Potential antivirals and antiviral strategies against SARS coronavirus infections

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There are a number of antivirals as well as antiviral strategies that could be envisaged to prevent or treat severe acute respiratory syndrome (SARS) (or similar) coronavirus (CoV) infections. Targets for the prophylactic or therapeutic interventions include interaction of the spike (S) glycoprotein (S1 domain) with the host cell receptor, fusion of the S2 domain with the host cell membrane, processing of the replicase polyproteins by the virus-encoded proteases (3C-like cysteine protease [3CLpro] and papain-like cysteine protease) and other virus-encoded enzymes such as the NTPase/helicase and RNA-dependent RNA polymerase. Human monoclonal antibody blocking S1 may play an important role in the immunoprophylaxis of SARS. Fusion inhibitors reminiscent of enfuvirtide in the case of HIV may also be developed for SARS-CoV. Various peptidomimetic and nonpeptidic inhibitors of 3CLpro have been described, the best ones inhibiting SARS-CoV replication with a selectivity index greater than 1000. Human interferons, in particular α- and β-interferon, as well as short interfering RNAs could further be pursued for the control of SARS. Various other compounds, often with an ill-defined mode of action but selectivity indexes up to 100, have been reported to exhibit *in vitro* activity against SARS-CoV: valinomycin, glycopeptide antibiotics, plant lectins, hesperetin, glycyrrhizin, aurintricarboxylic acid, chloroquine, niclosamide, nelfinavir and calpain inhibitors.

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Severe acute respiratory syndrome (SARS) is a new infectious disease that is mainly characterized by influenza-like symptoms, high fever, myalgia, dyspnea, lymphopenia and lung infiltrates (pneumonia) leading to acute breathing problems, with an overall mortality rate of approximately 10% (in the elderly as high as 50%). The disease appeared in the Guandong province of southern China at the end of 2002 from where it swept into 29 countries. When the epidemic finally waned after more than 100 days, the WHO had counted a cumulative number of more than 8000 probable SARS cases with almost 800 deaths. The etiological agent of SARS has been unequivocally identified as the SARS-associated coronavirus (SARS-CoV) [1–6].

SARS-CoV was, following the human CoVs 229E and OC43, the third CoV to be identified in humans. Subsequently, a fourth human CoV, NL63, was isolated from individuals suffering from respiratory illness [7], and it is likely that additional human CoVs may be uncovered in the future. This underscores the importance of the search for inhibitors of the SARS-CoV, and given the multitude of targets that could be envisaged for chemotherapeutic intervention [8], numerous approaches have already been proposed to inhibit SARS-CoV replication and spread [9,10].

The organization of the SARS-CoV genome (29,740 bases long plus-stranded RNA) is similar to that of other CoVs (FIGURE 1). There are essentially two regions, the replicase region and the structural region. The replicase region encompasses two overlapping open reading frames (ORFs 1a and 1b). A translational readthrough by a -1 position ribosomal frameshift allows the translation of the overlapping reading frames into a single polyprotein. Virus-encoded proteases, namely the papain-like cysteine protease (PLpro) and the picornavirus

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3C-like cysteine protease (3CLpro) cleave the polyprotein into the individual polypeptides required for replication and transcription [8]. The remaining one third of the genome encodes for at least four structural proteins: spike protein (S), envelope protein (E), membrane glycoprotein (M) and nucleocapsid protein (N). Several additional genes encoding additional nonstructural proteins are known as 'accessory genes'.

This review will evaluate the different gene products encoded by the SARS-CoV genome as possible points of attack for chemotherapeutic (or prophylactic) agents, thereby reviewing the various strategies that have already been proposed to curb (treat or prevent) a potential SARS-CoV infection. These approaches have to be viewed in the framework of antivirals and antiviral strategies against virus infections at large [11].

Virus entry into the host cell

The metallopeptidase angiotensin-converting enzyme 2 (ACE2) has been identified as a functional receptor for the SARS-CoV [12]. ACE2 interacts with the S1 domain of the viral S glycoprotein, and this interaction and the subsequent infection can be blocked by both a soluble form of ACE2 and their anti-ACE2 antibody [12]. ACE2 expression in cell lines correlates with their susceptibility to SARS-CoV S-driven infection, suggesting that ACE2 must be a major receptor for SARS-CoV. This, in turn, suggests that the SARS-CoV S protein may be considered as an attractive target for therapeutic intervention [13]. Interestingly, ACE2 expression positively correlated with the differentiation state of human airway epithelia; undifferentiated cells expressing little ACE2 were poorly infected with SARS-CoV, while well differentiated cells expressing more ACE2 were readily infected [14].

A 193-amino acid fragment of the S protein (corresponding to residues 318–510) binds to ACE2 more efficiently than the full S1 domain, and, in fact, the 193-residue fragment blocks S protein-mediated infection with an inhibitory concentration of 50% (IC_{50}) of less than 10 nM (the IC_{50} of the full S1 domain being ~50 nM) [15]. Also, human monoclonal antibodies to the S1 protein domain block the association of SARS-CoV with ACE2, indicating that the ACE2 binding site of S1 could be a target for drug development [16]. A small-molecular-weight inhibitor that was found to interact with the ACE2 active catalytic site, (*S*,*S*)-2-(1-carboxy-2-[3-[3,5-dichlorobenzyl]-3H-imidazol-4-yl]-ethylamino)-4-methyl-pentanoic acid (MLN-4760) has been described [17]. Whether MLN-4760 inhibits SARS-CoV infection has not, as yet, been demonstrated.

Whereas the S1 domain of the S glycoprotein determines virus attachment to the host cells, the subsequent virus–cell fusion process is governed by conformational changes of the two heptad regions (HRs)-N (or HR1) and HR-C (or HR2) within the S2 domain, resulting in the formation of a 6-helix bundle (trimer of dimers). Systemic peptide mapping has shown that the site of interaction between the HR1 and HR2 regions is between residues 916 and 950 of HR1 and residues 1151–1185 of HR2 [18]. It has also been shown that a peptide, CP-1, derived from the HR2 region, inhibits SARS-CoV infection in the micromolar range: CP-1 bound with high affinity to a peptide from the HR1 region, NP-1 (FIGURE 2) [19]. CP-1 could bind to the HR1 region, thereby interfering with the conformational changes leading to the 6-helix bundle formation (FIGURE 3) and the therewith associated virus–cell fusion process. Obviously, the HR1–HR2 interaction may be viewed as an attractive target for the design of potent peptide-type SARS-CoV entry inhibitors [20,21], reminiscent of the HIV-1 gp41 HR2-derived peptide T20 (enfuvirtide) which has been developed as a HIV-1 fusion inhibitor [22]. The latter could inhibit the fusion of SARS-CoV with target cells, but apparently with too low efficiency to be therapeutically meaningful.

