The present invention provides methods for locating critical portions or sites on the spike protein (S protein) of SARS-associated coronavirus (SARS-CoV) responsible for the viral infection that causes Severe Acute Respiratory Syndrome (SARS). The present invention also provides novel synthetic peptides targeting such critical portions or sites of the S protein of SARS-CoV for preventing or treating of SARS-CoV infection or subject. The present invention further provides methods of testing antiviral activity exerted by antiviral agents using real-time quantitative PCR.
Figure 1

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<tr>
<th>No.</th>
<th>Sequence</th>
<th>Position</th>
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</tr>
<tr>
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</tr>
<tr>
<td>P4</td>
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</tr>
<tr>
<td>P5</td>
<td>RDSVSDTJTTXVRDFKTSFHI1</td>
<td>553-572</td>
</tr>
<tr>
<td>P6</td>
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<td>598-617</td>
</tr>
<tr>
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<td>690-709</td>
</tr>
<tr>
<td>P8</td>
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<td>727-756</td>
</tr>
<tr>
<td>P9</td>
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</tr>
<tr>
<td>P10</td>
<td>IQR(E)KIDRLNEVAKNLNES1</td>
<td>1161 1180</td>
</tr>
</tbody>
</table>
Figure 4
Figure 5A

Figure 5B
Figure 8F
SYNTHETIC PEPTIDE TARGETING CRITICAL SITES ON THE SARS-ASSOCIATED CORONAVIRUS SPIKE PROTEIN RESPONSIBLE FOR VIRAL INFECTION AND METHOD OF USE. THEREOF.

This application is a continuation-in-part and claims priority of U.S. Ser. No. 10/855,354, filed Dec. 22, 2004, the contents of which are incorporated herein by reference.

Throughout this application, several publications are referenced herein by Arabic numerals with parentheses. Full citations for the references referenced by Arabic numerals with parentheses may be found at the end of the specification immediately preceding the claims. Disclosure of these references in their entirety is hereby incorporated by reference into this application to more fully describe the state of the art to which the present invention pertains.

FIELD OF THE INVENTION

The present invention relates to locating and targeting critical portions or sites on the spike protein ("S protein") of the SARS-associated coronavirus ("SARS-CoV") responsible for the viral infection which causes Severe Acute Respiratory Syndrome ("SARS"). The present invention also relates to new synthetic peptides for the prevention and treatment of SARS. The present invention further relates to testing antiviral activity exerted by antiviral agents using real-time quantitative PCR.

BACKGROUND OF THE INVENTION

SARS spread to over thirty countries in 2003. There were more than 8,000 individuals infected and 800 lives were lost. A novel SARS-CoV was subsequently identified as the etiological agent of SARS (1-3), and it was further confirmed that the virus caused a similar disease in cynomolgus macaques (4). Although SARS appears to have been successfully contained, re-emergence of this life threatening disease remains a significant possibility. There have been three laboratory-acquired and four community-acquired SARS cases that have been recently reported in Singapore, Taiwan and China (46). Therefore, effective vaccines and antiviral drugs against this disease are urgently needed.

SARS-CoV-like viruses have been isolated from and characterized in small animals such as civet cats and raccoon dogs, implying that these animals may be the source of SARS (5). Important factors associated with the emergence of novel infectious diseases from animal sources include extensive exposure and rapid virus evolution (6). Phylogenetic analysis has revealed that although humans SARS-CoV and animal SARS-CoV-like viruses are related to the three groups of the previously found coronaviruses, they are different enough to make up their own, the fourth group, which may be a big family in wildlife. Increasing consumer demands for wild farmed animals in Guangdong, China in the past 15 years has provided an incubator to facilitate interspecies virus transmission from wild farmed animals to domestic animals and humans. The mutation rate will increase in interspecies transmitted viruses due to novel selection pressure in the new hosts.

In SARS-CoV infection, the spike protein ("S protein") recognizes and binds to host cell receptors, and the conformational changes induced in the S protein would then facilitate the fusion between the viral envelope and host cell membranes. Previous studies have clearly identified that there are significant sequence variations in the region encoding the S protein, with minor variable nucleotide substitutions changes (5). These substitutions may hold the key to understanding why and how the virus crossed the species barrier from animals to humans in the recent outbreak. The rapid mutation of these sites was further elucidated in a recent study for SARS surveillance. By comparing animal SARS-CoV-like viruses isolated in May 2003 (5) and those isolated after October 2003 (unpublished data), further variations in these sites are identified, which are completely identical to the human SARS-CoV isolated from a patient in December 2003 in Guangzhou (7) (Table 1). The rapid variations in these sites suggest that at least some of these mutations would play a crucial role in viral transmission across the species barrier from animals to humans. It is hypothesized that agents that interfered with, or bound competitively with these protein domains would be able to inhibit SARS-CoV infections by disrupting the function of the S protein. To test this hypothesis, ten peptides that spanned these variable regions were synthesized and their antiviral effects in a cell culture system were investigated.

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<th>P2</th>
<th>P3</th>
<th>P4</th>
<th>P5</th>
<th>P6</th>
<th>P7</th>
<th>P8</th>
<th>P9</th>
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<tr>
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</tr>
</tbody>
</table>

*Unpublished data.

**Abbreviations used:**

HCV ("HCV")

Humarus ("HR")

Chinese fowl ("CF")

Uruguay ("UR")

Shanghain ("SH")

P1-70 are SIQ (ID NO): 1-10, respectively.

[0007] Antiviral peptides targeting HIV-1 (8, 9), gp40 of feline immunodeficiency virus (FIV) (10), and the coniled-cell domain of human T-cell leukemia virus type-1 (HTLV-I) (11) have been demonstrated to be effective inhibitors of these viral infections, with potential therapeutic value in the treatment of the viral diseases. The inhibitory effects of these synthetic peptides were mediated by blocking the interaction of viral proteins with their cellular receptors, or alternatively, by preventing membrane fusion. Based on these findings, a recent study has demonstrated that a peptide targeting the heptad repeat 2 region of the SARS-CoV S protein inhibits virus infection in the micromolar range (12).

