Severe Acute Respiratory Syndrome Coronavirus Entry into Host Cells: Opportunities for Therapeutic Intervention

Kap-Sun Yeung,[1] Gregory A. Yamanaka,[2]* Nicholas A. Meanwell[1]

[1]Department of Chemistry, The Bristol-Myers Squibb Pharmaceutical Research Institute, 5 Research Parkway, P.O. Box 5100, Wallingford, Connecticut 06492
[2]Department of Virology, The Bristol-Myers Squibb Pharmaceutical Research Institute, 5 Research Parkway, P.O. Box 5100, Wallingford, Connecticut 06492

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Abstract: A novel human coronavirus (CoV) has been identified as the etiological agent that caused the severe acute respiratory syndrome (SARS) outbreak in 2003. The spike (S) protein of this virus is a type I surface glycoprotein that mediates binding of the virus to the host receptor and the subsequent fusion between the viral and host membranes. Because of its critical role in viral entry, the S protein is an important target for the development of anti-SARS CoV therapeutics and prophylactics. This article reviews the structure and function of the SARS CoV S protein in the context of its role in virus entry. Topics that are discussed include: the interaction between the S1 domain of the SARS spike protein and the cellular receptor, angiotensin converting enzyme 2 (ACE2), and the structural features of the ectodomain of ACE2; the antigenic determinants presented by the S protein and the nature of neutralizing monoclonal antibodies that are elicited *in vivo*; the structure of the 4,3-hydrophobic heptad repeats HR1 and HR2 of the S2 domain and their interaction to form a six-helical bundle during the final stages of fusion. Opportunities for the design and development of anti-SARS agents based on the inhibition of receptor binding, the therapeutic uses of S-directed monoclonal antibodies and inhibitors of HR1–HR2 complex formation are presented. Med Res Rev, 26, No. 4, 414–433, 2006

**Deceased June 27, 2005. We dedicate this review to the memory of Gregory A. Yamanaka, our friend, colleague, and collaborator, who was very much a scholar and a gentleman. Greg's life was taken unexpectedly whilst this article was in the final stages of revision. He was a biochemist who participated in antiviral drug discovery for 20 years, bringing unique insights to bear on problems with a passion that few can match. During this time, he contributed significantly to the understanding of atazanavir, a human immunodeficiency virus protease inhibitor marketed by Bristol-Myers Squibb as Reyataz™, and entecavir, an inhibitor of hepatitis B virus recently marketed by Bristol-Myers Squibb as Baraclude™.*

Correspondence to: Dr. Kap-Sun Yeung, Departments of Chemistry, The Bristol-Myers Squibb Pharmaceutical Research Institute, 5 Research Parkway, P.O. Box 5100, Wallingford, Connecticut 06492, USA. E-mail: KapSun.Yeung@bms.com

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1. INTRODUCTION TO THE SARS CoV

Severe acute respiratory syndrome (SARS) is an atypical pneumonia that initially emerged from Guangdong Province in the southern region of China around November 2002. This virus subsequently spread rapidly to 25 countries around the world within a very short period of time, infecting more than 8,000 people and causing more than 700 deaths by the time the outbreak ended in July 2003.[1,2] The mortality rate from SARS is approximately 15%, which compares with less than 1% for influenza infection, and shows a marked dependency on age. The unanticipated outbreak caused enormous anxiety and uncertainty for public health management authorities based on the unknown cause and the paucity of effective therapeutics available. The SARS outbreak was ultimately contained by a concerted effort that included patient isolation, intensive control of infection in hospitals, traditional quarantine measures, and the issuing of a travel advisory that was enforced by the World Health Organization.

The etiological agent of SARS was quickly identified as a coronavirus (CoV) in March 2003 after a unique, global collaborative effort between health and scientific organizations. Phylogenetic analysis of the amino acid sequence of this newly discovered CoV revealed it to be distinct from the previously characterized group 1, 2, and 3 coronaviruses,[3,4] although extensive genome and proteome analyses suggested that it is distantly related to group 2 coronaviruses, for example human CoV OC43 and murine hepatitis virus (MHV).[5] Like other coronaviruses, the SARS CoV is an enveloped, positive-stranded RNA virus that contains a large genome, comprised of 29,740 bases that constitute 14 open reading frames. The two large, overlapping open reading frames, ORF1a and 1b, that comprise approximately two-thirds of the genome, encode a single polyprotein containing the replicative enzymes, while the downstream open reading frames encode the structural proteins in the following order: spike (S), envelope (E), membrane (M), and nucleocapsid (N). In between these four structural genes are eight predicted open reading frames, referred to as accessory genes, that encode non-structural proteins of unknown function.[3,4,6,7]

Interferons, ribavirin, and some HIV protease inhibitors, particularly Kaletra, a combination of lopinavir and low-dose ritonavir, were used empirically to treat SARS-infected patients in some affected countries during the outbreak.[8] SARS CoV vaccines, which offer the promise of an effective means of preventing and controlling potential future outbreaks, remain under active development.[9] To date, there are no specific and efficacious antiviral drugs readily available to prevent or treat SARS. Although widespread SARS CoV infections have not re-emerged following the initial outbreak, sporadic cases were reported in 2004[1] and the possibility of an outbreak emerging from the natural reservoir remains unpredictable.[2] Consequently, effective antiviral agents against SARS CoV are desirable and, in contrast to influenza infection, viral load in the respiratory tract of a SARS Co V-infected individual peaks at day 10 following the onset of clinical symptoms, providing a wider window for treatment.

The SARS CoV surface glycoprotein, designated the spike or S-protein, is responsible for attachment and entry of the virus to host cells, the critical early step in the life cycle of the virus, and represents an important potential target for therapeutic intervention. Similar to HIV,[10] the entry of the SARS CoV into host cells takes between 10 and 20 min, based on observations viewed by electron microscopy during infection of Vero E6 cells in culture.[11] Although detailed kinetic studies have not been reported, there continues to be tremendous interest in understanding precisely the mechanistic steps associated with SARS CoV entry, and several significant findings have been reported within a

short period of time. This article summarizes developments in this area, reviewing the structure and function of the SARS S protein,[12,13] its antigenic determinants and the mechanism of viral entry as it is presently understood. We highlight the opportunities for therapeutic intervention in this process that may potentially provide a treatment for SARS CoV infection.[14–16]

