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(71) Applicant (for all designated States except US): **VAN-
DERBILT UNIVERSITY** [US/US]; Box 6009, Station
B, Nashville, TN 37235 (US).

(72) Inventor; and

(75) Inventor/Applicant (for US only): **DENISON, Mark**
[—/US]; 2509 Barton Avenue, Nashville, TN 37212 (US).

(74) Agent: **HIGHLANDER, Steven, L.**; Fulbright & Ja-
worski L.L.P., Suite 2400, 600 Congress Avenue, Austin,
TX 78701 (US).

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(54) Title: METHODS OF TREATING SEVERE ACUTE RESPIRATORY SYNDROME VIRUS INFECTIONS

(57) Abstract: The present invention provides methods for inhibiting severe acute respiratory syndrome (SARS) virus infections. Specifically, cysteine proteinase inhibitors are proposed as therapeutics for SARS, potentially used in combination with interferons.



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DESCRIPTION

METHODS OF TREATING SEVERE ACUTE RESPIRATORY SYNDROME VIRUS INFECTIONS

BACKGROUND OF THE INVENTION

The government owns rights in the present invention pursuant to grant number 5RO1 A126603-15 of the National Institutes of Health and National Institute Allergy Infectious Disease. The present invention claims benefit of priority to U.S. Provisional Serial No. 60/471,604, filed May 19, 2003, the entire content of which is hereby incorporated by reference.

1. Field of the Invention

The present invention relates generally to the fields of microbiology, immunology and virology. More particularly, it concerns methods of treatment for severe acute respiratory syndrome (SARS) virus infections.

2. Description of Related Art

Coronaviruses have been long known to cause important diseases in a wide variety of animal species, including humans, cattle, swine, chickens, dogs, cats and mice. Coronavirus diseases in non-human species may be quite severe, and devastating in domestic livestock such as pigs, cattle and chickens. The characterized human coronaviruses - HCoV-229E and HCoV OC43 - are significant causes of upper respiratory infections, responsible for 10-35% of human colds. Studies of human coronaviruses have been limited by their lack of growth in culture from primary isolates, and by the lack, until recently, of reverse genetic approaches for their study. Thus, while the human coronaviruses are arguably two of the most economically important viruses in humans, ongoing research has been pursued only by a handful of dedicated investigators.

An outbreak of atypical pneumonia, designated "severe acute respiratory syndrome" or "SARS" was first identified in Guangdong Province, China, last year. It now has spread to several countries, including Canada and the United States, although it remains much more prevalent in China. The mortality rate appears to be as high as about 6%. Testing has identified the causative agent as a new human coronavirus. This occurrence surprised many scientists and

public health officials, but has highlighted the potentially dangerous characteristics of coronaviruses well known to investigators: high rates of mutagenesis and homologous RNA recombination. In fact, template switching and recombination are essential to the normal life cycle of the viruses. In addition, the species barrier for coronaviruses has been predicted to be tenuous. Studies of coronaviruses in culture have demonstrated the ability of coronaviruses to adapt for replication in cells of different species. In addition, some studies have demonstrated that the murine coronaviruses may cause disease in primates following direct inoculation into brain. Finally, coronaviruses have been proposed, based on evolutionary studies, to have acquired genes from other viruses or cells, probably by recombination events. The emergence of a new coronavirus pathogenic for humans, by either adaptation of an animal virus, or by recombination of two coronaviruses during a coinfection, is consistent with these features of coronavirus evolution, replication and maintenance in populations.

A treatment protocol consisting of antibacterials and a combination of ribavirin and methylprednisone was recently proposed. However, according to U.S. Army Medical Research Institute of Infectious Disease, the therapeutic value of ribavirin remains unclear, as it has no activity against SARS virus *in vitro*. Thus, the potentially lethal nature of SARS infection provides a compelling argument for new approaches in the development coronavirus therapies.

SUMMARY OF THE INVENTION

Thus, in accordance with the present invention, there is provided a method of inhibiting replication of severe acute respiratory syndrome (SARS) virus comprising contacting a cell infected with the SARS virus with a first cysteine proteinase inhibitor. The first cysteine proteinase inhibitor may be selected from the group consisting of L-trans-epoxysuccinyl-leucylamido(4-guanidino)butane) (E64), E64d, and E64c, and any other cysteine proteinase described in U.S. Patents 6,331,542; 6,297,277; 6,287,840; 6,284,777; 6,232,342; 6,180,402; 6,162,791; 6,147,188; 6,057,362; 6,034,066; 6,004,933; 5,998,470; 5,976,858; 5,925,772; 5,776,718; 5,766,609; 5,714,484; 5,663,380; 5,618,966; 5,486,623; 5,317,086; and 4,891,356. The method may further comprise contacting the cell with a second cysteine proteinase inhibitor. The second cysteine proteinase inhibitor may be contacted with the cell at the same time as the first cysteine proteinase inhibitor or at a different time from the first cysteine proteinase inhibitor.