[0008] In this invention, peptides which target four regions of the S protein were synthesized and identified to effectively inhibit SARS-CoV infection in a monkey kidney (MKK-4) cell line. Synergistic antiviral effects were observed when cells were treated with combinations of two or three of these peptides prior to infection. 3D modeling indicated that these antiviral peptides map to a conserved interface present for the correct assembly of the trimeric peplomer. The results suggest a novel inhibitory mechanism distinct from the previously reported end-SARS-CoV peptide, which disrupted the heptad repeat 2 (HR2) interaction.

[0009] “Peplomer” described herein means layers of proteins which surround the capsid in animal viruses with tubular nucleocapsids. The envelope has an inner layer of lipids and virus-specific proteins also called membrane or envelope proteins. The outer layer has one or more types of morphological subunits called peplomers which project from the viral envelope; this layer always is composed of glycoproteins.

[0010] “Subject” shall mean any animal, such as a mammal or a bird, including, without limitation, a cow, a horse, a sheep, a pig, a dog, a cat, a rodent such as a mouse or rat, a turkey, a chicken and a primate. In the preferred embodiment, the subject is a human being.

[0011] “Pharmacologically acceptable carrier” shall mean any of the various vehicles or carriers known to those skilled in the art. For example, pharmaceutically acceptable carrier includes, but is not limited to, 0.01-0.1 M and preferably 0.05 M phosphate buffer or 0.8% saline. Additionally, such pharmaceutically acceptable carriers can be aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions and suspensions, including saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer’s solution, dextrose and sodium chloride, lactated Ringer’s and fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers such as those based on Ringer’s solution, and the like. Preservatives and other additives may also be present, such as, for example, antimicrobials, antioxidants, chelating agents, inert gases, and the like.

[0012] Peptides described herein are represented by “one-letter symbols” for amino acid residues as follows:

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<th>Letter</th>
<th>Amino Acid</th>
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<tr>
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<td>His</td>
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<td>F</td>
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<td>Y</td>
<td>Tyr</td>
</tr>
<tr>
<td>V</td>
<td>Val</td>
</tr>
</tbody>
</table>

**SUMMARY OF THE INVENTION**

[0013] It is an object of this invention to develop agents to interfere with, or bind competitively with the variation sites...
on the S protein of SARS-CoV. Another object of this invention is to identify regions of the S protein of SARS-CoV that is important for peptidomer function using these agents. Yet another object of this invention is the use of these agents to inhibit SARS-CoV infection by disrupting the function of the S protein. The agents described herein are 20mer peptides designed to span the twelve identified variations of the S gene based on the genome sequences between human SARS-CoV and animal SARS-CoV-like virus isolates. The peptides of this invention are represented by the following formulas (SEQ ID NO: 1-10):

- X-PHELlQGEPRNAFPLP-Z (SEQ ID NO: 1)
- X-FTPVl4PKELCID-2-Z (SEQ ID NO: 2)
- X-Ll88HtREITEGF-2-Z (SEQ ID NO: 3)
- X-RKQclicGQTFSI-2-Z (SEQ ID NO: 4)
- X-K6QDIEPEKEKQD-2-Z (SEQ ID NO: 5)
- X-FQQEEEEFESM-2-Z (SEQ ID NO: 6)
- X-VQGQCKYKMQELQSQ-2-Z (SEQ ID NO: 7)
- X-KQGCMC-2-Z (SEQ ID NO: 8)
- X-QEQGCM-2-Z (SEQ ID NO: 9)
- X-EEGCMC-2-Z (SEQ ID NO: 10)

in which amino acid residues are represented by the one-letter symbols, wherein X is an amino group, an acetyl group, a 6-fluoroisocaproxy-carboxylic group, or a hydrophobic group, and Z is a carboxyl group, an acetyl group, or a hydrophilic group.

[0014] The invention provides a method for locating sites of SARS-CoV S protein responsible for causing viral infection in a cell comprising contacting the cell prior to SARS-CoV infection with a peptide having the formula X-PTFEMKTIEGINTTVDSDK-Z (SEQ ID NO: 23); X-TPFEMMKTIEGINTTVDSDK-Z (SEQ ID NO: 25); X-TPFEMKTIEGINTTVDSDK-Z (SEQ ID NO: 26); X-TPFEMKTIEGINTTVDSDK-Z (SEQ ID NO: 27); X-TPFEMKTIEGINTTVDSDK-Z (SEQ ID NO: 28); X-TPFEMKTIEGINTTVDSDK-Z (SEQ ID NO: 29); or a combination thereof.

[0015] The invention further provides a method for preventing or inhibiting SARS-CoV infection in a subject comprising administering to the subject an effective amount of a pharmaceutical composition comprising SEQ ID NO: 2, SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 10, or a combination thereof, and a pharmaceutically acceptable carrier.

[0016] The invention also provides a method of testing antiviral activity exerted by antiviral agents using real-time quantitative PCR with a specific forward primer, reverse primer, and a fluorescence-labeled probe.

**BRIEF DESCRIPTION OF THE DRAWINGS**

[0017] The patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawings will be provided by the Office upon request and payment of the necessary fee.

[0018] The foregoing and additional features and advantages of the present invention will be better understood in light of the accompanying "Detailed Description Of The Invention" and the drawings described in this section.