2. SARS CoV SPIKE PROTEIN

As a newly discovered virus that is distinct from the three known groups of cornonavirus, the SARS CoV S protein shares only 20%–27% amino acid identity with the group 1, 2, and 3 cornonaviruses.[3] Figure 1 shows a schematic organization of the SARS CoV S protein, noting the approximate positions of the various functional domains that have been identified to date. At the *N*-terminal is a short secretory signal sequence thought to be cleaved co-translationally. The S1 domain is a globular structure that is responsible for binding to the host cell receptor, which has been identified as angiotensin converting enzyme 2 (ACE2)[17] (Section 4). Sequence analysis has predicted that the S protein contains heptad repeat regions of the type found in several other viral fusion proteins.[3] Computer sequence predictions together with systematic amino acid mapping studies of the stalk-like S2 domain have located the HR1 region to amino acids 896–972, while the HR2 region extends from amino acids 1142 to 1188 (Fig. 1).[3,18–23] Biophysical analysis showed that the isolated HR1 domain is highly helical and itself assembles into a thermally stable coiled coil, whereas the HR2 domain has low helical content and is less stable.[18,21] The HR1 and HR2 elements associate into a stable, antiparallel, six-helix bundle in which the HR1 is assembled centrally as a triple-stranded coiled coil, and to which the HR2 elements are bound[18–23] (Section 3). This intramolecular interaction is common to several other viruses and is thought to be the mechanism by which viral and host membranes are brought into close proximity as a prelude to membrane fusion. As a consequence, the SARS CoV S protein is characterized as a class I integral membrane protein, sharing membership with other class I viral envelope glycoproteins, including the S protein of related group 2 cornonaviruses, MHV,[24,25] HIV gp41, influenza hemagglutinin HA2,[26] the respiratory syncytial virus (RSV) F protein,[27] and the simian parainfluenza virus 5 (SV5) F protein.[28] The S2 domain of the SARS CoV is more conserved than the S1 domain, with the HR1 and HR2 regions exhibiting higher identity with other cornonaviruses, 50% and 32%, respectively.[19] Interestingly, circular dichroism (CD) analysis and proteinase K digestion studies have suggested that the HR2 domain binds to HR1 with a combination of helical and extended conformations in the HR1–HR2 core complex.[18,20] Such a structural feature, which resembles those of MHV[25] and SV5,[28] was subsequently confirmed by X-ray crystallographic studies (Section 3). The HR1 and HR2 regions are separated by a 170 amino acid interdomain loop that is similar in length to that of the corresponding region in the SV5 F protein. This relatively long interdomain loop is thought to counterbalance the short region of the S protein

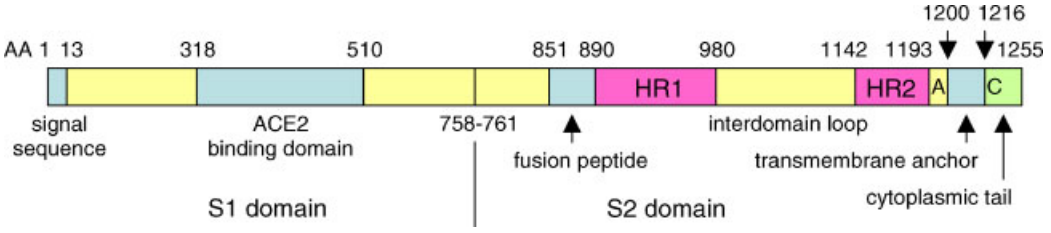


Figure 1. Schematic organization of SARS CoV S protein, showing the S1 and the S2 domains, and the approximate amino acid positions of the various functional domains (the size of each domain is not drawn to exact scale).[18–22,30,61,62] A: A trytophan and tyrosine rich aromatic region.[29,37] C: A cysteine rich region. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

that is proximal to the viral membrane, providing the flexibility required for the conformational change that occurs during the membrane fusion process.[21] This region is also noted for its high hydrophobicity and high activity in a membrane leakage model, which measures the activity of S protein-derived synthetic peptides to rupture phospholipid vesicles encapsulating a fluorescence probe molecule, suggesting an important role in facilitating the fusion process.[29]

Type I surface glycoproteins are typically processed proteolytically into individual subunits comprised of a membrane-bound fusion protein and a receptor-binding subunit that are associated non-covalently or through the agency of disulfide bonds and hydrogen-bonding interactions. Proteolytic cleavage usually occurs proximal to the fusion peptide, unmasking but not exposing this critical mediator of the fusion process. However, proteolytic cleavage of the SARS CoV S protein into separate S1 and S2 domains has not been observed, consistent with the fact that it does not contain the basic amino acid cleavage site (e.g., RRXRR) that is found in group 2 and 3 cornonaviruses.[3] Consequently, the boundary of the S1 and S2 domains has not been precisely located but it is predicted to be in the vicinity of amino acid 758 based on sequence analogy to MHV.[30] Cleavage of the S protein is not a necessary prerequisite for viral entry,[20,30,31] although cleavage of the S protein appears to enhance cell–cell fusion, as observed between 293T cells expressing the SARS CoV S protein and Vero E6 cells.[32] In the absence of proteolytic cleavage of the S protein, SARS CoV–host membrane fusion will depend on the activity of an internal fusion peptide that has been predicted to reside between amino acids 851 and 890,[20,21] a region rich in alanine and glycine residues located near the *N*-terminal of HR1. However, results from membrane partitioning and leakage studies using S protein-derived synthetic peptides suggest that this fusion peptide may reside further towards the *C*-terminal, starting from amino acid 770.[33] Possible proteolytic processing of the S protein was proposed based on the detection of S2 fragments in the cell lysates of SARS CoV-infected Vero E6 cells by S2-specific monoclonal antibodies.[34–36] However, it should be noted that isolation of the corresponding S1 fragments were not reported.

A small stretch of approximately 12 amino acids rich in tyrosine and trytophan residues has been identified in between HR2 and the transmembrane anchor. This highly hydrophobic, aromatic region shows a high propensity to partition into a lipid bilayer in a membrane leakage model system.[29,37] This region, together with the interdomain loop mentioned above, may help to destabilize lipid bilayers, providing an additional impetus for fusion between the viral and host membranes. Immediately following the transmembrane domain is a short, cysteine-rich region within the cytoplasmic domain that is conserved among coronaviruses[3,31] and is required for fusion activity.[38] In its native form, the full length S protein exists as a stable and non-covalently complexed homotrimer with *C*-terminal deletion studies suggesting that the transmembrane and/or cytoplasmic domains are important for trimerization.[31,39]

Enveloped viruses expressing surface glycoproteins enter host cells by two well-established pathways. One is through receptor binding-induced conformational changes of the virus glycoproteins, which leads to direct fusion of the viral and host membranes at the cell surface. This process is used by HIV and RSV. The second pathway is one of receptor-mediated endocytosis in which the virion is taken into the cell in an endosomal compartment. As the pH of the endosome falls during transit to the lysozome, the acidic environment triggers the conformational changes of the virus glycoproteins that are required for membrane fusion. This paradigm is used most prominently by influenza virus. Whether the entry of SARS CoV is pH dependent or pH-independent remains to be established, with current experimental evidence not definitive. The entry of the SARS CoV S protein-pseudotyped virus on a HIV vector into Vero E6 cells, 293T cells, or Huh7 cells was inhibited by high (millimolar) concentration of NH_4Cl, suggesting a pH-dependent endocytotic process.[32,40–42] However, these results were contradicted by another study[43] using lower, μM concentrations of NH_4Cl and the observations from an assay in which fusion between cells separately expressing the SARS CoV S protein and the ACE2 receptor occurred efficiently at neutral pH.[30] Moreover, entry of the SARS CoV into Vero E6 cells *via* membrane fusion at the cell surface rather than an endocytotic

pathway was observed by electron microscopy.[44] It should also be noted that CD-analysis showed that the helicity of a HR1 peptide (amino acids 882–973) did not increase significantly at pH 4.5 when compared to neutral pH,[18] suggesting that low pH did not induce a significant conformational change. Very recent studies on the entry of Ebola virus into host cells, a process mediated by the disulfide-linked surface glycoprotein GP1-GP2, using a pseudotyped system to infect Vero cells, have revealed a third process for triggering virus and host membrane fusion.[45] For Ebola virus entry, this process appears to be dependent on an initial proteolytic cleavage of GP1 by the endosomal cysteine proteases, capthepsin B, or L followed by a second proteolytic step mediated by capthepsin B.[45] The pH studies on SARS CoV entry as described above do not exclude such a mechanism. It is also intriguing, as noted, that the detection of S2 fragments in the cell lysates of SARS CoV-infected Vero E6 cells were reported.

