combined as a cocktail to more effectively combat the pathogen.

The technique of generating MAbs from a patient's immune repertoire has several advantages, including the delivery of large numbers of potential antibodies for selection according to criteria such as affinity, epitope specificity, and propensity to generate escape mutants [49]. In addition, such antibodies have undergone affinity maturation in human lymphoid organs, are fully human (not chimeric), and include post-translational modifications. However, the repertoire is not unlimited. For example, a patient who recovered from humanized SARS-CoV does not produce antibodies specific for civet SARS-CoV. This phenomenon was demonstrated in the partially humanized GD03T0013 strain of SARS-CoV (GD03) isolated during the forme fruste re-emergence of SARS in Guangdong, China, in late 2003 [66].

2.1.4.2 Antibody-dependent enhancement of infection

Antibody-dependent enhancement (ADE) of infection is recognized in a number of viral infections, including

coronaviral infection in the cat [67]. Experimentally, Yang *et al.* demonstrated that neutralizing immunoglobulins (isolated from a SARS-convalescent patient) directed against a human strain of SARS-CoV (S111, S127, and S3.1) potentiated infection by civet SARS-CoV [66].

The problems of ADE and escape mutants are not insurmountable, as shown by the identification of neutralizing MAb S110, which did not enhance entry of civet SARS-CoV [66], and m396 and S230.15, which neutralized all known epidemic and zoonotic strains except bat SARS-CoV [68]. MAb m396 had an IC₅₀ of 0.1 and 0.01 µg/ml against the partially humanized GD03 and the fully humanized Tor2 strains of SARS-CoV, respectively. In laboratory mice, it was shown that the serum neutralizing antibody titers correlated with *in vivo* protection against recombinant SARS-CoV from both the 2002 – 03 (icUrbani) and 2003 – 04 (icGD03) outbreaks, as well as against civet SARS-CoV (icSZ16). By systematically evaluating mutated S proteins generated by alanine-scanning site-directed mutagenesis, and in combination with analysis of the crystal structure of the RBD.m396 complex, it was concluded that the highly conserved Ile-489 and Tyr-491 residues probably account for the broad spectrum of neutralizing activity. The relative potencies (IC₅₀) of m396 and S230.15 compared with other published MAbs are tabulated by Zhu *et al.* (see Table 1, ref. 68). Thus, despite the potential for antibody-enhanced infection, passive immunoprophylaxis with carefully selected MAbs or combinations holds great promise as a potent medicine against SARS-CoV. However, the specificity precludes its application to other coronaviruses or mutants. Indeed, genotype monitoring is imperative as part of the overall strategy if MAb is to be employed successfully. Although no SARS-CoV vaccine has yet been developed, it is conceivable that passive immunoprophylaxis would be combined with active immunization should such a vaccine become available for this significantly fatal disease [69].

2.1.4.3 Perspective

Could MAb be harmful? The catastrophe of clinical trial TGN1412 illustrates the potential danger of MAb therapy, especially when directed against host molecules [70], and demands careful planning of clinical trial. Even well tested MAb might have unexpected side effects, for example, cardiac toxicity in the case of Trastuzumab® (anti-HER₂ MAb) [71,72].

Although only a handful of anti-SARS-CoV MAbs are currently being patented, and progress appears to be stalled, the entire process of development, patenting, clinical validation, regulatory approval, and deployment is likely to be expedited should SARS return. Monoclonal antibody technology certainly is in the spotlight, along with the threat of pandemic influenza [73].

In the decades to come, we should expect a proliferation of know-how and an increase in the number of MAb

anti-infectives. The careful combinations of MAb cocktails [58,65], MAb with other anti-infectives or vaccines, and the fine-tuning of the Fc-effector and other molecular attributes, such as glycosylation, will permit safer and more effective medications.

2.2 Membrane fusion inhibitors targeting S2 domain

SARS-CoV is an enveloped virus and utilizes a similar mechanism as HIV1 to achieve membrane fusion with the host cell and effect entry. Heptad repeats (HR; N-terminal HR₁ and C-terminal HR₂), located in the S2 domain of SARS-CoV spike (S) protein, interact with each other in an oblique, antiparallel manner, as revealed by the crystal structure [74], resulting in a hairpin configuration, following the engagement of the S1 RBD with ACE2. The conformation change in the S2 protein is followed by its oligomerization into a six-helix bundle fusion core and fusion of the viral envelope with the cell membrane [75].