**FIG. 1** Diagram of SARS-CoV Genomic and Locations of the Synthetic Peptides. The S protein of SARS-CoV includes of 1255 amino acid residues. Ten peptides (PS-20001-SEQ ID NO: 16-25, respectively) were designed to block viral entry based on the hypothesis that the residue variations between human SARS-CoV and animal SARS-CoV-like viruses might determine the preference of viral infection between human and animals. Amino acid variation(s) in each peptide are highlighted by indices and the alternative amino acid(s) identified from animal SARS-CoV-like viruses is shown in Table 1. The arrows indicate the location of each peptide in the S protein. Peptides with strong anti-SARS-CoV activities are shown in SEQ ID NO: 17 ("P2"), SEQ ID NO: 23 ("P6"), SEQ ID NO: 24 ("P7") and SEQ ID NO: 25 ("P8"). Abbreviations used: angiotensin-converting enzyme 2 binding region ("ACE2"), heptad repeats ("HR1" and "HR2"), open reading frame ("ORF"), and trans-membrane domain ("TM"). Results of ELISA and candididal production analysis are also listed.

**FIG. 2A-2F** PeptidesMediated Antiviral Effects Determined by Cytotoxic Effects of SARS-CoV Infection. F9806.4 cells were treated with (FIGS. 2A, 2C, and 2E) or without peptides (FIGS. 2B and 2F) for one hour before viral infection. Thirty-six hours post-infection, photos were taken to show cell morphology using phase-contrast microscopy (FIGS. 2A-2D, 400x) or virus morphology using electron microscopy (FIGS. 2E and 2F). The cells were incubated with peptides and did not show interference with the expression vector (FIG. 2A), while the untreated cells showed typical CPE after infection with SARS-CoV (FIG. 2B). No visible effect was observed when the cells were treated with SEQ ID NO: 8 at 50 μg/ml (FIG. 2C), or the CPE was significantly reduced by the treatment of SEQ ID NO: 8 at 25 μg/ml (FIG. 2D). Electron microscopy shows typical morphology of SARS-CoV (highlighted by arrows) within the infected cell (FIG. 2E), compared to an absence of virus visible in the cells pretreated with SEQ ID NO: 8 at 100 μg/ml (FIG. 2F). **FIG. 3A and 3B** Antiviral Effects Determined Mediated by Single Effective Peptide The antiviral activities of the effective peptides SEQ ID NO: 2, SEQ ID NO: 6, SEQ ID NO: 8, and SEQ ID NO: 10 were determined by measuring the viral titers in the culture media (FIG. 3A) and viral RNA copies in the cells (FIG. 3B). F9806.4 cells were pre-incubated with different concentrations of peptides (i.e., 100 μg/ml, 50 μg/ml and 25 μg/ml, respectively). Thirty-six hours post-infection, relative virus yield in the culture media was titrated by TCID₅₀, and compared between peptide pretreated and non-pretreated groups (FIG. 3A); relative viral genome copies in the cells were determined by real-time quantitative RT-PCR in triplicates and the standard deviations were illustrated (FIG. 3B). The relative viral titers or RNA copies of control were defined as 100%. The experiments were repeated at least twice.

**FIG. 4** Active Peptides Inhibit Virus Entry of the Cells Copies of the viral RNA were determined by Q-RT-PCR at different time points post-infection as indicated. Cells untreated by peptides, designated as virus controls ("VC"), were infected with the same dose of virus as the others. The uninfected cells (labelled as "UVC") were treated with SEQ ID NO: 8 and used as negative controls.
[0023] FIGS. 5A and 5B: Synergistic Antiviral Effects Mediated by Combination of Two Active Peptide FRK4-4 cells were incubated with different concentrations of two-peptide mixtures for one hour before viral infection. In each experiment, equal amount (by weight) of two peptides were mixed, and the final concentration of total peptides used were 100 µg/mL, 50 µg/mL, and 25 µg/mL, respectively. A combination of non-active peptide SEQ ID NO:9 and active SEQ ID NO:18 was used as a control. The antiviral effects were determined by titration of the yield of virus in culture media (FIG. 5A) and measuring intracellular viral RNA copies (FIG. 5B) as described in FIGS. 3A and 3B.

[0024] FIGS. 6A and 6B: Synergistic Antiviral Effects Mediated by Combination of Three Effective Peptides FIGK4-4 cells were incubated with different concentrations of three-peptide mixtures for one hour before SARS-CoV infection. In each experiment, equal amount (by weight) of three peptides were used, and the final concentrations of total peptides used are indicated in FIGS. 6A and 6B. Antiviral antiviral effects were determined by titration of the released virus in culture media (FIG. 6A) and measuring intracellular viral RNA copies (FIG. 6B) as described in FIGS. 3A and 3B.

[0025] FIGS. 7A and 7B: Comparison of Anti-Human Virus Effects Between Human Virus-Derived Peptides and Animal Virus-Derived Peptides FRK4-4 cells were incubated with 100 and 50 µg/mL of human or animal virus-derived peptides for one hour before SARS-CoV infection. The antiviral effects were evaluated by titration of the released virus in culture media (FIG. 7A) and measuring intracellular viral RNA copies (FIG. 7B) as described in FIGS. 3A and 3B. Abbreviations used: virus controls (*V*).
[0033] This invention provides a method for locating sites of SARS-CoV-2 S protein responsible for causing viral infection in a cell comprising contacting the cell prior to SARS-CoV-2 infection with a peptide having the formula X-PITFMRKDYFNTNDV-C-Z (SEQ ID NO: 2), X-QGGQYQCTQDGAYIDNETD-P-Z (SEQ ID NO: 9), X-QGGQYQCTQDKAlSAGLAIQ-S (SEQ ID NO: 3), X-QGGQYQCTQC1AIKENAVKAVLS11-Z (SEQ ID NO: 10), or a combination thereof. The peptide can be a combination of two peptides (e.g., SEQ ID NO:64) SEQ ID NO:8). The peptide may be positioned at SARS-CoV-2 S protein amino acid residue 239-278, amino acid residue 598-647, amino acid residue 737-756, or amino acid residue 1161-1186. The peptide binds either on or adjacent to the ACE2 binding region or the S1 and S2 domains of SARS-CoV-2 S glycoprotein. In this way, the peptide helps to prevent, inhibit, or reduce SARS-CoV-2 infection.