Recently, Simmons and colleagues suggested that following receptor binding and induced conformational changes in the S glycoprotein, a third step would be involved in the viral entry process, namely cathepsin-L proteolysis within endosomes [23]. They demonstrated that a cathepsin-L-specific inhibitor, MDL 28170 (also known as calpain inhibitor III or Z-Val–Phe[CHO]), at the same time inhibited cathepsin-L activity and S protein-mediated infection (at an IC_{50} of 2.5 nM and 0.1 μM, respectively). In addition to calpain inhibitor III,

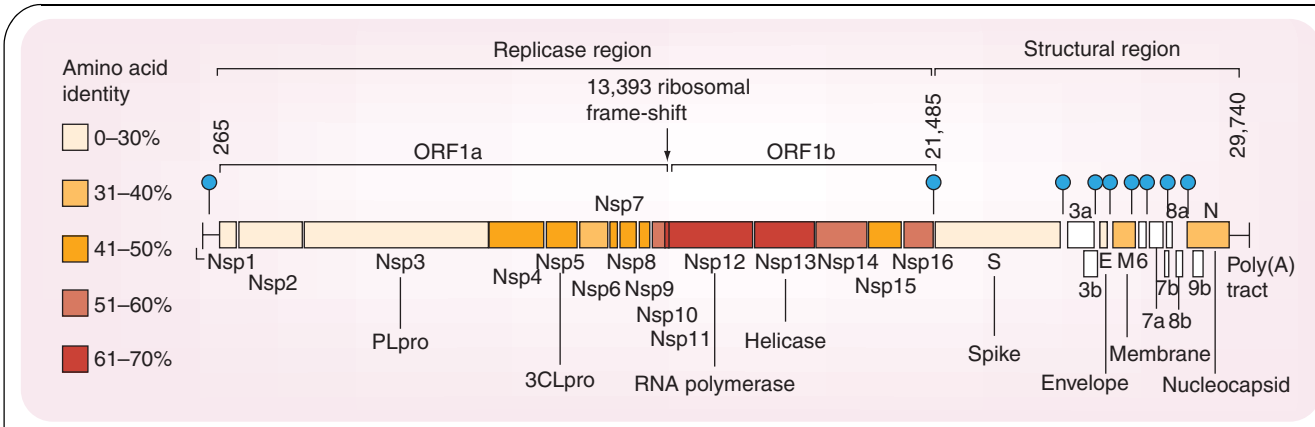

Figure 1. Genome structure of severe acute respiratory syndrome coronavirus. Reprinted with permission from [8].
3CLpro: 3C-like cysteine protease; Nsp: Nonstructural protein; ORF: Open reading frame; PLpro: Papain-like cysteine protease.

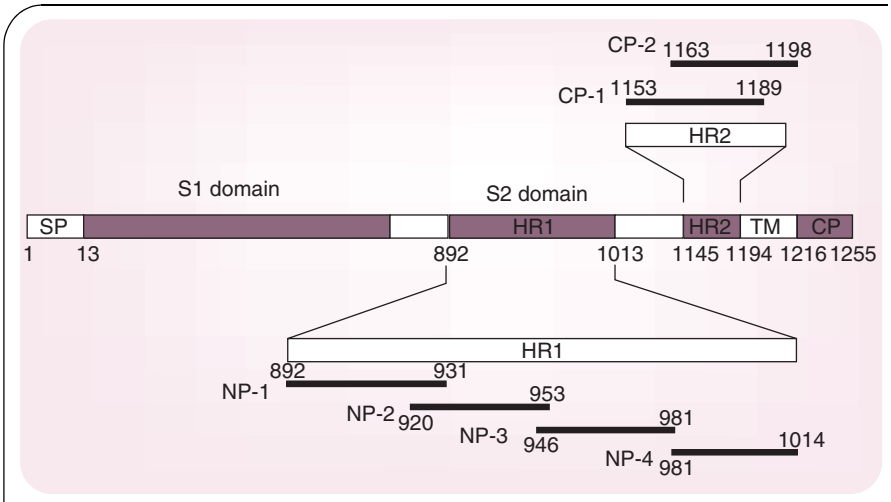

Figure 2. Severe acute respiratory syndrome coronavirus (SARS-CoV) spike protein. Residue numbers of each region correspond to their positions in the spike protein of SARS-CoV. Six peptides corresponding to the sequences of HR1 and HR2 regions are also shown. Reprinted with permission from [19]. CP: Cytoplasmic domain; HR: Heptad region; SP: Signal peptide; TM: Transmembrane domain.

some other calpain inhibitors have been described as inhibitors of SARS-CoV replication, the most selective (selectivity index >100) being calpain inhibitor VI (4-fluorophenylsulfonyl Val–Leu[CHO]) (FIGURE 4) [24].

Virus-encoded proteases

Following entry of SARS-CoV into the host cell, the genomic plus-stranded RNA is translated to produce two large overlapping replicase polyproteins, which are further processed to functional polypeptides through extensive proteolytic cleavage, mainly by the 3CLpro. The SARS 3C-like protease, also called main protease (Mpro) cleaves the replicase polyproteins at as many as 11 conserved sites, so as to generate the functional proteins necessary for virus replication. Mpro was quickly recognized as an attractive target for the development of anti-SARS-CoV agents [25]. It was proposed that compounds such as AG-7088, which had proven to be active against the rhinovirus 3C protease, could be modified in order to make them active against CoVs such as SARS-CoV [25]. As a first modification of AG-7088, the methylene group of the *p*-fluorophenylalanine residue was removed, and the resulting KZ7088 was modeled into the structure of the SARS-CoV Mpro [26].

The crystal structures of the SARS-CoV Mpro complexed with various substrate analogs, such as a hexapeptidyl chloromethyl ketone (FIGURE 5) [27] or azapeptide epoxide [28] have been determined, and the recombinant SARS-CoV Mpro has been successfully cloned and expressed [29]. The information thus gathered on the mode of inhibitor binding and enzyme catalysis should help to provide a structural basis for rational drug design.