3. THE S2 DOMAIN AND 4,3-HYDROPHOBIC HEPTAD REPEATS (HR1, HR2) OF THE SARS CoV S PROTEIN

Very recently, X-ray crystallographic structures of a complex of HR1 and HR2 that represent the fusion active form of the S protein of the SARS CoV have been solved at 1.6,[46] 2.2,[47] and 2.8 Å[48] resolution. These structures validated conclusions drawn earlier from biophysical studies. As depicted in Figure 2, the three HR1 units (amino acids 890–973) associate into a central, parallel trimeric coiled coil structure to which the three HR2 elements (amino acids 1147–1188) bind into grooves in the HR1 complex in an oblique, antiparallel direction.[47] The HR2 units each consist of a short five-turn α-helix (amino acids 1161–1177), which contrasts with the longer nine-turn helix observed in the HR2 elements of both HIV gp41[26] and the RSV F protein.[27] At each end of the *N*- and *C*-terminus of the HR2 helix, the peptide chains exist in an extended conformation. As revealed by another structure of the HR1–HR2 complex (PDB 2BEZ),[46] the extended HR2 region at the *N*-terminal spans five additional residues from Asn1142 to Thr1160, which complements residues 962–929 of the HR1 coiled coil. The interaction between HR1 and HR2 is largely hydrophobic, with the HR2 helical region binding into deep grooves in the complex while the extended regions bind in shallower grooves.[48] Both of the extended regions of HR2 consolidate their interaction with HR1 by several β-sheet-like hydrogen bonds formed between the primary amide groups of the side chains of Asp and Gln of HR1, and the main chain carbonyls and amide NHs of HR2.[47] The *N*-terminal residues of HR1, amino acids 890–892 in the complex shown in Figure 2, are in an extended conformation, while residues 885–889 of the HR1 construct used in these crystallographic studies are disordered, suggesting that this region, which connects to the fusion peptide, is flexible to allow the projection of the fusion peptide into the host membrane.

Using a strategy similar to that utilized in the discovery of inhibitors of HIV gp41-mediated fusion that ultimately produced the drug enfuvirtide (T-20), peptides derived from the heptad repeats HR1 and HR2 of the S2 domain of the SARS CoV virus were evaluated for their capacity to prevent infection of Vero cells. In this cell-based assay, the *C*-terminal HR2-derived peptides were inhibitory but peptides derived from the *N*-terminal HR1 were not, results consistent with observations from the related MHV CoV area.[24] A 68-mer peptide HR2-8 (amino acids 1126–1193) showed inhibitory activity, with an EC_{50} of 17 μM.[20] Similarly, a shorter HR2-derived peptide, CP-1 (amino acids 1153–1189) exhibited an EC_{50} of 19 μM.[19] A sequence derived from HR2-18 (amino acids 1161–1187) displayed an EC_{50} of 5.2 μM.[49] These HR2 peptides, which contain the key HR1-interacting region defined by amino acids 1142–1185, are thought to inhibit fusion by interfering with the formation of the fusion-active six-helix bundle, binding to HR1 during the pre-hairpin state. Inhibition of this type is sequence specific, since a peptide derived from the MHV HR2 region, designated MHV-HR2, was inactive against SARS CoV fusion.[20] However, the inhibitory potency of these SARS CoV HR2 peptides is considerably lower than the sub-micomolar activity measured

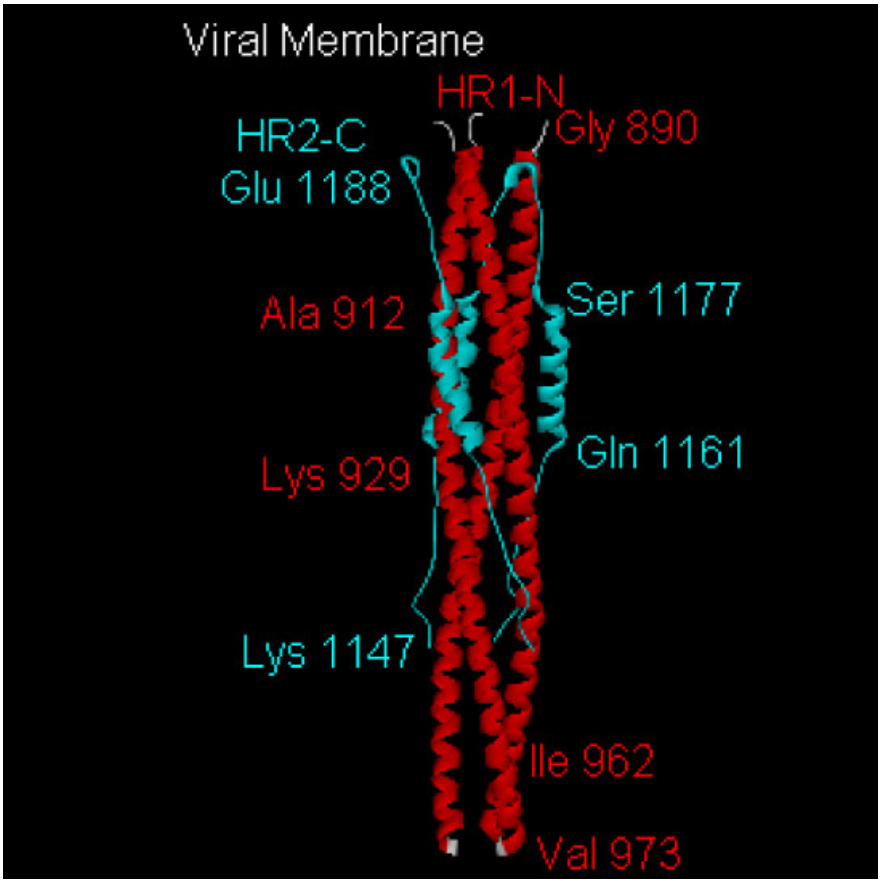


Figure 2. Crystal structure of the SARS CoV S2 HR1-HR2 core complex (HR1 (red): amino acids 890–973; HR2 (blue) amino acids 1147–1188). HR1-N: HR1 N-terminus; HR2-C: HR2 C-terminus. The structure is adapted from PDB 1WYY[47] and by using WebLab ViewerPro 5.0 (Accelrys). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