[0034] In the method above, the cell may be from a primate, a monkey cell line (e.g., 1Bak-4 and Caco2), a human, or a human cell line (e.g., Caco2, HeLa, CNE1 and CNE2).

[0035] In one embodiment, the peptide is SEQ ID NO:2 and interferes with ACE2 binding site conformation change.

[0036] In another embodiment, the peptide is SEQ ID NO:2, SEQ ID NO:6, SEQ ID NO:8, or SEQ ID NO:10, and the peptide interferes with polymerase function by competitive binding to an S protein monomer by mimicking regions exposed after ACE2 binding induced conformation change. The affected sites are preferably S protein amino acid residues 259-278, 598-647, 737-756, or 1161-1180.

[0037] The invention also provides a method for preventing or inhibiting SARS-CoV-2 infection in a subject comprising administering to the subject an effective amount of a pharmaceutical composition comprising SEQ ID NO:2, SEQ ID NO:6, SEQ ID NO:8, and/or SEQ ID NO:10, or a combination thereof, and a pharmaceutically effective carrier. The pharmaceutical composition may comprise a single peptide (e.g., SEQ ID NO:8), a dual peptide (e.g., SEQ ID NO:6,SEQ ID NO:8), or a combination thereof. In the preferred embodiment, the subject is a human being.

[0038] The following delivery systems, which employ a number of routinely used pharmaceutically acceptable carriers, are only representative of the many embodiments envisioned for administering the above pharmaceutical compositions.

[0039] Injectable drug delivery systems include solutions, suspensions, gels, microspheres and polymeric in situ gels, and can comprise excipients such as solubility-shifting agents (e.g., ethanol, propylene glycol, and sucrose) and polymers (e.g., polyethylene oxides and PLGA’s). Implantable systems include rods and discs, and can contain excipients such as PLGA and polyethylene oxide.

[0040] Oral delivery systems include tablets and capsules. These can contain excipients such as binders (e.g., hydroxypropylmethylcellulose, polyvinyl pyrrolidone, other cellulosic materials, and starch), diluents (e.g., lactose and other sugars, starch, dicalcium phosphate, and cellulosic materials), disintegrating agents (e.g., starch polymers and cellulose materials) and lubricants (e.g., stearates and tucks).

[0041] Transmucosal delivery systems include patches, tablets, suppositories, pastes, gels, and ointments, and can contain excipients such as solubilizers and enhancers (e.g., propylene glycol, boric acid, and acids) and other vehicles (e.g., polyethylene glycol, fatty acid esters, and derivatives, and hydrophilic polymers such as hydroxypropylmethylcellulose and hydroxyacids).

[0042] Dermal delivery systems include, for example, aqueous and nonaqueous gels, creams, multiple emulsions, microemulsions, liposomes, vesicles, solutions, suspensions and nonaqueous solutions, lotions, aerosols, hydrocarbon bases, and powders, and can contain excipients such as solubilizers, permeation enhancers (e.g., fatty acids, fatty acid esters, fatty alcohols and amino acids), and hydrophobic polymers (e.g., polycarboxylates and polyvinylpyrrolidones). In one embodiment, the pharmaceutically acceptable carrier is a liposome or a transdermal enhancer.

[0043] Solutions, suspensions and powders for reconstitutable delivery systems include vehicles such as corresponding agents (e.g., gums, surfactants, cellulosics, and sugars), humectants (e.g., sorbitol), solubilizers (e.g., ethanol, water, PEG, and propylene glycol), surfactants (e.g., sodium lauryl sulfate, Spans, Tweens, and cetyl pyridine), preservatives and antioxidants (e.g., parabens, vitamins E and C, and ascorbic acid), anti-caking agents, exciting agents, and eluding agents (e.g., EDDA).

[0044] The invention further provides a method of testing antiviral activity exerted by antiviral agents using real-time quantitative PCR with a specific forward primer, reverse primer, and a fluorescent-labeled probe. The forward primer can be a fragment of the DNA sequence of the SARS-CoV-2 S protein gene, such as 5’-AGTTTACAGCTTTGAGAGACA-3’ (SEQ ID NO:11). The reverse primer can be a fragment of the DNA sequence of the SARS-CoV-2 S protein gene, such as 5’-GCAATGGGCM- TAGTGGTGTTAAA-3’ (SEQ ID NO:12). The fluorescent-labeled probe, such as 5’-CCGTGACAGCAGACATCTGCAC- 3’ (SEQ ID NO:13), can also be labeled by any fluorescence as reporter in any real-time PCR detection system such as 5’-TACTCTAAAGC6GCGTTTTCCUCUG- AG-3’. In addition to the specific forward primer, reverse primer, and the fluorescent-labeled probe, a phosphate probe, such as 5’-6FAMTCMCTTCTTAATTTTATCCCA-3’ (SEQ ID NO:15), can also be used to test antiviral activity exerted by antiviral agents using real-time quantitative PCR.

[0045] The present invention will be better understood from the “Experimental Section” that follows. However, one
skilled in the art will readily appreciate that the specific methods and results discussed are merely illustrative of the present invention as described more fully in the claims which follow thereafter.

Experimental Section

Cell Culture and Viral Strains

[0046] The above-mentioned peptides were tested in fetal rhinos kidney (FRhK-4) cell culture described as follows.

[0047] Fetal rhinos kidney (FRhK-4) cells were cultured and maintained in MEM medium with 10% fetal bovine serum (10% MEM; Invitrogen, USA) at 37°C, with 5% CO2. SARS-CoV strain GZ09 was isolated from the nasopharyngeal wash fluid of a patient who suffered from SARS in Guangzhou, China in February 2003, and maintained in the FRhK-4 cells (13, 14). Serial passages of the GZ09 strain in FRhK-4 cells consistently yielded cytopathic effects (CPEs) with a titer of $10^{4.6}$ TCID$_{50}$/ml. Genome sequencing (accession number AY304495) and phylogenetic analysis showed that GZ09 lay between the reported Hong Kong strains, and the Canadian and U.S. strains (15).