Of a number of peptidomimetic compounds (aziridinyl peptides [30], keto-glutamine analogs [31], chymotrypsin-like protease inhibitors [32] and peptide anilides [33]) that have been reported as inhibitors of the SARS-CoV Mpro, the niclosamide anilide (FIGURE 6), with a K_i = 0.03 µM (IC_{50} = 0.06 µM), proved to the most potent (competitive) inhibitor [33]. Also, several nonpeptidic compounds have been described as inhibitors of the SARS-CoV Mpro: that is, etacrynic acid derivatives such as etacrynic acid amide (K_i = 35.3 µM) [34], isatin derivatives (IC_{50} values ranging from 0.95 to 17.50 µM) [35], hexachlorophene derivatives (IC_{50} values ranging from 7.6 to 84.5 µM) [36] and natural products from teas such as theaflavin-3,3´-digallate (IC_{50} = 9.5 µM) [37]. However, in none of these cases [30–37] was it ascertained whether the compounds were also effective in inhibiting SARS-CoV infection in cell culture, except for two of the chymotrypsin-like protease inhibitors [32] which were found to inhibit SARS-CoV in cell culture at a relatively high concentration (45 and 70 µM, respectively) [32].

There are only a few cases where the 3CL protease inhibitors were shown to inhibit both the SARS-CoV protease activity and virus replication in cell culture. The Phe–Phe dipeptide inhibitor shown in FIGURE 7 was found to inhibit the 3CL protease at an IC_{50} of 1 µM (K_i = 0.52) and inhibited

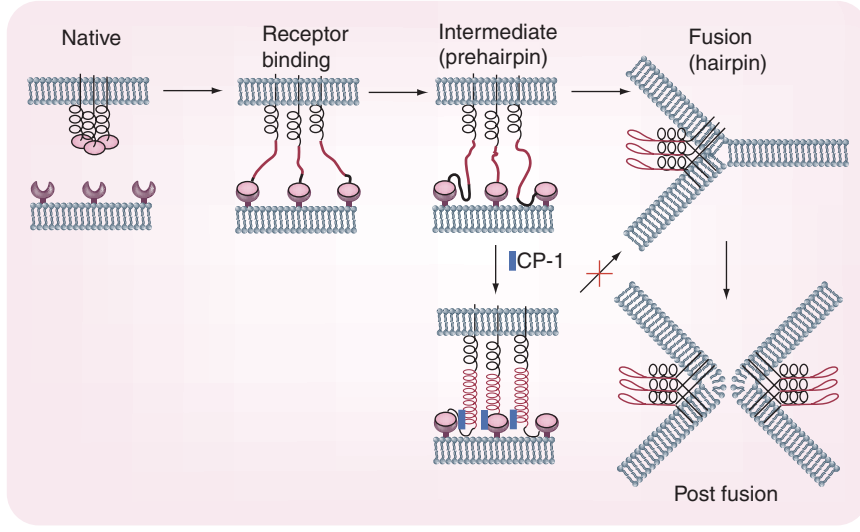

Figure 3. Conformational changes of severe acute respiratory syndrome coronavirus spike protein during the process of fusion between the virus and target cell membranes. Reprinted with permission from [19].

virus replication in Vero cells at an effective concentration of 50% (EC_{50}) of 0.18 µM, while not being toxic to the host cells at a concentration of 200 µM (selectivity index: >1000) [38]. Cinanserin (SQ 10,643, a well characterized serotonin antagonist) (FIGURE 8) is another example of an inhibitor of SARS-CoV replication which may act via inhibition of the 3CL protease (IC_{50} for the enzyme = 5 µM; EC_{50} values for reduction of viral RNA and infectious particles ranging from 19 to 34 µM) [39]. Finally, an octapeptide, designed for the SARS-CoV Mpro, namely AVLQSGFR, was reported to inhibit SARS-CoV replication in Vero cells at an EC_{50} of 0.027 µg/ml, while not being cytotoxic at 100 µg/ml, thus establishing a selectivity index of greater than 3700 [40]. Whether this highly selective antiviral effect was actually mediated by an inhibition of the SARS-CoV Mpro was not ascertained in this study [40].

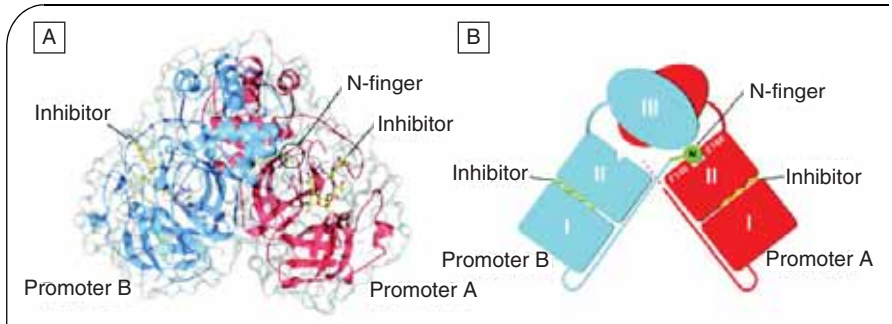

Figure 5. The severe acute respiratory syndrome coronavirus (SARS-CoV) main protease (Mpro) dimer structure complexed with a substrate–analog hexapeptidyl chloromethyl ketone inhibitor. (A) The SARS-CoV Mpro dimer structure is presented as ribbons, and inhibitor molecules are shown as ball-and-stick models. Promoter A (the catalytically competent enzyme) is red, promoter B (the inactive enzyme) is blue and the inhibitor molecules are yellow. The N-finger residues of promoter B are green. The molecular surface of the dimer is superimposed. (B) A cartoon diagram illustrating the important role of the N-finger in both dimerization and maintenance of the active form of the enzyme. Reprinted with permission from [27].

In addition to 3CLpro, a PLpro is encoded by the SARS-CoV genome. SARS-CoV PLpro processes the replicase polyprotein at three conserved cleavage sites, thus generating the nonstructural proteins NSP1, NSP2 and NSP3. It was recently demonstrated that SARS-CoV PLpro also had deubiquitinating activity [41,42]. The possibility that SARS-CoV PLpro could deubiquitinate host or viral proteins has added a higher level of functional complexity to this enzyme, and should, in principle, elevate the value of SARS-CoV PLpro as a potential target for therapeutic intervention.