for the corresponding inhibition of MHV fusion by the MHV-HR2 peptide, which exhibits an EC_{50} of 0.9 μM.[20] This is attributed, in part, to a weaker interaction within the SARS CoV six-helix bundle, as indicated by the lower thermal stability when compared to that of MHV. A comparison of the SARS CoV six-helix bundle structure with that of MHV[25] revealed the structural basis for the weaker interaction.[46] First, a larger void volume exists inside the SARS CoV coiled coil due to the presence of the smaller side chain of Leu at position 920 of HR1, instead of the larger side chain of the conserved Phe at this position (SARS CoV numbering) among other cornonaviruses. Second, the SARS CoV HR1 has shallower hydrophobic pockets that interacts with smaller side chains of HR2 residues in a few positions, when compared to MHV (e.g., Ala in SARS CoV instead of Val as in MHV). Third, the buried surface area of the HR1-HR2 groove interface of SARS CoV is 10% less than in the MHV complex. Nevertheless, it should be noted that a peptide derived from HR2 of the SARS CoV, HR2-38, (amino acids 1149–1186) was shown to have EC_{50} below 5 nM in an infection assay using Vero E6 cells.[23] This HR2 peptide is almost identical to the one shown in Figure 2 and the potency suggests that this peptide represents the optimal interaction with HR1 domain. Another contradictory result, that the HR1-derived peptide HR1-1 (amino acids 889–926) exhibited inhibitory activity with an EC_{50} of 3.7 μM, has also been reported.[49] These discrepancies may be due to physicochemical properties of peptides used and different assay conditions, including longer incubation times and different read-out methods.

In addition to the peptide-based inhibitors, small molecule inhibitors have been identified using an assay that measures the binding of compounds to a S2 protein (amino acids 733–1190) expressed in *E. coli.* Tetra-*O*-galloyl-β-D-glucose and luteolin were found to be inhibitors in the binding assay, with micromolar EC_{50}s, and shown to prevent infection of Vero E6 cell by both pseudotype and wild-type SARS CoV.[50] An examination of the six-helix bundle crystal structure identified a hydrophobic pocket located in HR1 as a potential target for the design of small molecule inhibitors.[46] This pocket is occupied by the side chains of Leu1148 and Val1146 from the extended conformation region of HR2 in the six-helix bundle. The deeper grooves into which the HR2 helical regions bind could also be considered as a potentially viable target for small molecule inhibitors.[48] The feasibility of this approach has been demonstrated recently with the discovery of small molecules that target a similar hydrophobic pocket in HR1 of the RSV F protein.[51] BMS-433771 is a potent, orally bioavailable RSV fusion inhibitor that exhibits antiviral efficacy in murine model of RSV infection.[52,53]

4. THE CELLULAR RECEPTOR FOR THE SARS CoV: ANGIOTENSIN CONVERTING ENZYME 2 (ACE2)

The ACE2 protein has been identified as the host receptor for the SARS CoV, recognizing the S protein and mediating virus binding to the host cell and subsequent entry.[17,54,55] Interestingly, ACE2 itself was discovered only recently (in 2000) using a genomic-based approach and characterized as an enzyme homologous to ACE.[56,57] Although both enzymes are zinc metalloproteases that function as carboxypeptidases, ACE is a peptidyl dipeptidase while ACE2 is a type I integral membrane protein that cleaves only one amino acid from the *C*-terminal of substrates. ACE2 is mainly expressed in the heart, kidney, and testes and, like ACE, appears to be an important enzyme in the renin-angiotensin cascade. While ACE hydrolyses the decapeptide angiotensin I (AI) to the vasoconstrictor AII, ACE2 acts in conjunction with ACE to convert AI to the heptapeptide A1-7, which is a vasodilator. While it has been established that ACE2 plays an essential role in the regulation of cardiac function,[58] other physiological functions of this enzyme remain to be discovered. More recently, ACE2 was found to be highly expressed on lung alveolar epithelial cells and enterocytes of the small intestine,[59,60] locations consistent with the pathogenesis of SARS CoV infection. Although SARS appears mainly as a respiratory disease, the SARS CoV was shown to infect the human liver cell line Huh7,[41,42] which expresses high levels of ACE2.

In the absence of an X-ray crystal structure of the SARS CoV S protein bound to ACE2, biochemical studies are being used to discern the nature of the interaction between these proteins. In the process of identifying ACE2 as the SARS CoV receptor, the binding of the S1 domain to the receptor was clearly demonstrated, with amino acids 318–510 of S1 shown to be a critical recognition element.[30,54,61,62] Point mutation studies indicated that Glu452 and Asp454 in this region are responsible for key interactions with the ACE2 receptor, while five Cys residues, 348, 366, 419, 467, 474, are also essential elements in the association with ACE2.[61] A soluble form of the S1 domain associates into a dimer which binds to ACE2 more avidly than the monomer. The S1 dimerization domain has been mapped to amino acids 17–217, a region closer to the amino terminus of S1 that is also essential for membrane fusion.[63]

The peptidase activity of ACE2 was shown to make no contribution to the S protein-mediated entry of the SARS CoV. An ACE2 variant, in which the active site histidine residues 374 and 378 were modified to asparagines, proved to be equally effective as a receptor for virus entry as the wild-type ACE2.[64] Moreover, truncation of the cytoplasmic domain of ACE2 from 42 to 9 amino acids did not affect the efficiency of SARS CoV infection in a pseudotype assay, suggesting that this domain was not essential for ACE2-mediated entry of the virus.[65] A soluble form of ACE2 conjugated to an human IgG1 fragment was shown to bind with high affinity to S1, Kd = 1.7 nM, as measured by surface plasmon resonance (Biacore) methodology.[66] More recently, crystallographic structures of

the ectodomain of ACE2 in native form and bound to the active site-directed inhibitor MLN-4760 have been reported.[67] As shown in Figure 3, the zinc-containing active site of ACE2 is located deep inside and the near the bottom of a long canyon formed between the *N*-terminal subdomain I and the *C*-terminal subdomain II. A notable structural difference between the native and inhibitor-bound forms is that there is an inhibitor-induced repositioning of the two subdomains by 16° relative to each other, such that the open cleft closes to wrap around the inhibitor. The outer edge region of one subdomian moved as much as 13 Å. These crystal structure data, together with the mapping studies that have identified the location of the receptor-binding domain on S1 of the CoV S protein, provide a basis for molecular modeling of the binding interaction between the two proteins. Although the sequence similarity between the SARS CoV S protein and the S protein of other known coronaviruses is very low and X-ray structures of these proteins are not yet known, attempts have been made to use predictions of secondary structure and fold recognition approaches to construct a model for the SARS CoV S protein.[68–70] Early modeling studies based on a homology model of human ACE2 and a