Peptide Incubation And Viral Infection

[0048] Approximately 5,000 FRhK-4 cells per well were seeded in 96-well plates in 10% MEM and cultured overnight. After the cells were washed twice with PBS, peptides diluted to different concentrations with MEM (0% FBS), were added to the cultures and incubated at 37°C for one hour. The cultures were then infected with SARS-CoV GZ09 strain at a multiplicity of infection (MOI) of 0.05. CPE appeared after ~36 hours and peaked 72 hours post-infection. All experiments were performed in triplicate and were repeated at least three times.

Quantitative RT-PCR

[0049] Infectivity of SARS-CoV to the FRhK-4 cells in the presence of the ten 20mer peptides was assessed by real-time PCR. Cells were washed twice with PBS, and total RNA was extracted using RNeasy Mini kit (Qiagen, Hilden, Germany) in accordance with the manufacturer’s instructions. Reverse-transcription was performed using random hexamers with the ThermoScript RT system (Invitrogen, CA). Intracellular viral RNA was quantified using quantitative RT-PCR (QF-RT-PCR) (13, 16, 17), using a forward primer 5'-GCTTAGGCCTTGGAGAGAGAC3' (SEQ ID NO: 11) and a reverse primer 5'-GCTAATCGTCTAGTAGTATTTATTGTA3' (SEQ ID NO:12) (final concentration 200 nM), a fluorescent-labeled probe 5'-ACCTGACCTGCCGCGACGATGGTGTATGTA3' (SEQ ID NO: 13) and a phospholipid probe 5'-GCAAACCCTGCTTCTCCTTATAATGTTGCC-3' (SEQ ID NO:15) (final concentration 800 nM). The real-time quantification was carried out using iC Faststart DNA Master Hyb Probes and LightCycler (Roche Diagnostics, Manchattan, N.J., USA). PCR conditions employed were 95°C for 10 min, and then 50 cycles at 95°C for 10 sec, 60°C for 5 sec, 72°C for 5 sec, and 40°C for 30 sec. Ten-fold serial dilutions of plasmid ranging from 1.5 pg/ml to 1.5x10-6 pg/ml were used as standard and housekeeping gene beta-actin was used as an endogenous control to normalize for inter-sample variation in the amount of total RNA.

Determination of Viral Titers and Identification of Peptides with Antiviral Properties

[0050] In addition, the infectivity of SARS-CoV to the FRhK-4 cells in the presence of the ten 20mer peptides was assessed by the quantification of viral particles released into the culture medium using a CPE-based TCID$_{50}$/ml test (17, 18). Culture supernatant collected from SARS-CoV-infected cells 36 hours after viral infection was serially diluted at 10 fold with 1% MEM and inoculated into FRhK-4 cells in 96-well plates incubated with each peptide at a concentration of 25, 50 and 100 µg/ml for one hour before infection with SARS-CoV at 0.05 MOI. CPEs were determined 36 hours post-infection to evaluate peptide-mediated protection from viral infection.

[0051] Results were evaluated after 2 days of culture under phase-contrast microscopy, and viral titers were calculated. None of the 10 peptide-treated cells exhibited cytopathic effects at the concentrations tested, as represented by SEQ ID NO:8 to SEQ ID: 2A. In untreated cells, the CPEs observed after 36 hours were typified by cell rounding and detachment (Fig. 2B). Significant antiviral effects were observed for peptides SEQ ID NO:2, SEQ ID NO:6, SEQ ID NO:8 and SEQ ID NO:10. The most efficacious peptide, SEQ ID NO:8, selectively protected cells from CPEs at 100 and 50 µg/ml (Fig. 2C), and significantly reduced CPEs at 25 µg/ml (Fig. 2D). Similarly, significant protection was observed for SEQ ID NO:10 at 100 and 50 µg/ml, and for SEQ ID NO:2 and SEQ ID NO:6 at 100 µg/ml, while the other six peptides did not detectably reduce the levels of CPEs, even at a concentration of 100 µg/ml (Table 2).

**TABLE 2**

<table>
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<tr>
<th>Peptide concentration (µg/ml)</th>
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<tbody>
<tr>
<td></td>
</tr>
<tr>
<td>100 µg</td>
</tr>
<tr>
<td>P1*</td>
</tr>
<tr>
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</tr>
<tr>
<td>P10</td>
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</table>

*P1-P10 are SEQ ID NO:8-1 to 10, respectively.

[0052] Moreover, the peptide-mediated protection of the FRhK-4 cells from SARS-CoV infection was further evidenced by electron microscopy. FRhK-4 cells with or without SARS-CoV infections were harvested and fixed in 2.5% glutaraldehyde (Electron Microscopy Sciences, Fort Washington, Pa., USA) for 4 hours and post-fixed in 1% osmium tetroxide for 1 hour. Cells were then transferred to a 1.5 ml tube and centrifuged at 1,000 rpm for 10 min. The supernatant was removed and a liquidated aqueous solution (2%/55-60°C) Sigma, St. Louis, Mo., USA) was added to the cell pellet. After sonication of the gel, approximately 1 mm cubes containing the cell pellet were cut and dehydrated in graded ethanol. The cubes were then embedded in epoxy resin (Polysciences, Warrington, Pa., USA). Ultrathin sections of 70 nm thickness were prepared and stained with...
urnel acetate (Electron Microscopy Sciences, Fort Washington, Pa, USA) and lead citrate (Electron Microscopy S, Vienna, Austria). Sections were examined under a Philips EM208S electron microscope at 80 kV, and images were marked with a 100 nm scale bar. Results revealed morphology typical of non-SARS-CoV infections in non-peptide-treated cells (FIG. 2B), whereas no virus was visible in SARS-CoV NO.5-treated cells (FIG. 2F).