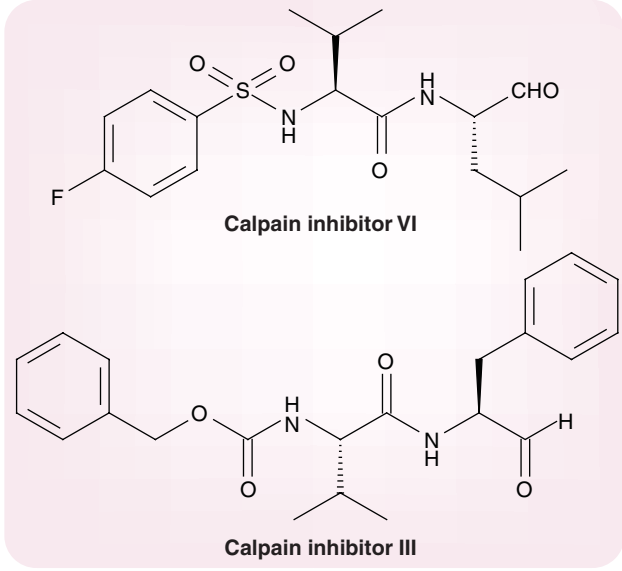

Figure 4. Calpain inhibitors. Calpain inhibitor VI: 4-fluorophenylsulfonyl-Val–Leu(CHO). Calpain inhibitor III: Z-Val–Phe(CHO).

Virus-encoded helicase, polymerase & endonuclease

SARS-CoV encodes for an helicase which must unwind the double-stranded (±)RNA helices during the viral replication cycle. This helicase, akin to the herpesviral DNA helicase, also possesses NTPase activity, and may therefore be termed an NTPase/helicase. The SARS-CoV NTPase/helicase has been considered a potential target for the development of anti-SARS-CoV agents [43]. These agents could, in theory, be targeted at any of the three major domains of the enzyme, the N-terminal metal-binding domain, the hinge domain and the NTPase/helicase domain. Bananin (FIGURE 9) and three of its derivatives (iodobananin, vanillinbananin and eubananin) were shown to inhibit both the ATPase and helicase activity of the SARS-CoV NTPase/helicase, with IC_{50} values (for the ATPase activity) in the range of 0.5–3 µM [44]. Bananin was also found to inhibit SARS-CoV replication in fetal rhesus kidney (FRhK)-4 cells at an EC_{50} of less than 10 µM and a 50% cytotoxicity concentration (CC_{50}) of over 300 µM, thus exhibiting a selectivity index of over 30 [44]. Whether the antiviral effect obtained in cell culture was causally linked to the inhibition of the NTPase/helicase was not ascertained.

The SARS-CoV RNA-dependent RNA polymerase (RdRp), due to its pivotal role in viral replication, represents another potential target for anti-SARS therapy. This enzyme (FIGURE 10) does not contain a hydrophobic pocket for non-nucleoside inhibitors similar to those that have proven effective against the hepatits C virus (HCV) polymerase or HIV-1 reverse transcriptase [45]. In fact, non-nucleoside HIV-1 reverse transcriptase inhibitors were shown to have no evident inhibitory effect on SARS-CoV RdRp activity [46]. It is intriguing that during purification, the full-length SARS-CoV RdRp was unstable and was hydrolytically cleaved into three fragments, a N-terminal p12 fragment, a middle p30 fragment and a C-terminal p64 fragment comprising the catalytic domain. The cause of the cleavage is unclear. Nor is it clear whether this cleavage also occurs in the viral life cycle [46]. At present, few, if

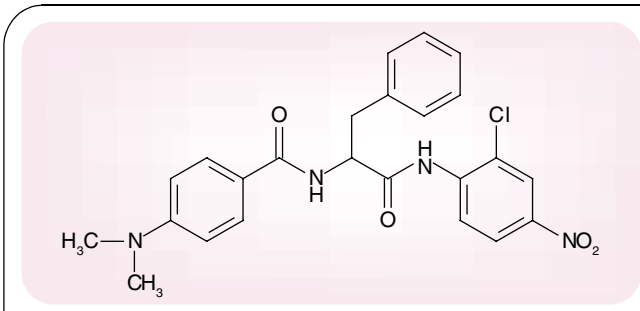

Figure 6. Niclosamide anilide (JMF 1507).

any, nucleoside analogs have been recognized as specific inhibitors of the SARS-CoV RdRp. There is N^4-hydroxycytidine, which has been accredited with both anti-HCV and anti-SARS-CoV effects. Against SARS-CoV it proved active at an EC_{50} of 10 µM (selectivity index ≥ 10) [24]. However, whether this antiviral effect was mediated by an inhibition of the viral RdRp was not ascertained.

Given the large genome of SARS-CoV (and other CoVs), and the need for discontinuous transcription to generate subgenomic transcripts, they could be expected to encode novel RNA-processing functions. Indeed, a number of such proteins have been identified: NSP14, a putative exonuclease; NSP15, an endoribonuclease and NSP16, a putative RNA methyltransferase. RNA endonuclease activity is unusual among positive-strand RNA viruses, suggesting that, because it specifically occurs during SARS-CoV replication, it could be considered a target for antiviral drug development. This pertains, in particular, NSP15, to an Mn^{2+}-dependent endoribonuclease that specifically cleaves RNA at unpaired uridylate residues. The role of NSP15, which consists of six subunits (arranged as a dimer of trimers) [47] in the CoV infection process, still remains to be elucidated.

Human monoclonal antibody

As mentioned above, human monoclonal antibody (mAb) to the S1 domain of the S protein of SARS-CoV blocks its association with the host cell ACE2 receptor [16]. The mAb concerned, 80R immunoglobulin (Ig)G1, was further evaluated for its immunoprophylactic efficacy *in vivo* in a mouse model [48]. When 80R IgG1 was given prophylactically to mice at doses therapeutically achievable in humans, viral replication was reduced by more than four orders of magnitude to below assay limits. The results demonstrated that the vast majority of SARS-CoVs isolated thus far remain sensitive to 80R. In an outbreak setting, early and rapid genotyping of the S1 gene fragment encoding the 80R epitope should provide an accurate guide for installment of immunoprophylaxis with 80R [48].

Data generated with human mAb (CR304) against SARS-CoV in ferrets also point to the feasibility of immunoprophylaxis with human mAb for the control of human SARS-CoV infections [49]. Similar observations in a mouse model demonstrating that primary infection with SARS-CoV provides protection from reinfection and that antibody alone can protect against viral replication, suggest that vaccines that induce neutralizing antibodies and strategies for immunoprophylaxis or, perhaps, immunotherapy are likely to be effective against SARS [50].

Most of the SARS-CoV-infected patients spontaneously recover, and recovered patients have higher and sustainable levels of both N and S glycoprotein-specific antibody responses, suggesting that antibody responses are likely to play an important role in determining the ultimate disease outcome of SARS-CoV-infected patients [51]. It would, therefore, not be unreasonable to expect that antibody to SARS-CoV, as present in convalescent plasma [52], may favorably influence the course of SARS.