2.2.1 Heptad repeat peptide inhibitor

Inhibitors that block the non-covalent interaction of HR₁ and HR₂ can prevent the formation of the fusogenic complex and thus deny viral entry. Conservation of the HR regions suggests that it is a good drug candidate. Along this line, spike protein HR-derived peptides have been predicted and demonstrated to inhibit SARS-CoV infection of Vero cells [76,77].

HR₂-8, derived from heptad repeat 2 of SARS-CoV, has an EC₅₀ of 17 μ M; rather disappointing compared with the corresponding peptide for MHV [77]. Further efforts by others resulted in HR₁-1 and HR₂-18, with EC₅₀ of 0.14 and 1.19 μ M, and stable recombinant proteins, HR₁₂₁ and HR₂₁₂ [78], with IC₅₀ of 4.13 and 0.95 μ M, respectively, on entry of the HIV/SARS pseudoviruses [79]. The recombinant proteins are also more economical to produce than synthetic peptides.

US patent 7,151,163 from Sequoia Pharmaceuticals disclosed a SARS-CoV fusion inhibitor (heptad repeat inhibitor) comprising a peptide with 40 amino acids (VVNIQKEIDRLNEVAKNLNESLIDLQELGKYEQYIK) linked to human serum albumin for enhanced solubility, half-life, and tissue distribution [80]. Also emphasized was the potential use of peptides as vaccines, utilizing its tendency to elicit an immunogenic response, and to raise therapeutic antiviral antibodies.

2.2.2 Peptide inhibitors of loop region

Like the peptides that mimic and interfere with the HR regions, peptides analogous to the N-terminus or pretransmembrane domain of the S2 subunit also showed inhibitory activity (40 – 70% inhibition by SARS-CoV plaque reduction assay at 15 – 30 μ M) [81]. Peptides analogous to the loop region ('hinge' area between the two heptad repeats) of SARS-CoV or MHV S proteins are more potent. Peptides SARS_{WW-III} and SARS_{WW-IV} inhibited viral plaque formation at concentrations of 30 μ M by 90% and 83%, respectively

(IC₅₀ = 2 – 4 μ M), and represent new peptide inhibitors directed to regions outside the HR regions. Efforts to enhance the potency, such as introducing alanine residues to promote a secondary α -helical structural feature, are underway. In a related patent application, Gallaher disclosed antiviral peptides derived from the charged preinsertion (CPI) helix [82]. The CPI is located within 100 amino acids from the transmembrane domain of SARS-CoV S protein near the C-terminus. CPIs have been shown to be involved in the induction of fusion of viral envelope and cell membrane in a number of viral systems. A special di- or tripeptide motif 'nucleates' helix formation. In SARS-CoV, it is PEL (Pro-Glu-Leu), and in MHV, it is PDFKE. The patent specifications teach how to locate the sequences of the fusion peptide and how to design inhibitory peptides of the CPI helix. The CPI helix in SARS-CoV comprises 78 amino acids: P E L D S F K E E L D K Y F K N H T S P D V D L GDISGINASVVNIQKEIDRLNEVAKNLNESLI DLQELG KYEQUIKWPWYVWLGE. This length is considered impractical for synthesis of the corresponding inhibitory peptide. A shorter peptide of 38 residues, designed for MHV CPI helix was shown to reduce plaque formation by 40% at 25 μ M. There is also significant plaque inhibition with as low as 1 μ M concentration.