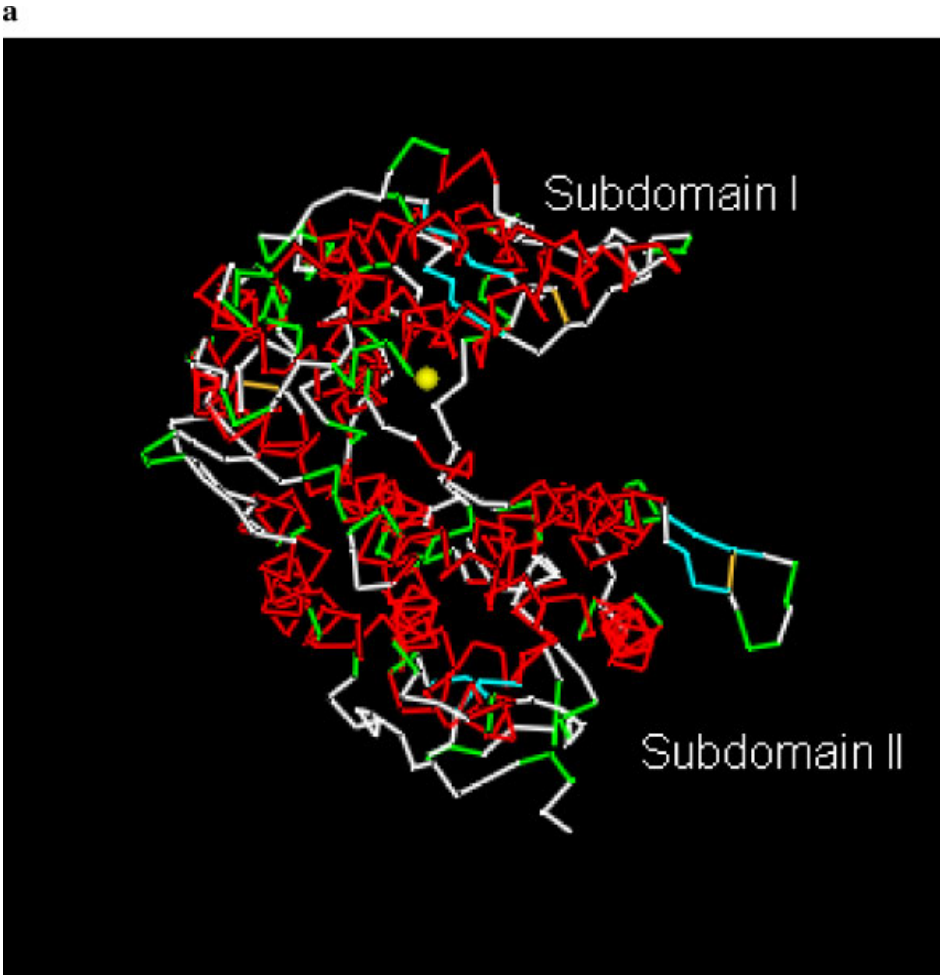
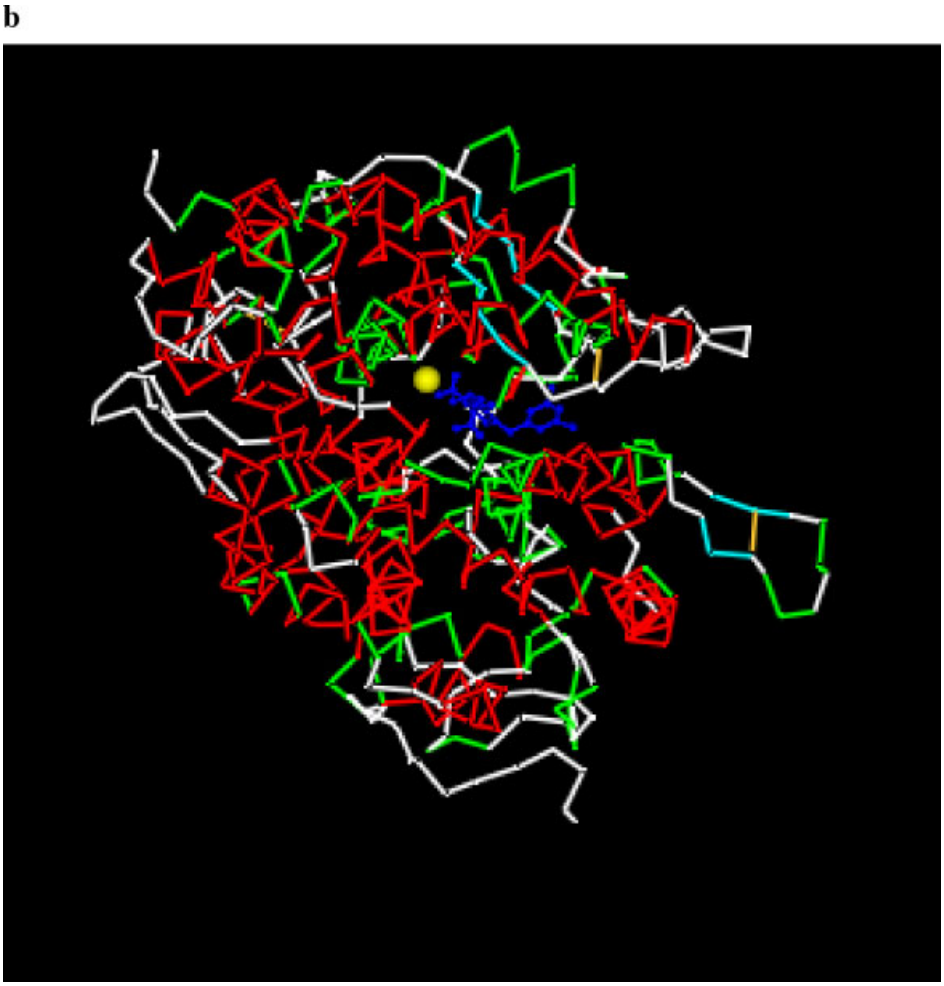


Figure 3. (a) Crystal structures of the ectodomain of human ACE2 in its native form, and (b) with inhibitor MLN-4760 (blue ball and stick) bound in the active site. The active site Zn^{2+} was shown as an enlarged yellow ball for clarity. The subdomain II in Figures (a) and (b) was drawn to the same orientation such that the relative conformational change of subdomain I is obvious. The structures are adapted from PDB 1R42 and 1R4L, respectively,[67] and by using WebLab ViewerPro 5.0 (Accelrys); (c) Structure of MLN-4760 and its ACE2 inhibitory potency. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]



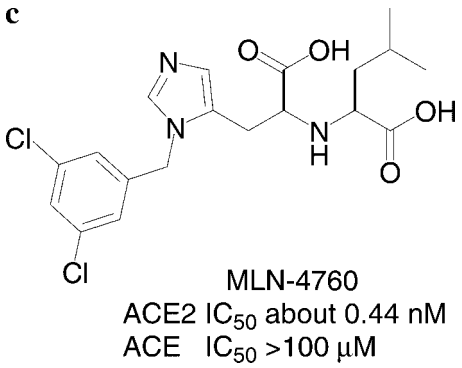


Figure 3. (*continued*)