As an interesting hypothesis, derived from the experience gathered with HIV [53], it may be proposed that antibody towards carbohydrate-hidden immunogenic epitopes on the SARS-CoV envelope may confer an immunoprophylactic, as well as an immunotherapeutic approach towards CoV and various other enveloped virus infections. To enable this approach, carbohydrate-binding agents, such as the *Hippeastrum hybrid* (amaryllis), *Galanthus nivalis* (snowdrop) and *Urtica dioica* mannose- or *N*-acetylglucosamine-binding lectins could be used to cause deletions, upon repeated exposure (passages), of the glycosylation sites in the SARS-CoV envelope in order to expose the carbohydrate-hidden epitopes to both active (vaccine) and passive (antibody) immune responses.

Human interferon

Shortly after SARS-CoV had been identified as the causative agent of SARS, Cinatl and colleagues [54] were the first to note that interferons (IFNs) inhibited the replication of SARS-CoV in cell culture *in vitro*; IFN-β being more potent than either IFN-α or -γ. These observations were subsequently confirmed in several other studies [55–58]. IFN-β exhibited potent antiviral activity at doses that had been shown to have acceptable safety profiles [55]. Also, IFN-α showed an *in vitro* inhibitory effect starting at concentrations of 1000 IU/ml [56]. In contrast with type I IFNs (α, β), type II IFN (γ) had little, if any, inhibitory effect on SARS-CoV replication [57]. The human MxA protein is one of the most prominent proteins induced by IFN-β. Nevertheless, no interference with SARS-CoV replication was observed in Vero cells stably expressing MxA, which implies that other IFN-induced proteins must be responsible for the strong inhibitory activity of IFN-β against SARS-CoV [58].

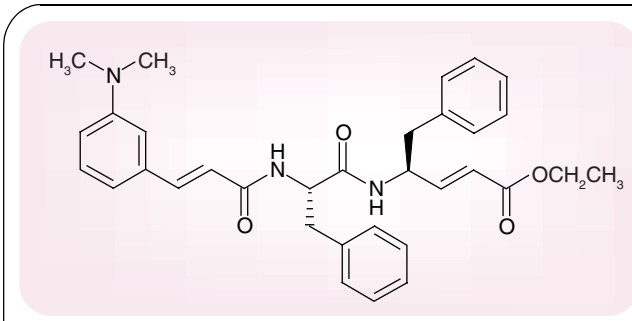

Figure 7. Phe–Phe dipeptide.

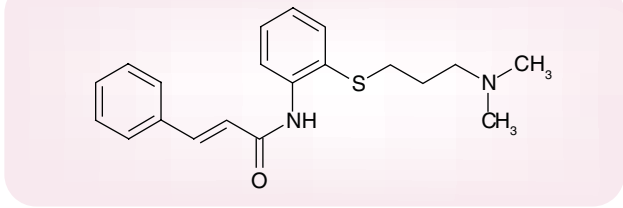

Figure 8. Cinanserin.

IFN-β, in conjunction with IFN-γ, was found to synergistically inhibit the replication of SARS-CoV in Vero cells [59], and a preliminary uncontrolled study with the IFN alfacon-1 (a synthetic IFN-α designed to represent a consensus IFN-α) suggested that this type of IFN, in combination with corticosteroids might be effective *in vivo* in the treatment of SARS [60]. Furthermore, a CpG oligodeoxynucleotide, which is able to induce human peripheral blood mononuclear cells (PBMCs) to produce high levels of IFN-α/β, has been accredited with strong activity against SARS-CoV *in vitro* [61].

Being a prophylactic rather than therapeutic agent, IFN(s) may have their highest utility in the prophylaxis or early postexposure management of SARS. Pegylated (PEG)-IFN-α has been shown to reduce viral replication and excretion, viral antigen expression by type 1 pneumocytes and the attendant pulmonary damage in cynomolgous macaques that were infected experimentally with SARS-CoV [62]. PEG-IFN-α is commercially available for the treatment of HCV (where it is generally used in combination with ribavirin) and hepatitis B. PEG-IFN-α as well as the other commercially available IFNs (e.g., IFN-β and alfacon-1) could be considered for prevention and/or early postexposure treatment of SARS should it re-emerge.

RNA interference

RNA interference (RNAi) can be defined as silencing of gene expression through degradation of (the target) RNA [63]. RNAi can be broken down into two main phases. In the first phase, long double-stranded RNA (dsRNA) is processed by Dicer, an RNAse III enzyme into duplexes of short interfering RNA (siRNA) of 21–24 nucleotides in length. Exogenous synthetic siRNAs can also be incorporated into the RNA-induced silencing complex (RISC), thereby bypassing the requirement for dsRNA processing by Dicer. In the second phase, a helicase present in RISC unwinds the duplex siRNA, which then pairs by means of its unwound antisense strand to its target messenger RNA (mRNA) that bears a high degree of sequence complementarity to the siRNA. An RNAse (Slicer) within RISC then proceeds to degrade the target mRNA at sites not bound by the siRNA, that is, 10 nucleotides upstream of the 5´-most residue of the siRNA-target mRNA duplex [63].

Thus, siRNAs have been developed that target the replicase [64] and S [65] genes of the SARS-CoV genome, thereby silencing their expression in cell culture. Potent siRNA inhibitors of SARS-CoV *in vitro* (i.e., the siRNA duplexes siSC2 [forward sequence: 5´-GCUCCUAAUUACACUCAACdtdt-3´] and siSC5 [forward sequence: 5´-GGAUGAGGAAGGCAAUUUAdtdt-3´], targeting the SARS-CoV genomeat S protein- and NSP12-coding regions, respectively) were further evaluated for their efficacy in a rhesus macaque SARS model [66], and were found to provide relief from SARS-CoV infection-induced fever, diminish SARS-CoV levels and reduce acute diffuse alveolar damage. No sign of toxicity was observed with the siRNA concerned [66]. Whether SARS can be conquered by the siRNA approach remains to be proven, however [67]. Therefore, the siRNA delivery forms should be further optimized, and the most effective target within the SARS-CoV genome should be identified. Since siRNAs, at least in their second stage of action, obey the antisense principle, antisense strategies such as those based on peptide-conjugated antisense phosphorodiamidate morpholino oligomers (P-PMOs) also deserve closer attention [68].