([0635] Since peptides SEQ ID NO:5 and SEQ ID NO:4 that respectively covered variations F680S and N479K (human to animal) located in the identified virus receptor (ACE2) binding region as described (23, 27), and the results showed that peptides SEQ ID NO:5 and SEQ ID NO:4 were inactive in this invention, indicating that either these two specific sites are not actively involved in splice-ACE2 binding, or the peptides cannot compete with the S protein for binding to ACE2. The active SEQ ID NO: 10 (residues 1101-1109) is derived from the HR2 region (residues 1153-1196) of the S protein. Similar to HIV-1 gp41, the S2 domain of SARS-CoV S protein contains HR1 and HR2 sequences, which are proposed to form a coiled-coil structure (12, 34) that is important for virus-host membrane fusion. The antiviral activity modeled by SEQ ID NO: 10 may be due to peptides blocking the interaction of HR1 and HR2, thereby interrupting membrane fusion. This is consistent with the reports by Liu et al. (12) and Triplet et al. (34). However, SEQ ID NO:9 (residues 800-909) derived from the HR1 region (residues 892-1013) did not exhibit antiviral activity. The other active peptides SEQ ID NO:2 (residues 259-276), SEQ ID NO:6 (residues 288-307) and SEQ ID NO:8 (residues 737-756) are located neither in the S1-ACE2 binding site (318-510) (24) nor in the HR1 and HR2 regions.

Active Peptides Reduce Viral Titer as Well as Intracellular Viral RNA Level

([0634] The antiviral efficacies of the four active peptides were further investigated using TCD_{50}, assays with the titration of virus released in the culture media, and by Q-RT-PCR to quantify the levels of cytopathic viral RNA in the samples harvested at 50 hours post-infection. As shown in FIG. 3A, the active peptides reduced the number of infective viruses in a dose-dependent manner. The released viral titer was reduced by over 5-fold (18%) of the untreated control) by treatment with either peptide SEQ ID NO:2 or SEQ ID NO: 6 at 100 μg/mL, and by about a half at 50 μg/mL; however, no antiviral effects were detected at 5 μg/mL. Peptide SEQ ID NO: 10 reduced the viral titer by over 15-fold (to 4% of the control at 100 μg/mL by 10-fold at 50 μg/mL and by a half at 25 μg/mL). Peptide SEQ ID NO:3 exhibited the strongest antiviral activity, with the viral titer reduced 50-fold (to 2% of the control), 15-fold, and 10-fold at a concentration of 100, 50, and 25 μg/mL, respectively. The 90% inhibition concentration (IC_{90}) for these peptides was calculated, and is shown in Table 3. The antiviral efficacy of peptide SEQ ID NO:3 is the highest, indicated by the lowest IC_{90} value, which is over 4.5-fold lower than that of SEQ ID NO:2, SEQ ID NO:6, and 3-fold lower than that of SEQ ID NO:10. FIG. 3B shows that the intracellular viral RNA levels were also reduced after treatment with peptides SEQ ID NO:2, SEQ ID NO:6, SEQ ID NO:3 and SEQ ID NO:10. Consistent with the results from the viral titration assays, SEQ ID NO:3 exhibited the highest antiviral potency.

([0655] The IC_{90} values were defined as the peptide concentrations that reduced the viral titer by 90%, relative to the unregulated control (standard deviation ['SD']). IC_{90} values were calculated by fitting the logistic equation to data determined for each peptide concentration (performed in triplicate). In a peptide combination, each peptide is in equal amount (weight). Active Peptides Inhibit The Entry of SARS-CoV Into Cells

([0656] To detect whether entry of SARS-CoV into cells is blocked by the peptides, peptide incubation started one hour before viral inoculation. Viral RNA copies in the media and inside the cells were determined by Q-RT-PCR at different time points post-infection. As shown in FIG. 4, compared to peptide-ununtreated cultures (labeled as the virus control ("Virus")), the viral RNA copies were higher in the media of peptide-treated cultures at 1 hour post-infection (1 h-S), but lower in those cells harvested at different time points post-infection. The virus growth curves in the treated cultures were similar to that in virus control, although they were at lower levels. Furthermore, these active peptides did not show antiviral effects when they were applied to the cultures 1 hour after the virus inoculation and compared viral RNA copies with untreated virus culture control at 6 and 12 post-infection (does not shown). The results indicated that the active peptides could block the entry of the virus to the cells but not inhibit the virus replication. Synergistic Antiviral Effects Mediated by Peptide Combinations

([0657] Combinations of peptides targeting different domains which exhibited synergistic antiviral effects were also investigated. Four peptides were co-treated with mixtures of two active peptides one hour before infection, with equal amount of each peptide and total concentrations of 100, 50 and 25 μg/mL, respectively. Samples were collected at 36 hours post-infection for viral titer determination (FIG. 5A) and viral RNA quantification (FIG. 5B). Titration experiments revealed that peptide SEQ ID NO:2 in combination with SEQ ID NO:6 reduced the viral titer 1000-fold (to 0.1% of the control) at 100 μg/mL and 10-fold at 50 μg/mL (even at a concentration of 25 μg/mL, where no antiviral activities were observed for either peptide individually (FIG. 5A), dual peptide treatment still reduced the viral titer 3-fold (to 32% of the control). The viral titer was reduced 10,000 fold by peptide SEQ ID NO:6 in combination with SEQ ID NO at 100 μg/mL. The IC_{90} of SEQ ID...
NO₃⁻, and SEQ ID NO:8 is reduced by about 12-fold, as compared with SEQ ID NO:6 alone (IC₅₀ 13.0 μM vs. IC₅₀ 113.0 μM), and 2.5-fold as compared to SEQ ID NO:8 alone (IC₅₀ 9.6 μM vs. IC₅₀ 24.9 μM) (Table 5). Significant synergistic effects were also observed for other active peptide combinations, but not in active plus inactive peptide combinations (e.g., SEQ ID NO:6, SEQ ID NO:9, as shown in both the calculated IC₅₀ values (Table 5) and the relative viral titers (FIG. 5A). These results were further confirmed by Q-RT-PCR assays (FIG. 5B).