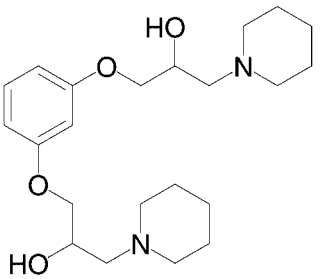
threading model of the known receptor-binding domain of S1 suggested that negatively charged ridges surrounding the ACE2 catalytic site are complementary to the largely positively charged surface of the receptor-binding domain of the S protein.[69]

Inhibition of the attachment of the SARS CoV S protein to ACE2 is a strategy with potential to identify inhibitors for the treatment of SARS infection. A peptide comprised of amino acids 318–510

of the S protein conjugated to the Fc domain of human IgG1 (RBD-Fc), inhibited S protein-mediated viral entry with an EC_{50} of < 10 nM in a pseudotype assay. This hybrid peptide was more potent than the corresponding conjugate of the full length S1, which showed an EC_{50} of 50 nM.[61] The difference in activity may due to a difference in physicochemical properties, for example, solubility or the possibility that the receptor-binding domain in the full length S1 is obscured and thus less accessible. A soluble form of ACE2 was shown to block the association of the S1 domain with Vero E6 cells and an anti-ACE2 antibody inhibited SARS CoV replication in Vero E6 cells with an EC_{50} of 1.5 μg/mL.[17] The soluble ectodomain of ACE2 was shown to inhibit S protein-mediated viral entry in a pseudotype assay, [65] while the catalytically inactive form of ACE2 conjugated to the Fc domain of human IgG1 potently inhibited SARS CoV infection in Vero cells, with an EC_{50} of 2 nM.[64] Similar receptor-based approaches using the HIV cellular receptor CD4 are being developed as anti-HIV therapies. PRO-540 is a CD4-IgG heteroconjugate that has completed a phase II clinical trail sponsored by Progenics,[71] while TNX-355 is a humanized IgG4 anti-CD4 monoclonal antibody that is in phase II clinical studies.[72]

The ACE2 protein is a potential target for therapeutic intervention but given the importance of this protein in cardiovascular function and its other unknown physiological roles, there is the potential for mechanism-based toxicities. Potent and selective small molecule inhibitors of ACE2 have been discovered, including MLN-4760 (Fig. 3c), which is a picomolar inhibitor of ACE2 with >5,000 fold selectivity over ACE.[73,74] On the other hand, ACE2 is insensitive to the inhibition by captopril and related ACE inhibitors.[56,57,74] The large conformational changes associated with the outer edge of the two ACE2 subdomains, likely S1-recognizing regions, upon binding of MLN-4760 could disrupt the interaction between the S protein and ACE2 and, hence, interfere with viral entry. However, this hypothesis remains to be tested. Another small molecule, VE607 (Fig. 4), was identified using a phenotype-based screen in which Vero cells were infected with SARS CoV. This compound was subsequently evaluated in a Vero cell-based plaque reduction assay as well as pseudotype viral S protein/ACE2-mediated entry assay.[75] VE607 inhibited in both the viral entry and plaque reduction assays at micromolar concentrations and did not inhibit either SARS CoV 3CL protease or helicase. However, confirmation of the mode of action awaits the selection of resistant virus and resistant mutation mapping.

The observation that SARS CoV does not infect certain cells that abundantly express ACE2 and also infects cells devoid of ACE2 expression[2,76] suggests that other receptors or co-receptors are required for viral entry. Very recently, CD290L, a C-type lectin, type II transmembrane glycoprotein also known as L-SIGN, DC-SIGNR, and DC-SIGN2, was identified as a potential receptor that



VE607

IC_{50} (viral entry) = 3.0 μM

EC_{50} (plaque reduction) = 1.6 μM

Figure 4. Structure of VE607 and its anti-SARS CoV activities.

interacts with amino acids 1–590 of the S1 domain of the S protein of SARS CoV. However, CD290L is much less efficient than ACE2 in promoting SARS CoV entry[77] but, importantly, this protein is expressed on type II alveolar cells and endothelial cells in human lung. Binding to the CD290L homolog, dendritic cell-specific intracellular adhesion molecule 3-grabbing non-integrin (DC-SIGN), was also observed using S protein pseudotyped virus.[40] Dendritic cells promoted SARS CoV infectivity by transporting the virus to target cells but these cells themselves were not susceptible to infection.

5. ANTIGENIC DETERMINANTS AND NEUTRALIZING MONOCLONAL ANTIBODIES

The SARS CoV S protein contains 23 potential *N*-linked glycosylation sites[3] and surface carbohydrates are important for recognition by neutralizing antibodies.[31] Antigenic determinants of the S protein that can elicit potent neutralizing antibodies were found to be located in both the S1 and S2 domains. The S1 peptide fragment represented by amino acids 441–700 expressed in an *E. coli* system[78] and a synthetic peptide comprised of amino acids 603–634[79] reacted with serum samples from convalescent SARS patients, but not with those from healthy donors. A peptide from the ACE2-binding domain, amino acids 318–510, conjugated to the Fc domain of human IgG1 (RBD-Fc), obtained by expression of 293T cells transfected with the corresponding plasmid, induced potent antibody responses in immunized rabbits.[80] These ACE-binding domain-directed antibodies inhibited the association of S1 to ACE2 and neutralized the infection of SARS CoV of Vero E6 cells *in vitro*.[80] In more detailed studies, 27 monoclonal antibodies were isolated from mice immunized with RBD-Fc, 25 of which were found to recognize 6 different conformationally-defined epitopes while two recognized linear epitopes within the receptor-binding domain, amino acids 435–451 and 449–458, respectively. Interestingly, only two groups of conformation-dependent antibodies could inhibit RDB-Fc binding to soluble ACE2 and ACE2-transfected 293T cells. These antibodies potently neutralized SARS CoV pseudovirus infection of ACE2/293T cells with nanomolar EC_{50}s, while the two antibodies that recognized linear epitopes exhibited only weak neutralization properties.[81] These results indicate the antigenic heterogeneity of a receptor-binding domain that contains multiple neutralization epitopes. Human monoclonal antibody CR3014, which was identified through screening of an antibody phage display library for binding to γ-irradiated, whole SARS CoV virions, neutralized the infectivity of SARS CoV towards Vero cells with nanomolar potency.[82] The level of neutralization correlated with the binding affinity to a peptide comprised of amino acids 1–565 of the S1 protein. More specifically, CR3014 bound to peptides comprised of amino acids 318–510 from nine human SARS CoV isolates. Since there was approximately 50% reduction in the binding of CR3014 to the peptide derived from the BJ302cl.2 strain that possessed a N479S mutation, it was suggested that this residue was important for the binding of CR3014. These results also indicate the importance of evaluating the antiviral spectrum of anti-SARS CoV monoclonal antibodies. Human non-immune recombinant monoclonal antibody 80R neutralized SARS CoV infection of Vero E6 cells *in vitro*.[66] The 80R epitope was conformationally dependent and located between amino acids 324 and 503 of the S protein,[83] encompassing the ACE2 receptor-binding domain, a result consistent with the observation that 80R was competitive with soluble ACE2 for binding to the S1 domain of the S protein. The recorded K_d of 32.3 nM could be enhanced to 1.59 nM, affinity comparable to the K_d of native ACE2, by conjugation to whole human IgG1. This 80R-IgG1 variant was 20-fold more effective in neutralizing the SARS CoV infection of Vero E6 cells, with an EC_{50} of 0.37 nM, which compares to an EC_{50} of 7.43 nM for 80R.[66] MAb 201 is a fully humanized monoclonal antibody that was isolated from transgenic mice with human Ig genes immunized with a recombinant ectodomain of the S protein, amino acids 1–1190. This antibody neutralized infection Vero E6 cells by SARS CoV at nM concentrations.[84] MAb 201 bound to full