Miscellaneous compounds

A growing number of compounds have been identified as SARS-CoV replication inhibitors exhibiting mechanisms of action which are both diverse and largely unexplored. Of greater than 10,000 agents tested against SARS-CoV in Vero cells, approximately 50 compounds were found active at less than or equal to 10 µM [69]; as the most potent inhibitor, with an EC_{50} of 0.85 µM (selectivity index = 80), emerged valinomycin, a peptidic insecticide acting as a potassium ion transporter (FIGURE 11). Inhibitory effects on SARS-CoV replication, with selectivity indexes of up to 100, and EC_{50} values as low as 1 µg/ml, have been observed for a variety of compounds including the vancomycin, eremomycin and teicoplanin aglycon derivatives [70], and the mannose-specific plant lectins derived from *G. nivalis*, *Hippeastrum hybrid* [71] and *Allium porrum* (leek) [72]. The mode of action of these compounds has not been assessed, but it is tempting to speculate that they interfere with the binding of the S glycoprotein to the host cells.

Isatis indigotica root and phenolic Chinese herbs were frequently used for the prevention of SARS during the SARS outbreaks in China, Hong Kong and Taiwan. *I. indigotica* root (*Radix isatidis*) is native to China. From the *I. indigotica* root extracts several compounds, that is, indigo, sinigrin, aloe-emodin and hesperetin, were isolated that inhibited the cell-free and cell-based cleavage activity of the SARS Mpro (3CLpro) at IC_{50}

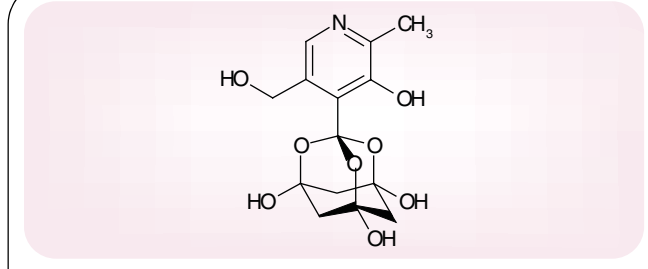

Figure 9. Bananin.

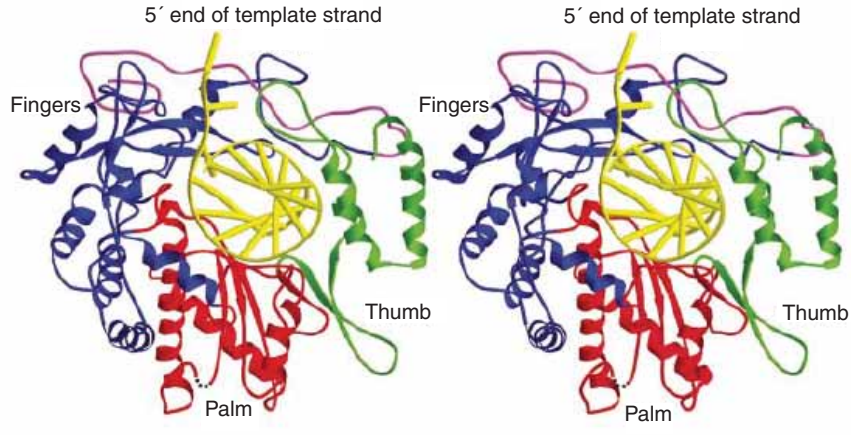

Figure 10. Ribbon diagram of the homology model of severe acute respiratory syndrome coronavirus RNA-dependent RNA polymerase with a docked RNA template primer. α-helices are shown as spirals and β-strands as arrows. The subdomains of the catalytic domain are colored as the N-terminal portion of the fingers subdomain (376–424) in magenta, the base of the fingers (residues 425–584 and 626–679) in blue, palm (residues 585–625 and 680–807) in red, and thumb (residues 808–932) in green. Reprinted with permission from [45].

values ranging from 10 to 1000 µM [73]. The inhibitory effects on SARS-CoV replication in cell culture (i.e., Vero cells) were not determined in this study. The cytotoxicity was determined, however, and, based on the ratio of the CC_{50} to the IC_{50} (cell-based cleavage), hesperetin appeared to be the most selective (selectivity index: ~300) [73].

Glycyrrhizin, another plant product that has been isolated from the licorice root (*Glycyrrhiza radix*), is known as an anti-inflammatory substance in Chinese medicine. Glycyrrhizin consists of one molecule of glycyrrhetinic acid linked to two molecules of glucuronic acid (FIGURE 12). It has long since been recognized as an antiviral substance, active *in vitro* against both DNA and RNA viruses, including HIV [74,75]. Glycyrrhizin has also been shown to inhibit the replication of SARS-CoV, but only at a concentration (EC_{50}: 300 µg/ml or ~365 µM) that would be difficult to achieve *in vivo* [76]. Through the introduction of certain chemical modifications it proved possible to increase the antiviral potency of glycyrrhizin, but, as these modifications also increased the cytotoxicity, the selectivity index of the glycyrrhizin derivatives was reduced as compared with that of glycyrrhizin (selectivity index: ≥65) [77].

Potent and selective inhibition of SARS-CoV replication has also been shown for aurintricarboxylic acid (ATA) [78]; reported values for its EC_{50} and CC_{50} (in Vero cells) were 0.2 and 37.5 mg/ml, respectively. Thus, the selectivity index of ATA was estimated to be 187 [78]. The anti-SARS-CoV activity of ATA was tentatively attributed to an inhibitory effect on the viral RdRp [79]. It should be recognized, however, that ATA, which is commonly represented in its monomeric structure but actually occurs as a heterogenous mixture of polymers (FIGURE 13), is able to bind to, and inhibit, a variety of proteins and cellular processes [80,81]. ATA has since long been known as an inhibitor of HIV replication [82], and its anti-HIV activity is at least partially mediated by a specific interaction with the HIV cell receptor CD4 [83].

Ribavirin, another compound that has long since been known as a broad-spectrum antiviral agent [84], targeted at inosine monophosphate (IMP) dehydrogenase (a key enzyme involved in the *de novo* biosynthesis of GTP) [85], did not show meaningful activity against SARS-CoV replication in cell culture [56,76]. Its EC_{50}, determined by virus yield reduction in Vero cells, was 40 µg/ml (CC_{50} > 200 µg/ml; selectivity index >5) [86]. In comparison, mizoribine, which is also assumed to act in its monophosphorylated form, as an inhibitor of the IMP dehydrogenase, exhibited an EC_{50} of 10 µg/ml (CC_{50} > 200 µg/ml; selectivity index > 20) [86]. Although these findings do not legitimate the use of ribavirin or mizoribine (FIGURE 14A) as single agents in the treatment of SARS, they point to IMP dehydrogenase as a potential target for the development of more potent anti-SARS-CoV agents.