In another embodiment, there is provided a method of treating a subject infected with a severe acute respiratory syndrome (SARS) virus comprising administering to the subject a first

cysteine proteinase inhibitor. The first cysteine proteinase inhibitor may be selected from the group consisting of L-trans-epoxysuccinyl-leucylamido(4-guanidino)butane (E64), E64d, and E64c, and any other cysteine proteinase described in U.S. Patents 6,331,542; 6,297,277; 6,287,840; 6,284,777; 6,232,342; 6,180,402; 6,162,791; 6,147,188; 6,057,362; 6,034,066; 6,004,933; 5,998,470; 5,976,858; 5,925,772; 5,776,718; 5,766,609; 5,714,484; 5,663,380; 5,618,966; 5,486,623; 5,317,086; and 4,891,356.

The method may further comprise contacting the cell with a second cysteine proteinase inhibitor. The second cysteine proteinase inhibitor may be contacted with the cell at the same time as the first cysteine proteinase inhibitor or at a different time from the first cysteine proteinase inhibitor. The first cysteine proteinase inhibitor may be administered more than once, such as daily for two weeks, for four weeks, or for eight weeks. The first cysteine proteinase inhibitor may be administered at about 1 to about 1000 mg/kg, and/or at daily dose of no more than about 2500 mg/kg.

The method may further comprise administering to the subject a non-proteinase antiviral composition. The method may also further comprising assessing viral load after administration of the first cysteine proteinase inhibitor, and/or adjusting the dosage of the first cysteine proteinase inhibitor. The first and/or second cysteine proteinase inhibitor may be administered orally, intravenously, intramuscularly, by inhalation or transdermally.

In other embodiments, methods are provided that included (a) a method of reducing virus load in a subject infected with a severe acute respiratory syndrome (SARS) virus comprising administering to said subject a first cysteine proteinase inhibitor; (b) a method of inhibiting virus replication in a subject infected with a severe acute respiratory syndrome (SARS) virus comprising administering to said subject a first cysteine proteinase inhibitor; (c) a method of limiting virus infection in a subject infected with a severe acute respiratory syndrome (SARS) virus comprising administering to said subject a first cysteine proteinase inhibitor; (d) a method of inhibiting the spread of severe acute respiratory syndrome (SARS) in a population comprising administering to members of said population a first cysteine proteinase inhibitor; and (e) a method of preventing severe acute respiratory syndrome (SARS) in a subject comprising (a) identifying a subject at risk of exposure to SARS virus and (b) administering to said subject a first cysteine proteinase inhibitor.

It is contemplated that any method or composition described herein can be implemented with respect to any other method or composition described herein.

The use of the word “a” or “an” when used in conjunction with the term “comprising” in the claims and/or the specification may mean “one,” but it is also consistent with the meaning of “one or more,” “at least one,” and “one or more than one.”

These, and other, embodiments of the invention will be better appreciated and understood when considered in conjunction with the following description and the accompanying drawings. It should be understood, however, that the following description, while indicating various embodiments of the invention and numerous specific details thereof, is given by way of illustration and not of limitation. Many substitutions, modifications, additions and/or rearrangements may be made within the scope of the invention without departing from the spirit thereof, and the invention includes all such substitutions, modifications, additions and/or rearrangements.

BRIEF DESCRIPTION OF THE DRAWINGS

The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

FIGS. 1A-C - E64-d inhibition of SARS-CoV cytopathicity and replication in Vero E-6 Cells. Vero cells were infected with Urbani strain of SARS-CoV and incubated for 48 h in the (FIG. 1A) absence or (FIG. 1B) presence of 400 µg/ml of E64-d. Cell rounding and death was prominent in absence of E64-d. No cell rounding, or death was noted in intact monolayer at 48 h p.i. in the presence of E64-d. (FIG. 1C) Vero E-6 cells were infected in the absence (control - black bars) or presence (white bars) of E64-d added at 1 h p.i. Samples of overlying media were obtained at 24, 48, and 72 h p.i. and analyzed by plaque assay on vero cells.