2.2.3 Peptides that inhibit oligomerization of S

Peptides derived from other areas of the coronavirus S protein, such as 20-mer peptides that mapped to the interface between the three monomers of the trimeric peplomers, also showed antiviral activity (Figure 3) [83]. The most potent peptide, P8, had an IC₉₀ of 24.9 μ g/ml by cytopathic effect-based assay of SARS-CoV on FRhK-4 cells. The combination of P6, P8, and P10 had an IC₉₀ of 0.9 μ g/ml. Zheng *et al.* hypothesized that difference in amino-acid sequences of animal and human SARS-CoV S proteins account for the species jump. They identified 12 variations between animal and human SARS-CoV S proteins. In their patent application, they disclosed ten 20-mer peptides, P1–P10, designed to span those areas [84]. P1–P6 target the S1 region and P7–P10 target the S2 region. P2, P6, and P8 are located outside the RBD and HR regions of S. Three-dimensional modeling indicated that peptides P6, P8, and P10 map to subunit interfaces putatively crucial for the correct assembly of the trimeric peplomers. They have a loop conformation as opposed to the helical conformation of inactive peptides, and interfere with peplomer function by competitive binding to the monomeric S and mimicking regions exposed after ACE2-binding-induced conformational change of S2. *In vitro* studies revealed that the viral cytopathic effect of SARS-CoV on FRhK-4 cells was completely prevented by 50 μ g/ml of P8. Moreover, electron microscopy shows absence of intracytoplasmic viral particles in cells protected by P8. The absence of cytotoxicity is demonstrated. The IC₉₀ of the most active peptide, P8, is 24.9 – 6.2 μ g/ml. These peptides show

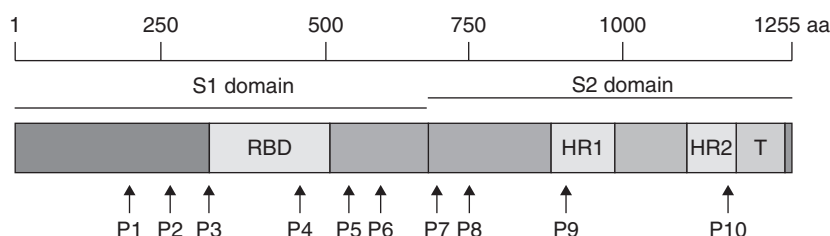


Figure 3. Peptides that inhibit oligomerization of S. Schematic of the 1255 amino acid SARS-CoV Spike (S) protein, with its S1 and S2 domains, receptor binding domain (RBD), heptad repeat (HR) 1, HR2, and transmembrane (T) domains. The locations in the S-protein that the 20-mer synthetic peptides (P1 - P10) correspond are indicated by arrows. Figure adapted from reference 83.

synergism when combined. This novel mechanism of viral inhibition is specific to the viral strain; peptides designed for animal strain of SARS-CoV have less inhibitory effect towards entry of the human strain.

2.2.4 Discussion

Peptides and recombinant proteins will need to be administered parenterally (or by inhalation), and face the same hurdles of competition as MAb with small molecules, which have the additional advantages of better stability, delivery across hydrophobic cell membranes, longer half-lives, and low risk of immunogenic effects. Peptide (and protein) drugs, however, are more active and specific and therefore have fewer nonspecific effects, minimal drug interactions, do not accumulate in the body, and have fewer toxicology issues from xenobiotic metabolism. Like other antiviral drugs, they are not exempt from viral escape, as reported recently [85].

Over 40 peptide drugs are now available in the market, including the blockbuster Humalog® (insulin lispro) from Eli Lilly. The HIV1 entry inhibitor, enfurvitide (Fuzeon™) [86], a 38-residue peptide based on the sequence of HR₂ of the HIV glycoprotein (gp41), approved by the US FDA in March 2003, is in use clinically.

2.3 Small molecular inhibitors of viral entry

Eighteen small molecules with activity against the entry of SARS-CoV pseudovirus into engineered 293T cells expressing ACE2 were identified in a phenotype-based high-throughput screen of 50,240 small molecules of diverse structures [87]. One of the molecules, VE607 (Figure 4), was active only during the crucial early phase of viral infection (0 – 2 h), indicating its inhibitory mechanism on viral entry; it did not nonspecifically inhibit poliovirus plaque formation. VE607 had an EC₅₀ of 3 μM and 1.6 μM with pseudotype virus entry assay and SARS-CoV plaque reduction assay, respectively. No published patent application is available.