The 4-aminoquinoline chloroquine, another 'old' compound best known for its antimalarial effects, but also accredited with antiviral and anti-inflammatory effects, has been recommended for its potential use, preferably in combination with other antivirals, in the treatment of AIDS as well as SARS [87]. Chloroquine (FIGURE 14B) was found to inhibit SARS-CoV replication in Vero cells at an EC_{50} of 8.8 µM (CC_{50} = 261 µM; selectivity index = 30) [88]. These inhibitory effects were observed when the cells were treated with the drug either before or after exposure to the virus [89]. The EC_{50} of chloroquine for inhibition of SARS-CoV *in vitro* approximates the plasma concentrations of chloroquine reached during treatment of acute malaria [88].

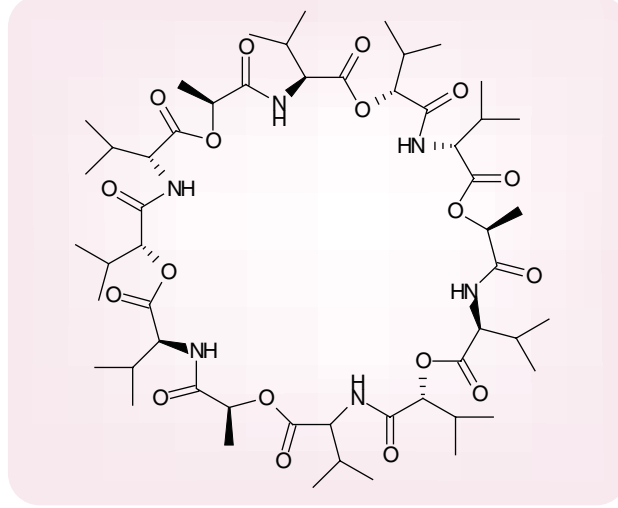

Figure 11. Valinomycin.

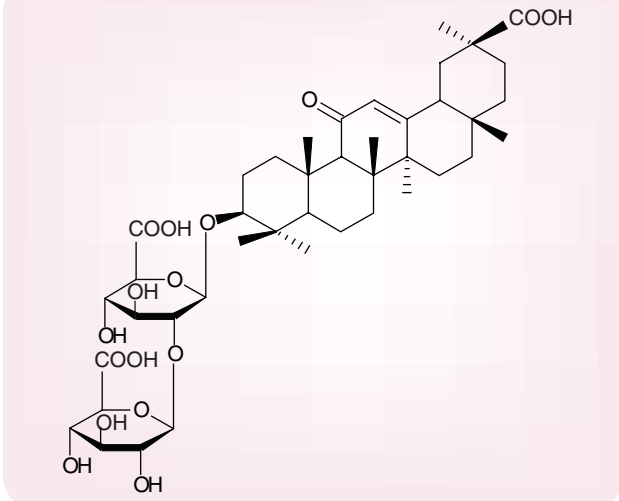

Figure 12. Glycyrrhizin.

Inhibition of SARS-CoV infection *in vitro* has also been reported for a nitric oxide (NO) generating compound, *S*-nitroso-*N*-acetylpenicillamine (SNAP), but only at an EC_{50} as high as 222 µM and a selectivity index as low as 2.6 [90]. Niclosamide (FIGURE 14C), an existing anti-helminthic drug, was able to inhibit the replication of SARS-CoV in Vero cells at an EC_{50} of 2 µM, while its CC_{50} was 250 µM; its selectivity could therefore be estimated at 125 [91].

Then there are the HIV protease inhibitors nelfinavir [92] and lopinavir [93] which have been reported to inhibit the replication of SARS-CoV in Vero cells and FRhK-4 cells, respectively. *In vitro* activity against SARS-CoV was demonstrated for lopinavir at a concentration of 4 µg/ml (in comparison with 50 µg/ml for ribavirin) [93]. Yamamoto and colleagues were not able to find a selective antiviral effect with lopinavir, but for nelfinavir they found an EC_{50} of 0.048 µM (CC_{50} = 14.5 µM; selectivity index = 300) [91]. Preliminary clinical trials in the treatment of SARS with lopinavir (boosted by ritonavir) point to an apparent favorable clinical response [93,94].

Expert commentary

Detailed knowledge on the molecular structure and functioning of most of the SARS-encoded proteins, except for the Mpro (3CLpro) is lacking, which means that thus far, few, if any, successful attempts have been made to rationally design anti-SARS-CoV compounds. Most of the antiviral compounds which have thus far been found effective against SARS-CoV, were evaluated because they had previously been shown (or were analogous to compounds that had been shown previously) to be effective against viruses other than SARS-CoV, in particular HIV.

It could be postulated that to become a potential anti-SARS candidate drug, the SARS-CoV inhibitor should possess a selectivity index of greater than 100, equally importantly an EC_{50} of not (much) higher than 1–3 µM, so as to be able to achieve sufficiently high plasma (and tissue) drug levels upon systemic administration. Thus, qualifying as potential anti-SARS drug candidates are some of the calpain inhibitors [23,24] and 3CLpro inhibitors, for example, the Phe–Phe dipeptide inhibitor shown in FIGURE 7 [38], which exhibited an EC_{50} of 0.18 µM and a selectivity index greater than 1000. Also, the octapeptide, AVLQSGFR, with an EC_{50} of 0.027 µg/ml (selectivity index >3700) [40] seems quite promising following the proposed criteria, and thus would be a number of the miscellaneous compounds such as valinomycin [69], niclosamide [90] and nelfinavir [91].