FIGS. 2A-F - Inhibition of multiple strains of SARS-CoV by E64d. Vero E6 cells were infected with SARS strains Urbani (FIG. 2A), Tor 2 (FIG. 2B) or Tor 7 (FIG. 2C) at an MOI of ~1 TCID₅₀ per cell. Cells were either mock treated (white bars) or pretreated with E64d 400mg/ml with a single dose 1 h prior to infection (black bars). Supernatant virus titers were measured by plaque assay at 24, 48 and 72 h p.i. Monolayers of Vero cells were infected with SARs strains Urbani (FIG. 2D), Tor 3 (FIG. 2E) or Tor 7 (FIG. 2F) as in FIGS. 2A-C, in the presence or absence of E64d. Images of cell monolayers were obtained at 48 h p.i.

DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

I. The Present Invention

Cysteine proteinase (CyP) inhibitors, as a class of drugs, have been well known for some time. The present inventor published, almost a decade ago, on the ability of a particular CyP inhibitor, E64d (an analog of E64), to block cleavage of a murine coronavirus (mouse hepatitis virus) polyprotein, and to inhibit virus replication (Kim *et al.*, 1995). The inhibition of replication was associated with a rapid shutoff of new viral RNA synthesis. CyP inhibitors have also been tested against reovirus, tobacco etch virus, sweet potato feathery mottle virus, HIV-1, herpesvirus, and European lake trout virus.

In the present invention, the inventor proposes the use of CyP inhibitors such as E64d to prevent and treat SARS. In testing this hypothesis, *in vitro* studies now have shown that E64d is able to reduce virus titers in culture by several logs, thereby confirming the efficacy of such drugs against the SARS virus. Thus, the use of CyP inhibitors provides a new approach to treatment of the emerging health crisis of SARS.

II. Severe Acute Respiratory Syndrome (SARS) Virus

Nidoviruses are positive-stranded RNA viruses infect a wide range of vertebrates. The virions are enveloped, pleomorphic, spherical, or kidney-shaped. Surface projections of envelope distinct; club-shaped; dispersed evenly over all the surface. Two families are established: Family *Arteriviridae* and Family *Coronaviridae*. The SARS virus is a member of the latter family, and is subcategorized as a coronavirus.

Virions are enveloped, slightly pleomorphic, spherical or kidney shaped, and about 120-160 nm in diameter. Surface projections of envelope are distinct, club-shaped, spaced widely apart and dispersed evenly over all the surface. Nucleocapsids are rod-shaped (straight or bent), about 9-13 nm in diameter. Virions associated RNA nucleocapsids exhibit helical or tubular symmetry.

Molecular mass (Mr) of the virion 400×10^6 . Buoyant density is 1.23-1.24 g cm⁻³ in CsCl, and 1.15-1.19 g cm⁻³ in sucrose. The sedimentation coefficient is 300-500S. Under *in vitro* conditions, virions are stable in acid environment (pH 3), relatively stable in presence of Mg⁺⁺. Virions are sensitive to heat, lipid solvents, non-ionic detergents, formaldehyde, and oxidizing agents.

Virions contain one molecule of linear positive-sense single stranded RNA with a total genome length is 20,000-33,000 nt. The 5' end of the genome has a cap, and the 3' end has a

poly(A) tract. Subgenomic mRNA is found in infected cells. The SARS genomic sequence has been deposited into GenBank (accession numbers AY274119.3 and AY278741) (Rota *et al.*, 2003; Marra *et al.*, 2003).

At the 5' end of the genome, a putative 5' leader sequence with similarity to the conserved coronavirus core leader sequence, 5'-CUAAAC-3' was observed. Putative TRS sequences were identified and scored as strong, weak or absent based on inspection of the alignments. The 3' UTR sequence contained a 32 base-pair region corresponding to the conserved s2m motif, believed to be a universal feature of astroviruses.

Open reading frames were predicted by comparing sequences of known coronavirus proteins, resulting in putative identification of replicases 1a and 1b, S protein, E protein, M protein and N protein. Based on further comparison to known proteins of the three known coronaviral groups reveals that SARS proteins do not readily cluster more closely with any one group. As such, it has been proposed that SARS is the first representative of "Group 4" coronaviruses. The 29,751-base genome also encodes nine novel ORFs.