length S protein expressed on transfected HEK-293T/17 cells with a 50% binding at 1 nM, and to a soluble fragment of S protein (amino acids 1–590) with a K_d of 34 nM, as measured by Biacore technology. The neutralization epitope of MAb 201 was mapped to amino acids 490–510 of the CoV S protein, again within the ACE2 receptor-binding domain. Although conformationally-defined epitopes are very important for eliciting neutralizing antibodies, as mentioned earlier, studies using denatured S protein fragments suggested that MAb 201 may recognize linear epitopes on the receptor-binding domain of the S protein.[84] Two *E. coli*-expressed peptide fragments of the S protein, amino acids 447–455 and 789–797, were recognized by mouse monoclonal antibodies D3D1 and D3C5, respectively.[85] These sub-segmenting studies suggested that the nine amino acid sequence 447–455 of the S protein may constitute the minimal epitope of the receptor-binding domain and signify that inhibition of receptor-binding constitutes a key neutralization mechanism for anti-SARS CoV vaccines. For example, the major neutralizing epitope of antibodies elicited in mice, rabbits, and monkeys by immunization with an anti-SARS vaccine (ADS-MVA), obtained using an attenuated vaccinia virus Ankara expressing the full length SARS CoV S protein, was mapped to amino acids 400–600. This region encompasses the ACE2 receptor-binding domain of S1 and these antibodies were shown to bind to RBD-Fc protein.[86] Administration of this vaccine to monkeys provided protection against a SARS CoV challenge, with no detectable virus shedding on days 4 and 6 post-challenge in all the four study animals. Monoclonal antibodies 1A5 and 2C5 were isolated from mice immunized with an inactivated SARS CoV, and their epitopes mapped to the amino acid sequence 310–535 of the S protein. These two antibodies showed much higher neutralizing titers against SARS CoV infection of Vero E6 cells than other antibodies that recognize other regions of the S1 domain or the S2 domain.[35] Vaccination of mice with a DNA plasmid encoding a form of the S protein that included the transmembrane domain, elicited humoral, and cellular immune responses.[87] However, it was demonstrated that neutralizing antibodies, not T-cells, provided the immune protection against pulmonary viral infection in murine challenge studies using SARS CoV. In this experiment, a greater than 6 $\log_{10}$ reduction in viral load in the lung was observed in treated mice compared to control animals. One of the neutralizing epitopes identified in antibodies from rabbits exposed to these DNA vaccines was mapped to amino acids 12–535.[36] A S1 domain peptide fragment (designated as S-II), comprised of amino acids 485–625, was obtained by expression in *E. coli*. S-II was shown to bind to Vero E6 cells and to induce monoclonal antibodies in mice. All of the four monoclonal antibodies isolated recognized both the monomeric and the trimeric native forms of SARS CoV S protein, and two of these completely inhibited the SARS CoV infection of Vero E6 cells at a concentration of 10 μg/mL. Interestingly, two of the neutralizing epitopes identified were in the region of amino acids 548–567 and 607–627 of the S-II peptide,[39] both of which are located outside of the receptor-binding domain.

The amino acid sequence 787–809 of the SARS CoV S protein was identified as one of the epitopes recognized by antibodies from convalescent SARS patients.[88] The S2 domain peptide fragment comprised of amino acids 803–828 elicited antibodies in rabbits and mice that have neutralizing effects towards a SARS-CoV pseudovirus infection of Huh-7 cells.[89] Other studies also suggested that epitopes in the *C*-terminal region of the HR2 domain are susceptible to antibody responses. Other neutralizing epitopes to emerge from DNA vaccine-induced antibody generation in rabbits were mapped to amino acids 797–1192, also outside the ACE2-binding region.[36] Antibodies raised in rabbit against an *E. coli*-expressed peptide comprising amino acid residues 1055–1192 that encompass the HR2 region of the S2 domain, specifically recognized the full length, mature S protein expressed on a cell surface, and were shown to possess strong neutralizing activity against a SARS-CoV infection of Vero E6 cells.[90] Since the antibodies raised in rabbit against another *E. coli*-expressed peptide based on amino acid residues 1061–1093 did not possess neutralizing activity towards a SARS-CoV pseudovirus infection of Huh-7 cells[89] and antibodies elicited by the peptide fragment of amino acids 798–1055 also did not neutralize SARS-CoV infectivity,[90] an epitope is likely located in the 100 amino acids residue 1093–1192 of the HR2 region.

In addition to antibody epitopes, T-cell epitopes are also located on the S protein. Transgenic mice treated with an adenovirus vector-based SARS CoV pseudovirus showed a CD8 T-cell response, which was found to be induced by H-2K^b-restricted epitopes (amino acids 436–443 and 525–532), and H-2K^b-restricted epitopes (amino acids 366–374) of the S1 domain.[91] Amino acid sequences 978–986 and 1203–1211 were identified as HLA-A2-restricted epitopes.[92] Synthetic peptides derived from these two sequences induced a high frequency of IFN-γ-secreting T-cell responses in the PBMCs of HLA-A2+ convalescent SARS patients. A nine amino acid peptide within the S2 domain, amino acids 1167–1175 (RLNEVAKNL), elicited-specific HLA-A*0201-restricted CD8 cytotoxic T lymphocytes both in peripheral blood lymphocyte preparations and transgenic mice.[93]

