# Review

# New antiviral drugs, vaccines and classic public health interventions against SARS coronavirus

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**Severe acute respiratory syndrome (SARS) is caused by one of two recently discovered coronaviruses. The virus is emergent from South East (SE) Asian mammals: either the civet cat, a related species or a rat species. The virus has a long incubation period and low reproduction number ($R_0$ value) and hence the first outbreak in 2004 was controlled by hygiene and quarantine. However, the healthcare system was compromised and the economic cost was extremely high. Fortunately, the virus is easily cultivated in Vero E6 cells and therefore the search for new antivirals and vaccines was initiated within weeks of the discovery of the virus using classic techniques of cell culture and electron microscopy. Molecular diagnostics facilitated rapid and accurate diagnosis, a key factor in containing the outbreak. The broad-spectrum molecule ribavirin was used in SE Asia in infected patients alongside corticosteroids. In retrospect, many patients survived due to careful nursing. The only currently accepted intervention is interferon. Coronavirus replicon systems should facilitate rapid screening of new inhibitors and the complex mechanism of viral replication will ensure that drugs are developed against at least five molecular targets, in particular the viral protease.**

**Keywords: SARS, coronavirus, antivirals, vaccines, replicon**

## Introduction

In the opening years of the 21st century, the world community has already experienced two new clinical syndromes caused by coronaviruses (Drosten *et al.*, 2003; Peiris *et al.*, 2003a; Ksiazek *et al.*, 2003; Lee *et al.*, 2003; van der Hoek *et al.*, 2004; Zhong *et al.*, 2003), three recurring and serious outbreaks of H5 (Claas *et al.*, 1998), H9N2 (Peiris *et al.*, 1999) and H7 chicken influenza A, which has spread to humans in South East (SE) Asia and Europe, an outbreak of monkey pox in the USA (Enserink, 2003), aerosolized anthrax in the USA and the prion protein in cows in the USA and Canada and a metapneumovirus (van den Hoogen *et al.*, 2001). Therefore, we can confidently predict more emergent viruses will cross the species barrier as the human population exceeds 6 billion and expands into newly deforested areas of the world, interacts with previously undisturbed viruses, changes agricultural methods and travels the world to the extent of 100 million journeys a day.

The focus on bioterrorism could beneficially be extended to much wider and more important public health problems. The recent events in SE Asia, in particular, the continuing spread of severe acute respiratory syndrome (SARS) outbreak (Parry, 2002) and chicken influenza A (H5NI), have shown how precise virological surveillance combined with the rapid use of molecular genetics (Marra *et al.*, 2003) can detect newly emergent viruses and identify them quickly (reviewed by Webby & Webster, 2003; Oxford *et al.*, 2003). This has led to confidence at the World Health Organization (WHO) and a realization that intense and speedy intervention could even prevent a world outbreak of newly emerged viruses. In fact, WHO has powers, possibly unique in the world today, to ensure governmental compliance to public health measures by restricting travel (Heymann, 2004). The threat of travel restrictions can affect an economy, even one as robust as that of Hong Kong. Also, the intense virological surveillance system established in Hong Kong by WHO in the last years of the 20th century is already repaying the investment by the discovery not of influenza (the raison d'être), but of an entirely new and unexpected emergent virus, namely SARS-associated coronavirus (SARS-CoV), which causes severe acute respiratory syndrome.

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It has been estimated that a serious epidemic of SARS in Hong Kong could have caused billions of dollars loss in GDP for China. The outbreak has led to estimates that China's economy growth would be 1% lower than expected if the Hong Kong economy was pushed back into recession. Tourism in Hong Kong was severely affected. The SARS epidemic might also have implications for the future plans to outsource in China, with companies wary that SARS and future diseases or conditions may affect corporate health.

Fortunately, there are clear signs that SARS is a virus with a low infectious nature. However, once a person is infected, the virus replication can trigger devastating disease. It is a focal virus at present, and the most at-risk population are workers in the healthcare sector worldwide and those in the animal and market trade in SE Asia. Public health measures have successfully blocked a wider epidemic, but at-risk members of society need to be protected. Quite rightly, the virus has become the focus of endeavours to find new vaccines and antivirals.