III. Cysteine Proteinase Inhibitors

The present invention makes use of cysteine proteinase (CyP) inhibitors. A variety of these inhibitors are known. One example, *L-trans*-Epoxy succinyl-leucylamido(4-guanido)butane, or E64, was reported to be a cysteine proteinase inhibitor (Barrett *et al.*, 1982; Mehdi, 1991). At 10 μ m, E64 rapidly inactivated cathepsins B, H and L as well as papain, yet had no effect on serine proteinases at concentrations 50-fold higher. The analog variant Ep-475 (*L-trans*-Epoxy succinyl-leucylamido(3-methyl)butane) was more effective than E64 at inhibiting cathepsins B and L, but Dc 11 was about 100-fold less reactive. Variants currently in use include E64c and E64d.

Grinde (1982) also reported the isolation of E64 as a thiol proteinase inhibitor of fungal origin, along with two synthetic analogs, Ep-459 and the above-noted Ep-475. All three inhibitors were found to act selectively on lysosomal protein degradation. Ep-475 and E64 were found to inhibit as much as 50% of total degradation (about 70% of lysosomal degradation) at concentrations which did not disturb protein synthesis.

A variety of other cysteine proteinase inhibitors are known and some are described in U.S. Patents 6,331,542; 6,297,277; 6,287,840; 6,284,777; 6,232,342; 6,180,402; 6,162,791; 6,147,188; 6,057,362; 6,034,066; 6,004,933; 5,998,470; 5,976,858; 5,925,772; 5,776,718; 5,766,609; 5,714,484; 5,663,380; 5,618,966; 5,486,623; 5,317,086; and 4,891,356. Each of the foregoing patents is hereby incorporated by reference in their entirety.

Other types of cysteine protease inhibitors include siRNA's, ribozymes and antisense molecules directed at cysteine protease sequences, proposed picornavirus 3CLpro and 2a proteinase inhibitors, Rhinovirus proteinase inhibitors, and drugs designed around 3CLpro crystal structure.

IV. Formulations and Administration

The present invention provides for the preparation and use of CyP inhibitor formulations. Such compositions will generally be dissolved or dispersed in a pharmaceutically acceptable carrier or aqueous medium. The phrases "pharmaceutically acceptable" or "pharmacologically acceptable" refer to molecular entities and compositions that do not produce an adverse, allergic, or other untoward reaction when administered to an animal, or human, as appropriate. As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. The drugs of the present invention can be formulated for parenteral administration, *e.g.*, formulated for injection *via* the intravenous, intramuscular, subcutaneous, or even intraperitoneal routes. Typically, such compositions can be prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for use to prepare solutions or suspensions upon the addition of a liquid prior to injection can also be prepared; and, the preparations can also be emulsified. The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions; formulations including sesame oil, peanut oil, or aqueous propylene glycol; and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases the form must be sterile and must be fluid to the extent that it may be easily injected. It also should be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi.

The carrier also can be a solvent or dispersion medium containing, for example, water, ethanol, dimethyl sulfoxide (DMSO), polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion, and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or

sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum-drying and freeze-drying techniques. In certain cases, the therapeutic formulations of the invention also may be prepared in forms suitable for oral or intranasal administration.

An effective amount of the drug is determined based on the intended goal. The term "unit dose" or "dosage" refers to physically discrete units suitable for use in a subject, each unit containing a predetermined-quantity of the composition calculated to produce the desired responses, discussed above, in association with its administration, *i.e.*, the appropriate route and treatment regimen. Precise amounts of the pharmaceutical composition also depend on the judgment of the practitioner and are peculiar to each individual. Generally, it is proposed that doses between about 1 and 1000 mg/kg may be utilized, including 5 mg/kg, 10 mg/kg, 25 mg/kg, 50 mg/kg, 100 mg/kg, 250 mg/kg, 500 mg/kg and 750 mg/kg, optionally with a maximal daily dose of about 500 mg/kg to 5000 mg/kg, such as 2500 mg/kg. Factors affecting dose include physical and clinical state of the patient, the route of administration, the intended goal of treatment (alleviation of symptoms *versus* cure) and the potency, stability, and toxicity of the particular substance.

V. Combined Therapy with Additional Agents

In addition to inactive agents, therapies may involve the use of a second agent. Thus, the formulations may comprise, or may be given in conjunction with, a second antiviral agent. One example is an interferon. There are three main types of interferons – α , β and γ .