The identification of the antigenic determinants of the S protein has implications for vaccine design and development, including avoiding antibody-enhanced viral infectivity. Indeed, the potential for escalation of viral infection by SARS vaccines is currently a major clinical concern. For example, recent immunization studies conducted in ferrets with a candidate SARS CoV vaccine based on vaccinia virus showed signs of worsening the liver inflammation induced by SARS CoV infection.[9] The strong neutralizing immune response induced by peptide fragments of the receptor-binding domain described above also point to the sub-unit vaccine approach. The antigenic determinant studies presented above also suggest that monoclonal antibodies that target the S protein could, in principle, provide yet another effective approach to the inhibition of SARS CoV viral entry. Further support for the use of anti-SARS CoV monoclonal antibodies, in addition to the results from the DNA vaccine methodology discussed above, come from experimental results in mice where antibodies alone can prevent SARS CoV replication in the lung, particularly in the lower respiratory tract.[94] These results correlate with the neutralizing effects observed with SARS CoV S pseudotype assays *in vitro*.[41] Moreover, S protein-specific neutralizing monoclonal antibodies were isolated from a patient who recovered from a SARS infection, among which S3.1 (IgG1 κ isotype) was shown to protect against SARS CoV replication in the respiratory tract in a mouse model.[95] More encouraging results came from three animal studies with CR3014, 80R-IgG1, and MAb 201. Ferrets administered 10 mg/kg of CR3014 had a 3.3 $\log_{10}$ lower viral load in the lung and better lung pathology in a SARS CoV challenge study.[96] Administration of 2.5 mg/kg of 80R-IgG1 to mice 1 day prior to a SARS CoV challenge produced a 4 $\log_{10}$ drop in viral load in the lower respiratory tract compared to control animals, whereas using a 12.5 mg/kg dose reduced the viral load to below the detection limit.[83] MAb 201 protected mice from infection by live SARS CoV, demonstrating a greater than 2 $\log_{10}$ reduction in viral load in lung tissue at a dose of 1.6 mg/kg, while undetectable levels of virus were observed at doses of 8 and 40 mg/kg of MAb 201. Consequently, clinical trials were planned for MAb 201.[84] However, further studies of this and other monoclonal antibodies will be needed in order to evaluate the immunosafety, anti-SARS CoV spectrum, and efficacy in additional animal models. Although the prophylactic efficacy of anti-SARS CoV monoclonal antibodies in animals has been established, future studies will also be required to assess the safety and therapeutic efficacy in treating established infections. A very recent report using a pseudotype assay revealed escape from antibody neutralization by a SARS CoV virus isolated from a patient in late 2003 (GD03T0013) and from two palm civets (SZ3 and SZ16), which are proposed to be the natural animal reservoir for human SARS CoV. The antibodies used were competent for neutralizing human strains identified early during the SARS outbreak and raise some cause for concern.[97] Interestingly, the S-mediated entry of the pseudotype virus derived from the late 2003 example was less sensitive to inhibition by a soluble, recombinant human ACE2 ectodomain, while that derived from the two civets was enhanced by antibodies through their interactions with the receptor-binding domain. Antibody enhancement of viral entry, however, has not been observed with human strains of SARS CoV.[97] A specific study using 80R-IgG1, showed that pseudotype SARS CoV derived from GD03T0013, but not SZ3, was completely resistant to neutralization as a result of the D480G mutation,[83] a residue critical for 80R binding to the S1 domain, The implications of genetic mutations in the S protein to vaccine development and the use of antibody therapeutics remains to be determined.

6. CONCLUSION

A model for the entry of the SARS CoV into host cells based on the studies reported to date can be envisaged. Upon binding of the S1 domain of the trimeric S protein to the host cell receptor ACE2, the S2 domain, which carries the internal fusion peptide, is exposed, leading to the insertion of the fusion peptide into the host cell membrane. The existence of other receptors or co-receptors will require further investigation. A conformational rearrangement of the S2 domain at this pre-hairpin state may be induced by further interaction between the S1 domain and the receptor, whereupon the three HR2 regions wrap around the trimeric HR1 coiled coil, bringing the viral and host cell membranes into close proximity. The question of whether endocytosis of the virus occurs, and whether or not the conformational changes are promoted by low pH or certain unidentified proteases in the endosome still need to be resolved.[98] However, it is conceivable that multiple entry mechanisms may be exploited by the SARS CoV to establish an infection. During the process of hairpin formation by the S protein, the lipid bilayer is destabilized, and membrane mixing occurs along a hydrophobic pathway guided by the fusion peptide, the viral membrane proximal aromatic region, and the transmembrane anchor. This results in the formation of a fusion pore through which the nucleocapsid containing the viral RNA enters the host cell. The SARS CoV entry process offers several opportunities for therapeutic intervention, including blockade of the binding of the S protein S1 domain to the ACE2 receptor, inhibition of the HR1-HR2 association, and hairpin formation or neutralization of functional epitopes by monoclonal antibodies. Structural information provided by crystallographic structures of ACE2, with and without bound inhibitor, the SARS CoV HR1–HR2 core complex, and the identification of the antigenic determinants on S protein offer potential to accelerate the design of effective therapeutic agents. Certainly, crystallographic structures of a complex of S1 and ACE2[99] as well as S protein-monoclonal antibody complexes, should they be obtained in the future, will provide critical insights. As described, the inhibition of the SARS CoV entry process is feasible, although the inhibitors identified to date are only preliminary in nature. The current understanding of the SARS CoV entry process, the structural information now available and the emergence of inhibitors together form a solid basis for future drug discovery, of importance if the global community is to be adequately prepared for another SARS outbreak.

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Kap-Sun Yeung was born in mainland China and grew up in Hong Kong. He graduated with a B.Sc. (1st class honors) in chemistry from the Chinese University of Hong Kong in 1990. After spending a year working as a research assistant at the chemistry department of the University of Hong Kong, he obtained a Croucher Foundation Scholarship to pursue graduate studies at Cambridge University, UK, and in 1994 he obtained a Ph.D. in synthetic organic chemistry. Following a brief post-doctoral research period at the chemistry department of the Scripps Research Institute, he joined the antiviral drug discovery group at the Bristol-Myers Squibb Pharmaceutical Research Institute in Connecticut, in 1996. Currently, he is a senior research investigator designing and synthesizing potential drug candidates for the treatment of HIV and hepatitis C virus.

Gregory A. Yamanaka received a B.S. degree in molecular biophysics and biochemistry at Yale University in 1976 and a Ph.D. in microbiology in 1982 at the University of California, Berkeley. Following a post-doctoral appointment at Stanford University, Greg joined the research laboratories of Merck Sharpe and Dohme as a senior research virologist. In 1988, Greg accepted a position in the Bristol-Myers Squibb Pharmaceutical Research Institute as a senior virologist where he participated in drug discovery programs directed towards the identification and development of candidates for the treatment of herpes, human immunodeficiency, and hepatitis B infections. Greg performed some of the fundamental biochemistry studies of the HIV protease inhibitor atazanavir and the HBV inhibitor entecavir, both of which have reached the market and are helping to treat infected individuals. This was one of Greg's objectives in life and his contributions will be of benefit to mankind long after his untimely passing on June 27, 2005.

Nicholas A. Meanwell is currently Executive Director of Chemistry at the Bristol-Myers Squibb Pharmaceutical Research Institute in Wallingford, Connecticut. He received his Ph.D. in 1979 from the University of Sheffield for studies conducted under the supervision of Dr. D. Neville Jones that focused on the application of alkenyl sulfoxides as synthetic precursors of prostaglandin analogues. A post-doctoral fellowship (1979–1982) with Professor Carl R. Johnson at Wayne State University was devoted to the development of new synthetic methodology based on sulfur chemistry and its application to total synthesis. In 1982, he joined Bristol-Myers Squibb where he designed and synthesized inhibitors of blood platelet aggregation as part of the cardiovascular therapeutic focus before contributing to the identification and development of neuroprotective agents. The large-conductance, Ca^{2+}-dependent potassium channel opener Maxipost™, currently in Phase 3 clinical trials, emerged from those studies. Since 1994, he has been responsible for a team of chemists designing and synthesizing antiviral agents directed towards developing new therapeutic options for the treatment of HIV, HCV, RSV, and influenza.