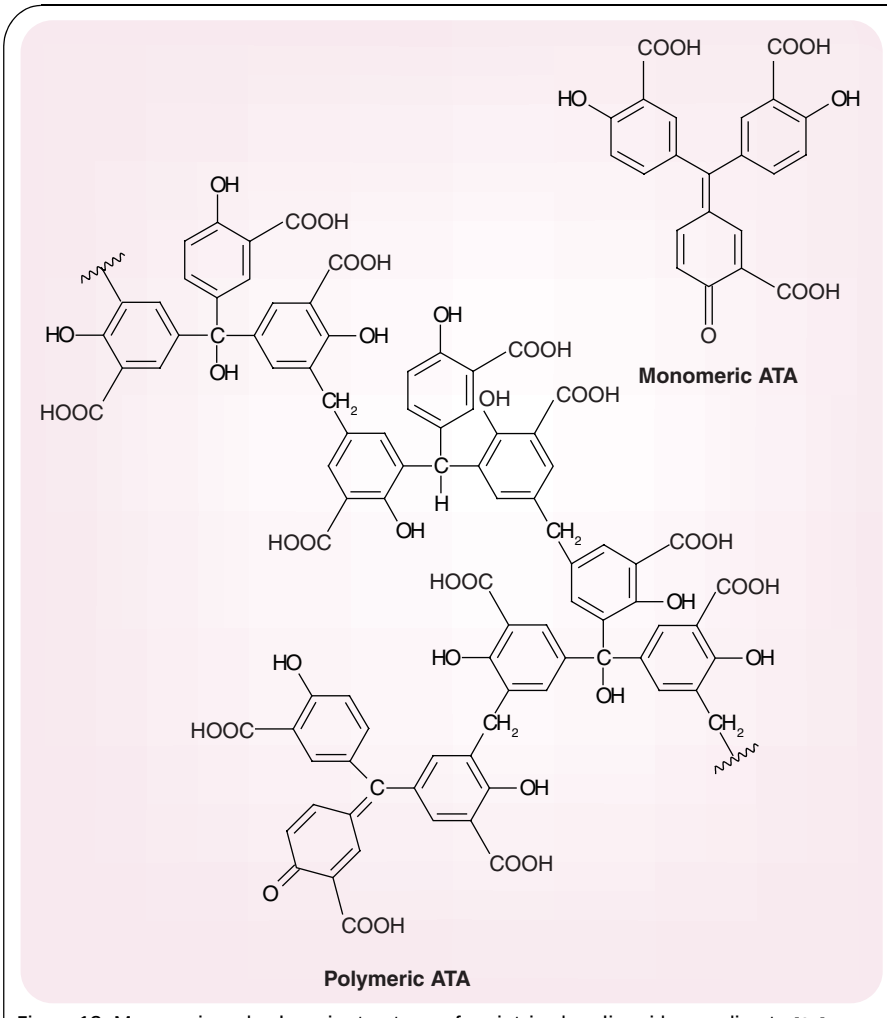

Figure 13. Monomeric and polymeric structures of aurintricarboxylic acid, according to [81].

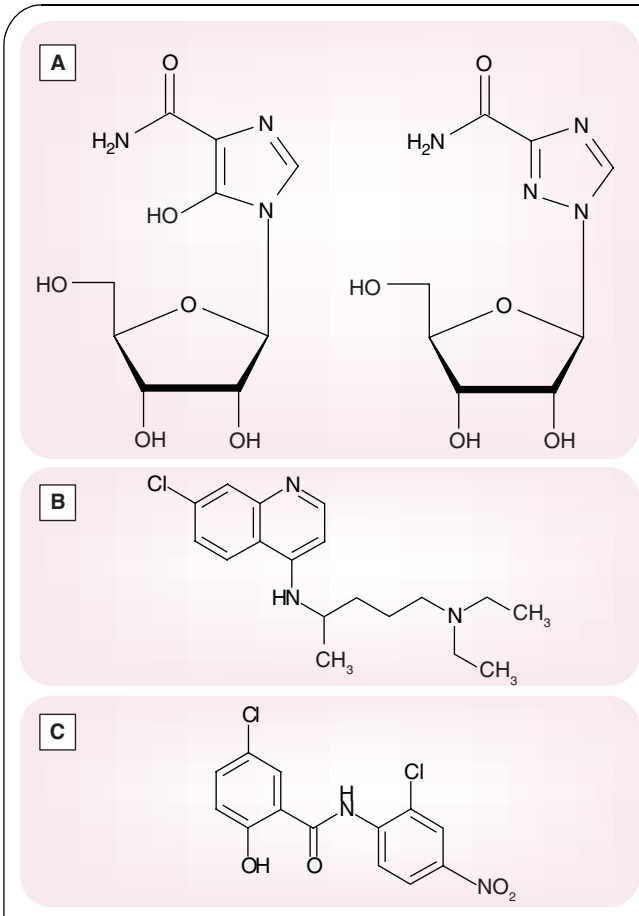

Figure 14. (A) Mizoribine and ribavirin, (B) chloroquine and (C) niclosamide.

Of equal, if not greater, importance is proof of efficacy against SARS in the *in vivo* setting, which remains to be provided with the aforementioned compounds. Such proof of principle exists for human mAbs [48,49], IFN-α [62] and siRNA [66], which means that, should SARS-CoV, or a similar CoV, re-emerge, attempts could be immediately undertaken to prevent and/or treat these infections with human mAbs, IFN and/or siRNA. Being commercially available at present, IFNs (PEG-IFN-α, IFN-β and alfacon-1 etc.) should possibly be considered the best choice to curb SARS, should it strike again.

Finally, as has thus far been applied in several viral (i.e., HIV and HCV) infections, and could be recommended for others (i.e., avian influenza), drug combination therapy certainly represents a valuable option for the management of SARS-CoV infection. As for any drug combination approach, this should allow the individual drugs to show a synergistic antiviral effect, thereby reducing the likelihood for drug resistance development (which, admittedly, has so far not been recognized as a problem in the treatment of SARS).

Five-year view

It is hard to predict whether SARS-CoV, or a similar CoV, would strike again in the future. This makes it impossible to speculate on how the field will evolve over the next 5 years. However, as for avian influenza H5N1, we ought to be better prepared. Therefore, attempts should continue to develop the appropriate means to prevent and/or treat SARS-CoV infections. The development of an adequate vaccine against SARS (not discussed here) remains, of course, mandatory, but, in addition, other prophylactic/therapeutic options should be duly explored, and these include, besides human mAbs, IFNs and siRNAs, also low-molecular-weight SARS-CoV inhibitors targeted at any of the specific processes involved in the viral replication cycle (i.e., viral entry into the cells, proteolytic cleavage, RNA replication and transcription).

Key issues

- Severe acute respiratory syndrome (SARS) is a new epidemic disease that emerged at the end of 2002, but subsequently subsided during the course of 2003.
- Measures should be undertaken to prevent or treat SARS should the SARS coronavirus (CoV) re-emerge.
- A first target for prophylactic or therapeutic intervention is the spike (S) glycoprotein involved in viral entry into the cells. This process can be hit by monoclonal antibody and fusion inhibitors.
- A second target is the processing of the replicase polyproteins by virus-encoded proteases. A number of protease inhibitors have been shown to interact at this level.
- Additional virus-encoded enzymes have been identified, among which are the NTP/helicase and RNA-dependent RNA polymerase, as potential targets for chemotherapeutic intervention.
- Human interferons (α and β) seem be indicated for the prophylaxis and early treatment of SARS-CoV infection.
- A possible approach to treat SARS may be based on RNA interference, using short interfering RNA duplexes.
- Other compounds that have been shown to inhibit SARS-CoV replication in cell culture include valinomycin, glycopeptide antibiotics, plant lectins, hesperetin, glycyrrhizin, aurintricarboxylic acid, chloroquine, niclosamide, nelfinavir and some of the calpain inhibitors.

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•• of considerable interest

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