At least 23 different variants of IFN- α are known (GenBank accession number M54886). The individual proteins have molecular masses between 19-26 kDa and consist of proteins with lengths of 156-166 and 172 amino acids. All IFN- α subtypes possess a common conserved sequence region between amino acid positions 115-151 while the amino-terminal ends are variable. Many IFN- α subtypes differ in their sequences at only one or two positions. Naturally-occurring variants also include proteins truncated by 10 amino acids at the carboxy-terminal end. Disulfide bonds are formed between cysteines at positions 1/98 and 29/138. The disulfide bond 29/138 is essential for biological activity while the 1/98 bond can be reduces

without affecting biological activity. All IFN- α forms contain a potential glycosylation site but most subtypes are not glycosylated.

β -interferon (IFN- β) is low molecular weight protein that is produced by many cell types, including epithelial cells, fibroblasts and macrophages. Cells that express endogenous IFN- β are resistant to viral infection and replication. The β -interferon genes from mouse (GenBank accession numbers X14455, X14029) and human (GenBank accession numbers J00218, K00616 and M11029) have been isolated and sequenced. IFN- β is a multifunctional glycoprotein that can inhibit tumor growth both directly, by suppressing cell replication and inducing differentiation or apoptosis and indirectly by activating tumoricidal properties of macrophages and NK cells, by suppressing tumor angiogenesis and by stimulating specific immune response.

IFN- γ (GenBank accession number X13274 and AF375790) has antiviral-, antioncotic- and immunoregulatory-activities. It is produced by immunocompetent cells stimulated with antigens and/or mitogens. Because of these biological activities, IFN- γ is expected to be used as both an antiviral and antitumor agent, and energetically studied on clinical trials as a therapeutic agent for malignant tumors in general including brain tumors. IFN- γ preparations now commercially available are roughly classified into 2 groups, *i.e.*, natural IFN- γ 's, produced by immunocompetent cells, and recombinant IFN- γ 's, produced by transformed cells.

Other compositions suitable for use in combination with a cysteine protease inhibitor include interfering RNAs, antisense molecules and ribozymes that target other SARS products, spike protein binding mimetics, nucleoside analogs, inhibitors of viral uncoating, polymerase inhibitors and inhibitors of viral release.

The combined agents may be given at the same time, even in the same formulation. Alternatively, the two interferon may precede or follow the CyP inhibitor treatment by intervals ranging from minutes to weeks, but including within about 6-12 h or 12-24 h of each other. In some situations, it may be desirable to extend the time period for treatment significantly, however, where several days (2, 3, 4, 5, 6 or 7) to several weeks (2, 3, 4, 5, 6, 7 or 8) lapse between the respective administrations.

Various repeated combinations may be employed, CyP inhibitor therapy is "A," and the interferon is "B":

A/B/A	B/A/B	B/B/A	A/A/B	A/B/B	B/A/A	A/B/B/B	B/A/B/B
B/B/B/A	B/B/A/B	A/A/B/B	A/B/A/B	A/B/B/A	B/B/A/A		
B/A/B/A	B/A/A/B	A/A/A/B	B/A/A/A	A/B/A/A	A/A/B/A		

VI. Examples

The following example is included to further illustrate various aspects of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques and/or compositions discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

Example 1

The severe acute respiratory syndrome (SARS) pandemic caused by a previously unidentified human coronavirus, SARS-CoV, has driven the search for effective treatments of SARS-CoV infected individuals. As of May 14, 2003, there have been 587 deaths among 7,628 probable cases of SARS. Despite an intensive effort, to date no effective antiviral treatments available against SARS have been described. Patients with SARS have been treated symptomatically according to the severity of the illness, and a treatment protocol consisting of antibiotics and a combination of ribavirin and methylprednisone was recently proposed. However, the therapeutic value of ribavirin remains unclear, as it has no activity against SARS-CoV *in vitro*. Here we show that a cysteine proteinase inhibitor previously demonstrated to block replication of the coronavirus, mouse hepatitis virus strain A59 (MHV-A59) (Kim *et al.*, 1995), effectively inhibits replication of SARS-CoV in Vero E-6 cells.

Examination of the genome sequences of SARS-CoV isolates reveals an organization that is typical of coronaviruses (Marra *et al.*, 2003; Rota *et al.*, 2003). The SARS-CoV replicase gene is predicted to encode two polyproteins in overlapping open reading frames, ORF1a and ORF1b. Coronavirus ORF1a and ORF1ab polyproteins of characterized coronaviruses are co-translationally processed by two or three virus-encoded cysteine proteinases (Marra *et al.*, 2003; Rota *et al.*, 2003; Ziebuhr *et al.*, 2000; Lu *et al.*, 1995). The SARS-CoV has been predicted to encode one papain-like cysteine proteinase and one 3C-like proteinase (3CLpro or Mpro), also containing a catalytic cysteine. The SARS-CoV 3CLpro has been demonstrated to have proteolytic activity against peptide substrates containing a known coronavirus replicase polyprotein cleavage site (Anand *et al.*, 2002).