2.3.1 Cathepsin L (CTSL) inhibitors

The cathepsins are a diverse group of endosomal and lysosomal proteases with endo- and exopeptidase activities and diverse functions. They can be divided according to their catalytic activities as aspartyl, serine, or cysteine

proteases. Cathepsin S plays a pivotal role in the maturation and peptide-binding competency of class II molecules and is inducible by interferon (IFN)-γ in major histocompatibility complex (MHC)-class-II-expressing cells [88]. Cathepsin L (CTSL) is one of the major proteases in mammalian cells with broad activity against a variety of extracellular matrix proteins and intracellular proteins. It is implicated in the transformed phenotype because of its co-regulation with cell growth [89]. It was recently found to be a requisite for neovascularization by endothelial progenitor cells after ischemia [90]. Because malignant tumors are also dependent on neovascularization, CTSL could be implicated in the malignant phenotype; it thus presents itself as an oncologic drug target. In viral infections, the cathepsins are known to play a role in cellular entry of reovirus and, recently, Ebola virus [91,92]. Simmons *et al.* found that CTSL is required for SARS-CoV entry into the cytosolic compartment via the endosomal pathway [93], although another novel coronavirus, HCoV-NL63, which also utilizes ACE2 as the cellular receptor, is not [94]. Recently, Bosch *et al.* have shown CTSL to functionally cleave SARS-COV S at T678, between S1 and S2 domains [20]. They thus demonstrated that CSTL activates the membrane fusion function of SARS-CoV S protein, supporting the hypothesis that the S protein is cleaved during cell entry rather than during virion biogenesis.

This new target to prevent viral entry was screened against a chemical library of 1000 pharmacologically active compounds. Simmons *et al.* uncovered a novel protease inhibitor, MDL28170 (other names: calpain inhibitor III and Z-Val-Phe-CHO). SARS-CoV (Tor 2 strain) and HIV(SARS S) pseudovirions were inhibited by this agent, which is known to nonspecifically inhibit cytosolic calpains and cathepsin B. Using a fluorogenic substrate (VEID-MCA), an IC₅₀ of 2.5 nM was determined [93]. There is no nonspecific inhibition of infection by HIV(VSV-G) pseudovirions.

The corresponding patent application filed by Diamond *et al.* [95] disclosed compounds containing a Val-Phe structure with CTSL inhibitory activity, such as Z-Val-Phe-CHO (MDL28170 or PN-001) (Figure 5), A-Val-Phe-FMK, Boc-Val-Phe-4-chlorobenzyl, Z-Val-Phe-NHO-enzy, Z-Val-Phe-NHO-4-methoxybenzyl, and Z-Val-Phe-NHO-4-methylbenzyl. The IC₅₀ of MDL28170 as determined using

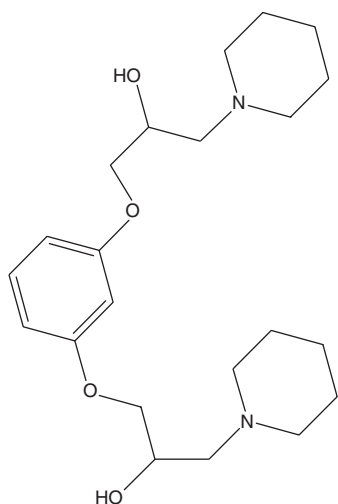


Figure 4. VE607. The mechanism of action of this SARS-CoV entry inhibitor is unknown.

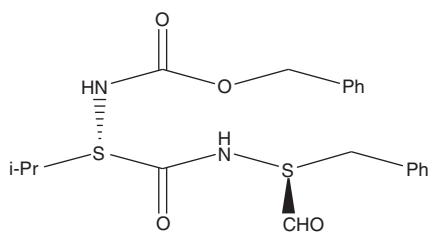


Figure 5. Z-Val-Phe-CHO (MDL28170 or PN-001). The IC_{50} of this cathepsin L inhibitor on pseudotype virion entry is approximately 100 nM.

pseudotyped virion entry into cells is approximately 100 nM. This compound also inhibited Ebola virus entry and had no inhibitory activity towards two viruses that do not require CTSL for entry (VSV and MLV pseudoviruses). Fourteen other formulae are also included in the claims. Notable compounds are 3,5 dinitrocatechol, pindobind, and U73122 (1-(6-((8R,9S,13S,14S)-7,8,9,11,12,13,14,15,16,17-decahydro-3-methoxy-13-methyl-6H-cyclopenta[a]phenanthren-17-ylamino)hexyl)-1H-pyrrole-2,5-dione), which inhibit CTSL with IC_{50} of 68 nM, 0.8 μ M and 0.7 μ M, respectively.