[0058] The synergistic antiviral effects were investigated by incubating the cells with mixtures containing equal amounts of three peptides applied one hour before viral inoculation. Interestingly, all peptide combinations exhibited greatly improved antiviral potencies (Table 3), reflected in both the levels of released virus (FIG. 6A) and in the quantitation of intracellular viral RNA (FIG. 6B). There was essentially complete inhibition of SARS-CoV-2 infection following pre-treatment with the three peptide mixtures at total concentrations of 50 and 25 μM. As SEQ ID NO:2 and SEQ ID NO:6 map to the S1 domain, which is involved in binding to the host receptor ACE2 (24, 39), while SEQ ID NO:8 and SEQ ID NO:10 map to the S2 region that plays a role in virus-membrane fusion (25, 26), these results clearly indicated that combinations of peptides that targeted different domains within the viral S protein significantly enhanced the antiviral effects.

Antiviral Effects of Peptides Derived from Animal Viral Sequences

[0059] To determine whether peptides derived from the sequences of animal SARS-CoV-like viruses can also inhibit human SARS-CoV-2 infection, peptides derived from the animal viruses were synthesized and used to treat FRH-4 cells before the cells were challenged with human SARS-CoV-2. Both virus titers in the supernatant (FIG. 7A) and viral RNA in the cells (FIG. 7B) were measured and compared to that of the controls treated with peptides derived from the human virus. Higher viral titers and RNA levels were observed in samples treated with animal peptides than in controls, indicating that the animal peptides were less potent against human SARS-CoV-2 than human peptides. The most significant reduction of the antiviral potency was observed in SEQ ID NO:6 that the antiviral efficacy of animal SEQ ID NO:6 is 2 to 4 folds less than that of the human SEQ ID NO:6 (FIG. 7).

Peplomer Model Construction and Putative 3D Locations of the Active Peptides

[0060] To explore how the active peptides mentioned above inhibit viral infections, a molecular modeling of the S glycoprotein in its peplomer quaternary assembly with the Protein Data Bank (PDB) ID code 1TFK, shown in FIGS. 8A-8F, was used to find a potential mechanism to related to active peptide activity.

[0061] The structures of the S1 and S2 domains of the SARS-CoV-2 peplomer were retrieved from the PDB (19), with the ID codes 1Q6Z and 1Q4Y, respectively. Docking of the two domains has been minimally performed and energetically optimized by using molecular dynamics simulations with Gromacs (20). The reliability of each of the possible peplomer assemblies was assessed using the Prosa II software package (21). Peplomer image construction and exposed surface area (ESA) calculations were performed using the program MOLMOL (22). The predicted peplomer assembly of the S glycoprotein was deposited in the PDB with the ID code 1TFK (39).

[0062] In a model, SEQ ID NO:2 ("P2") is found to be located on the surface of the S protein adjacent to the ACE2 binding region (FIG. 8A). This suggests that SEQ ID NO:2 does not directly compete for ACE2 binding, but it may hinder the binding site conformation change. The other three active peptides (SEQ ID NO:6 ("P6"), SEQ ID NO:8 ("P8") and SEQ ID NO:10 ("P10")) are located on the surface of the S1 and S2 domains of the S glycoprotein, and exhibit loop conformations (FIG. 8A). Since these peptides occupy the monomer-monomer interface regions of the S glycoprotein, these peptides may interact with the peplomer function via competitive binding to the S protein monomer or by mimicking regions exposed after ACE2-binding-induced conformation change. These regions may serve as co-receptor binding sites, proteinase cleaving sites, and/or virus-cell membrane binding sites. These peptides may therefore compete with the peplomer for virus-cell membrane interaction (FIGS. 9A-9C).

[0063] SEQ ID NO:8 (residues 737-750), which targets an unknown function region (residues 673-802) of the S2 domain, exhibited the highest inhibitory activity to the virus infection. Interestingly, it is very close to a putative receptor-binding site (residues 737-741) predicted by 6o by et al. (35). By the consideration of the similarity of the infection progression between HIV-1 and SARS-CoV, which involves viral protein-receptor binding, change of viral protein conformation and fusion of virus-cell membrane, residues 737-750 may be an undefined co-receptor (similar to CXCR4 for HIV-1) binding site. SEQ ID NO:8 may compete with the peplomer for co-receptor interaction (FIG. 9A). This is supported by results showing that SEQ ID NO:8 also appeared to exert the highest synergistic antiviral effects when used in combination with the other active peptides (Table 3).

[0064] Studies with other coronaviruses have established that binding of the S1 to soluble viral receptor, or exposure to 37°C and an anhydrous pH (pH 8.9) can induce a conformational change accompanied by the cleavage of S1 and S2, which might be involved in triggering virus-cell membrane fusion (36-38). The active peptide SEQ ID NO:6 (residues 598-617) is located on the surface of S1 domain where it is close to the S1-S2 connection site (FIGS. 8A and 8F). Its antiviral activity may be attributed to the binding of SEQ ID NO:6 to the S protein monomer interacting with the cleavage of S1 and S2, resulting in the failure of subsequent fusion between viral envelope and host cell membrane (FIG. 9B).

[0065] The synergistic effect of two and three peptide combinations suggests that increased disruption of peplomer function can be achieved by simultaneously blocking multiple peptide-protein or protein-membrane interaction sites. These peptides may disturb the peplomer assembly or conformation by competitive binding to the S protein monomer (FIG. 9C).