Proteolytic processing of the replicase polyprotein is required throughout the coronavirus life cycle for viral RNA synthesis and generation of infectious virus progeny (Kim *et al.*, 1995). For the model coronavirus mouse hepatitis virus, strain A59 (MHV-A59), the cysteine proteinase

inhibitor, E64-d ((2S, 3S)-*trans*-epoxysuccinyl-L-leucylamido-3-methylbutane Ethyl Ester) (Hanada *et al.*, 1978a; 1978b; Barrett *et al.*, 1981), has been shown to block processing of the replicase polyproteins and to inhibit or abort viral RNA synthesis and virus production in cultured cells when added at any time during infection (Kim *et al.*, 1995). Based on these data, the inventor hypothesized that E64-d would also interfere with processing of the SARS-CoV polyproteins and as a result should inhibit replication of SARS-CoV.

The *in vitro* efficacy of E64-d was evaluated against three different isolates of SARS-CoV (Tor2, Tor7, and Urbani) using yield reduction assays. Vero E-6 cells infected with the SARS-CoV (m.o.i. 0.1 pfu/cell) were treated at 1 h p.i. with E64-d (400 g/ml, 1.2 mM) and monitored for cytopathic effect (CPE) and production of infectious SARS-CoV at 24, 48, and 72 hours postinfection. In non-treated, SARS-CoV-infected Vero E-6 cells at 48 h p.i., extensive cell rounding and death was noted. In contrast, E64-d inhibited degenerative cyopathic effects at all time points with all three SARS-CoV isolates (FIGS. 1A-B). This lack of viral CPE was paralleled by a dramatic inhibition of new infectious SARS-CoV (all three isolates) for at least 72 h, with greater than 5-log, 4-log, and 3-log reductions in supernatant virus at 24 h, 48 h, and 72 h, respectively, following a single addition of E64-d at 1 h p.i. (FIG. 1C). No cellular toxicity or death was associated with E64-d treatment. The duration of inhibition in virus growth and virus CPE was significantly greater than that described for MHV at the same E64-d concentration. A variety of SARS strains were used to infect Vero cell monolayers, including Urbani (FIG. 2A), Tor 2 (FIG. 2B) or Tor 7 (FIG. 2C) (MOI of ~1 TCID₅₀ per cell), and supernatant virus titers were measured by plaque assay at 24, 48 and 72 h p.i. The PFU/ml produced was substantially reduced in the presence of E64d for all three strains at 24 and 48 h p.i., and treated/untreated infected monolayers showed similar relative CPE at 48 h p.i. (FIGS. 2D-F).

The current data, in combination with the studies of other coronaviruses, indicate that E64-d may inhibit SARS virus replication at any time during infection, a critical feature for possible treatment of ongoing severe disease and for diminution or elimination of transmission. Importantly, E64-d has been shown to be safe in mice at the concentrations used in this study when administered daily for one month (Komatsu *et al.*, 1986). In addition, the prolonged inhibition of virus replication following a single dose of E64-d suggests the possibility of sustained effect on virus replication, another valuable feature of a potential antiviral agent. Finally, the demonstrated ability of E64-d in the MHV model to inhibit multiple proteinases suggests that it might be less susceptible to virus escape by mutations in individual proteinases.

Faced with the continuing epidemic caused by the SARS-CoV in a completely non-immune human population, the rapid identification, development and implementation of antiviral strategies is of the highest priority. While targeted studies of specific proteinases are essential for development of selective, high activity inhibitors of SARS-CoV proteinases, the results reported here suggest that efforts should also focus on the testing in culture and in animals of E64-d, other E64 derivatives, and other broadly active cysteine proteinase inhibitors.

* * * * *

All of the compositions and methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the compositions and methods, and in the steps or in the sequence of steps of the methods described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the scope of the invention as defined by the appended claims.