2.3.2 Discussion

That HCoV-NL63 does not require CTSL or cellular protease for infection suggests that it employs a different endosomal enzyme for cleavage, and that SARS-CoV might mutate under selection pressure into escape mutants independent of CTSL for cellular entry. Also of note is that CTSL enhances HIV1 infection by disrupting lysosomal interference with productive HIV1 infection [94]. This has led to concerns about a similar effect with other viruses, and it makes definitive

viral identification before the institution of CTSL inhibitor therapy an absolute requirement. Furthermore, the endo-/lysosomal system is vital in antigenic processing and the adaptive immune system, as highlighted by recent findings that administration of a CTSL inhibitor, CLIK148 [96], shifted a protective Th1 antiparasitic (to *Leishmania*) response to a devastating Th2 response in laboratory mice, pointing to potential dangers with targeting host proteins [97].

Other infections must be ruled out. Because cathepsin inhibitors show cross-reactivity, inhibitors of CTSL should be tested against other cathepsins to prevent untoward effects. Beyond the stage of discovery, an issue with the development of CTSL inhibitors is druggability (aqueous solubility and intestinal permeability for oral administration), as can be estimated by Lipinski's rule of five [98], an issue also pertaining to peptide drugs. Nevertheless, these inhibitors have shown potent inhibitory effects on SARS-CoV and Ebola virus, and demand further preclinical studies.

3. Conclusions

Coronaviruses are RNA viruses that infect various animal species, including humans. SARS-CoV emerged and re-emerged for a span of a year or so in the winters of 2002 and 2003. The presence of a natural reservoir in bats suggested that it might emerge again. Because of this, specific therapy for SARS-CoV, and coronaviruses in general, are needed. Much progress has been made, as can be seen in this review of viral entry inhibitors targeted at SARS-CoV. Therapies targeting the latter part of the coronaviral lifecycle will be reviewed in a subsequent issue.

4. Expert opinion

Of the coronaviral entry inhibitors reviewed here, monoclonal antibody technology is the most promising. The combination of CR3014 and CR3022, developed by Ter Meulen (US20080014204), has a desired spectrum [56]. These were produced from nonimmune human antibody libraries, unlike the next contender, S3.1, which was produced from human memory B cells (from convalescent patients) using the novel technique developed by Lanzavecchia (WO2004076677) [48]. Producing human monoclonal antibody from transgenic mice immunized against SARS-CoV (US20050069869) suggests that such animals that survive infection by the wild-type virus could be used to derive that antibodies, thus bypassing the development of a vaccine [61]. Peptide inhibitors of coronaviral entry have shown much promise; with the EC_{50} activity of heptad repeat inhibitors in the low micromolar range. Peptides that inhibit the oligomerization of the S2 domain of SARS-CoV also exhibit potent inhibition of coronaviral entry, with the combination of P6, P8, and P10 having an IC_{90} of 0.9 μ g/ml. Cathepsin L inhibitors claimed by Diamond *et al.* (US20070203073) have an EC_{50} as low as a fraction of a micromole [95]. Despite potential problems with nonspecific inhibition of

related cathepsins and other concerns, the potency and applicability to other viruses make this class of drug attractive. Finally, soluble ACE2 and derivatives are still on the drawing board, whereas ACE2 inhibitor is relatively weak and perhaps detrimental, given the importance of ACE2 in normal physiology.

If SARS-CoV returns today, monoclonal antibody combinations, cathepsin L inhibitors, protease inhibitors, and

nucleic-acid-based therapies (see Part II) would be the favorite drug candidates.

Declaration of interest

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

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