[0066] Two common features were found to be associated with the active peptides (SEQ ID NO:8, 9, and 10). First, they are located on the surface of the S1 and S2 domains where the monomers contact each other. Second, these active peptides exhibit loop conformations. On the contrary,
the inactive peptides are located at exposed peplomer surfaces (SEQ ID NO: 3, SEQ ID NO: 2, SEQ ID NO: 4, not shown) and exhibit helical structures (SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 7 and SEQ ID NO: 9). Active peptides are the only ones which exhibit a loop conformation, suggesting a loop conformation is necessary for ease of access of the peptide to the S protein monomer or a co-receptor. Consistent with this model, the inactive peptides either belong to exposed surface regions of the peplomer (SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 7 and SEQ ID NO: 9).

[0667] SEQ ID NO: 6, 8 and 10, the only peptides which reproduce loop conformations of the peplomer, are the ones which can more easily interfere with the peplomer assembly by competitive binding to the S protein monomer. More ordered secondary structure elements, such as the proposed helices formed by SEQ ID NO: 1, 3, 4, 5, 7 and 9 have no antiviral activities. Peptides reproducing exposed surface regions of the peplomer (SEQ ID NO: 1, 3 and 4) are total inactive since no interference of the peplomer quaternary structure stability can be induced by their presence. Thus, active peptides bind to the peplomer in critical regions for the conformational changes that are necessary for the membrane fusion process.

REFERENCES


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<223> OTHER INFORMATION: Ser or Leu

<400> SEQUENCE: 22

Thr Gln Val Met
20

<210> SEQ ID NO: 22
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<212> TYPE: PRT
<215> LOCATION: Artificial Sequence
<221> OTHER INFORMATION: Description of Artificial Sequence: synthetic peptide
<226> PRT

<221> NAME/KEY: MOD.PRD
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<223> OTHER INFORMATION: Thr or Ala
<226> PRT

<221> NAME/KEY: MOD.PRD
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<223> OTHER INFORMATION: Ala or Val

<400> SEQUENCE: 23

Gly Tyr Gly Ser Phe Cys His Gin Leu Asn Arg Ala Leu Ser Gly Ile
10 15

<210> SEQ ID NO: 24
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<221> OTHER INFORMATION: Description of Artificial Sequence: synthetic peptide
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<221> NAME/KEY: MOD.PRD
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<223> OTHER INFORMATION: Thr or Ala

<400> SEQUENCE: 24

Gly Ile Gly Val His Gin Asn Val Tyr Gin Asn Gin Lys Gin Ile
10 15

<210> SEQ ID NO: 25
<211> LENGTH: 20
<212> TYPE: PRT
<215> LOCATION: Artificial Sequence
<221> OTHER INFORMATION: Description of Artificial Sequence: synthetic peptide
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<221> NAME/KEY: MOD.PRD
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<223> OTHER INFORMATION: Lys or Gin

<400> SEQUENCE: 25
What is claimed is:

1. A method for locating sites of SARS-CoV S protein responsible for causing viral infection in a cell comprising contacting the cell prior to viral infection with a peptide having the formula: X-PTTFMLKYDFPGITDIAVDC-Z (SEQ ID NO:2), X-QYQVNYCQTVSIAHADQYIP-Z (SEQ ID NO:6), X-QVGFCTCQQNRLSGRA-SA-Z (SEQ ID NO:8), X-IQKEEDRNIYKNIQNESLZ (SEQ ID NO:10), or a combination thereof.

2. The method of claim 1, wherein the peptide is a combination of SEQ ID NO:6 and SEQ ID NO:8.

3. The method of claim 1, wherein the peptide is positioned at SARS-CoV S protein amino acid residue 259-278, 506-617, 737-756, or 1161-1180.

4. The method of claim 1, wherein the peptide binds either alone or adjacent to the ACE2 binding region or the 34 and 82 domain of SARS-CoV S glycoprotein.

5. The method of claim 4, wherein the peptide prevents, inhibits, or reduces SARS-CoV infection.

6. The method of claim 5, wherein the peptide is SEQ ID NO:2 and interferes with ACE2 binding site conformation change.

7. The method of claim 5, wherein the peptide is SEQ ID NO:2, SEQ ID NO:6, SEQ ID NO:8, or SEQ ID NO:10, and the peptide interferes with protein function by competitive binding to an S protein monomer by mimicking regions exposed after ACE2-binding induced conformation change.

8. The method of claim 7, wherein the peptide binds to SARS-CoV S protein at SARS-CoV S protein amino acid residues 259-278, 506-617, 737-756, or 1161-1180.

9. The method of claim 7, wherein the cell is a primate or a human.

10. A method for preventing or inhibiting SARS-CoV infection in a subject comprising administering to the subject an effective amount of a pharmaceutical composition comprising SEQ ID NO:2, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, or a combination thereof, and a pharmaceutically acceptable carrier.

11. The method of claim 10, wherein the pharmaceutical composition comprises a combination of SEQ ID NO:6 and SEQ ID NO:8.

12. The method of claim 10, wherein the subject is a cat, a horse, a sheep, a pig, a dog, a cat, a rodent, a chicken, or a primate.

13. The method of claim 10, wherein the subject is a human being.

14. A method of testing antiviral activity from antiviral agents using real-time quantitative PCR with a specific forward primer, a reverse primer, and a fluorescent-labeled probe.

15. The method of claim 14, further comprising a phosphotase probe.

16. The method of claim 15, wherein the phosphotase probe is SEQ ID NO:15.

17. The method of claim 14, wherein the forward primer is a fragment of the DNA sequence of the SARS-CoV S protein gene.

18. The method of claim 17, wherein the forward primer is SEQ ID NO:11.

19. The method of claim 14, wherein the reverse primer is a fragment of the DNA sequence of the SARS-CoV S protein gene.

20. The method of claim 19, wherein the reverse primer is SEQ ID NO:12.

21. The method of claim 14, wherein the fluorescent-labeled probe is SEQ ID NO:13.

22. The method of claim 14, wherein the fluorescent-labeled probe is labeled by any fluorochrome as reporter in any real-time PCR detection system.

23. The method of claim 22, wherein the fluorescent-labeled probe is SEQ ID NO:14.