V. References

The following references, to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are specifically incorporated herein by reference:

U.S. Patent 6,331,542

U.S. Patent 6,297,277

U.S. Patent 6,287,840

U.S. Patent 6,284,777

U.S. Patent 6,232,342

U.S. Patent 6,180,402

U.S. Patent 6,162,791

U.S. Patent 6,147,188

U.S. Patent 6,057,362

U.S. Patent 6,034,066

U.S. Patent 6,004,933

U.S. Patent 5,998,470

U.S. Patent 5,976,858

U.S. Patent 5,925,772

U.S. Patent 5,776,718

U.S. Patent 5,766,609

U.S. Patent 5,714,484

U.S. Patent 5,663,380

U.S. Patent 5,618,966

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U.S. Patent 5,317,086

U.S. Patent 4,891,356

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CLAIMS

1. A method of inhibiting replication of severe acute respiratory syndrome (SARS) virus comprising contacting a cell infected with said SARS virus with a first cysteine proteinase inhibitor.
2. The method of claim 1, wherein said first cysteine proteinase inhibitor is selected from the group consisting of L-trans-epoxysuccinyl-leucylamido(4-guanidino)butane (E64), E64d, and E64c.
3. The method of claim 1, further comprising contacting said cell with a second cysteine proteinase inhibitor.
4. The method of claim 3, wherein said second cysteine proteinase inhibitor is contacted with said cell at the same time as said first cysteine proteinase inhibitor.
5. The method of claim 3, wherein said second cysteine proteinase inhibitor is contacted with said cell at a different time from said first cysteine proteinase inhibitor.
6. A method of treating a subject infected with a severe acute respiratory syndrome (SARS) virus comprising administering to said subject a first cysteine proteinase inhibitor.
7. The method of claim 6, wherein said first cysteine proteinase inhibitor is selected from the group consisting of L-trans-epoxysuccinyl-leucylamido(4-guanidino)butane (E64), E64d, and E64c.
8. The method of claim 6, further comprising administering to said subject a second cysteine proteinase inhibitor.
9. The method of claim 8, wherein said second cysteine proteinase inhibitor is administered at the same time as said first cysteine proteinase inhibitor.
10. The method of claim 8, wherein said second cysteine proteinase inhibitor is administered at a different time from said first cysteine proteinase inhibitor.
11. The method of claim 6, wherein said first cysteine proteinase inhibitor is administered more than once.

12. The method of claim 11, wherein said first cysteine proteinase inhibitor is administered daily for two weeks.
13. The method of claim 11, wherein said first cysteine proteinase inhibitor is administered daily for four weeks.
14. The method of claim 11, wherein said first cysteine proteinase inhibitor is administered daily for eight weeks.
15. The method of claim 6, wherein said first cysteine proteinase inhibitor is administered at about 1 to about 1000 mg/kg.
16. The method of claim 6, wherein said first cysteine proteinase inhibitor is administered at daily dose of no more than about 2500 mg/kg.
17. The method of claim 6, further comprising administering to said subject a non-proteinase antiviral composition.
18. The method of claim 6, further comprising assessing viral load after administration of said first cysteine proteinase inhibitor.
19. The method of claim 18, further comprising adjusting the dosage of said first cysteine proteinase inhibitor.
20. The method of claim 6, wherein said first cysteine proteinase inhibitor is administered orally, intravenously, intramuscularly, by inhalation or transdermally.
21. A method of reducing virus load in a subject infected with a severe acute respiratory syndrome (SARS) virus comprising administering to said subject a first cysteine proteinase inhibitor.
22. A method of inhibiting virus replication in a subject infected with a severe acute respiratory syndrome (SARS) virus comprising administering to said subject a first cysteine proteinase inhibitor.
23. A method of limiting virus infection in a subject infected with a severe acute respiratory syndrome (SARS) virus comprising administering to said subject a first cysteine proteinase inhibitor.

24. A method of inhibiting the spread of severe acute respiratory syndrome (SARS) in a population comprising administering to members of said population a first cysteine proteinase inhibitor.
25. A method of preventing severe acute respiratory syndrome (SARS) in a subject comprising (a) identifying a subject at risk of exposure to SARS virus and (b) administering to said subject a first cysteine proteinase inhibitor.

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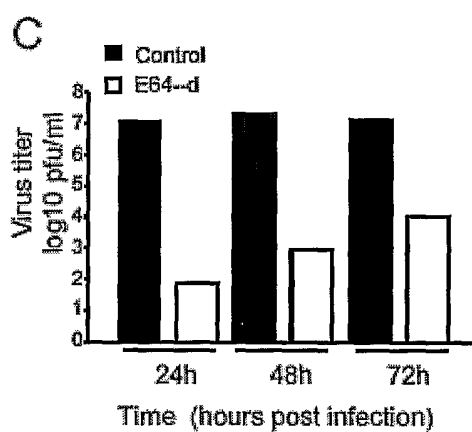
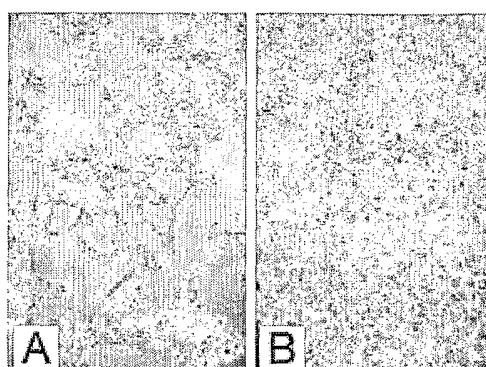


FIG. 1A-C

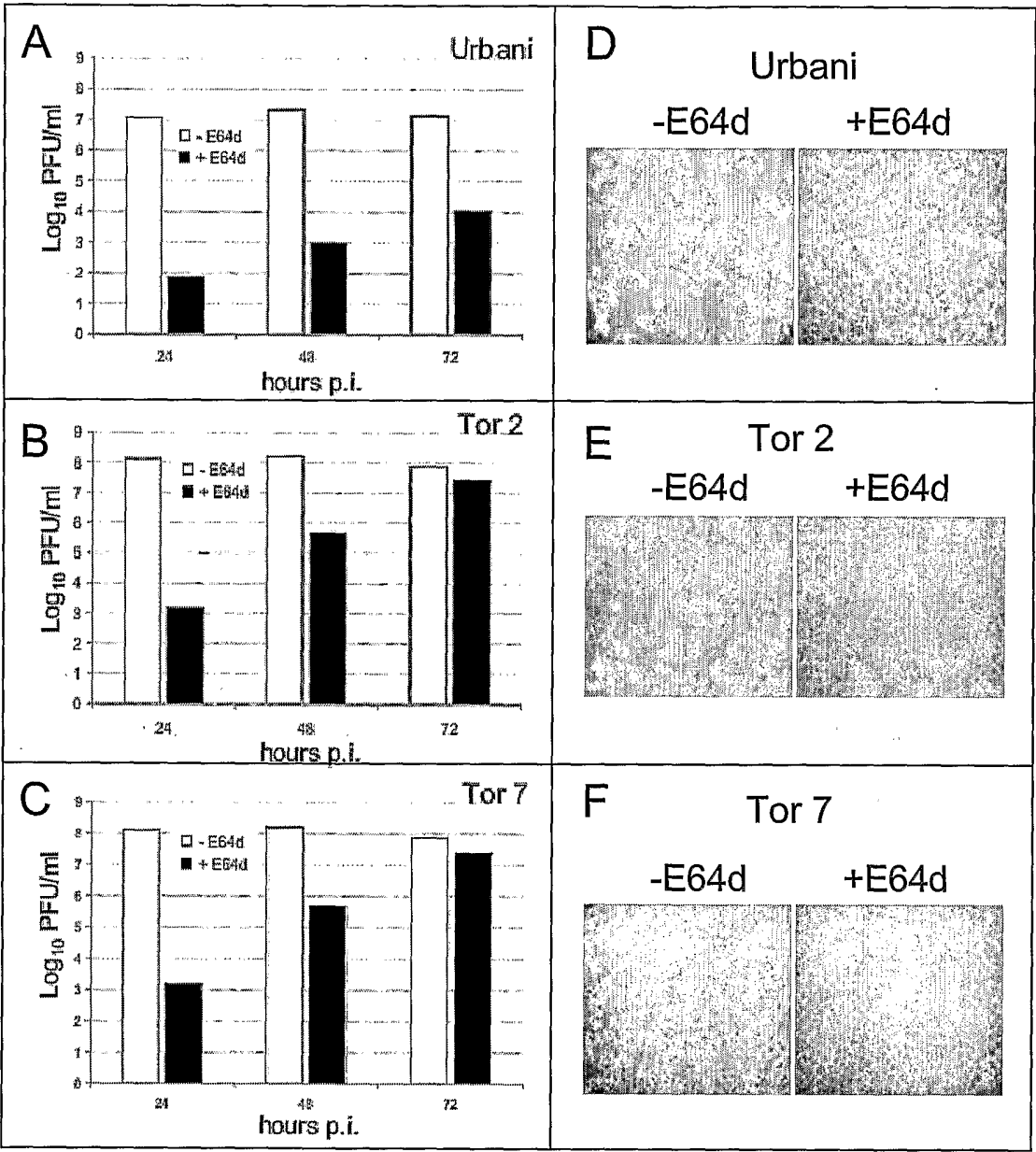


FIG. 2A-F