## SARS: the clinical disease

The relatively high occurence of community-acquired pneumonia in every country of the world is often under appreciated. SARS is only one of many pathogens that can invade the lower respiratory tract, most others being bacteria. Throughout the world, patients with pneumonia are first treated for 10 days with antibiotics such as cephalosporins and a macrolide or quinoline, and only when there is no or only partial resolution of fever is the possibility of a viral infection brought within the diagnostic spectrum. Obviously, the diagnosis can now be more refined, especially if there is a history of contact with a SARS case.

Most SARS patients have a deteriorating clinical course with dyspnoea, unproductive cough, oxygen dependence, unrelapsing fever and radiographic evidence of consolidation in the lung (Lee *et al.*, 2003; Booth *et al.,* 2003; Peiris *et al.,* 2003b). As with other virus-induced pneumonias, secondary infection with bacteria is a major complication of SARS and such organisms include *Streptococcus pneumonia*, methicillin-resistant *Staphylococcus aureus* (MRSA) and *Klebsiella pneumonia*. The usual factors influence the eventual clinical outcome such as the age of the patient, comorbidity and the use of any invasive mechanical ventilation. In essence, frail or otherwise compromised patients experience higher mortality.

The main route of entry of the virus is via the respiratory tract and droplet infection. SARS virus can also be transmitted via fomites and it can survive on physical objects – transmission may occur if individuals touch these objects. Lau *et al.* (2004) found that disinfecting objects reduced the risk of transmission by 70% and hand-washing 11 times a day reduced the chance of transmission by 55%. Faecal–oral transmission remains possible.

## Epidemiology of SARS

Initially, the virus mainly infected healthcare workers (Peiris & Guan, 2004). Such a trait suggested that prolonged contact with the virus on a medical ward was a risk factor for the virus and that the virus was not highly infectious in the wider community. Li *et al.* (2003) conducted an epidemiological investigation into hospital transmission and found that eight nurses had been infected from a single patient with a latent period of 12.6 days, whilst the 57 people in contact with these staff showed no signs of SARS. It was concluded that SARS patients have limited infective capability and that there would have to be prolonged exposure to contract SARS.

In a separate study, Lau *et al.* (2004) identified four groups who had caught SARS in Hong Kong: secondary and tertiary transmission cases in households, hospital workers, inhabitants of Amoy Gardens, Kowloon Bay, Hong Kong and cross-infected patients. The largest group was the hospital workers (26.6% of the sample). Their associated risk factors, as well as visiting mainland China [odds ratio (OR)=1.95], were visiting the Prince of Wales Hospital, Hong Kong (OR=7.07), visiting Amoy Gardens (OR=7.36) and visiting other hospitals and clinics (OR=3.7).

An analysis of the spread of SARS in Hong Kong showed that the epidemic had a period of exponential growth followed by a time of stability leading to decay thereafter (Donnelly *et al.*, 2003; Riley *et al.*, 2003*)*. Most patients had symptoms within 14 days of infection (range 6.3–16.7 days). Both the infection to onset time (incubation period) and the onset to hospital admission intervals are important factors in containing spread. The latter is crucial because along with isolation comes a reduction in the effective infectiousness and thereby a reduction of the risk of transmission to a new individual. Of course, the public health objective is to reduce the generation of secondary cases from each primary case to less than one ($R_0$) and thereby stop the outbreak. It appears that the duration of the infectious period is prolonged (10–14 days), peaks at the time of symptoms and thereafter declines for 7 days or more. Earlier studies showed that the estimated case fatality could reach 13.2%, or up to 43.3% in individuals over 60 years old. More recent estimates were significantly lower and it is apparent that subclinical infections outnumber those with the classic symptoms (Woo *et al.,* 2004). Thus, in Hong Kong at least 1728 patients from a population of 7 million developed SARS-CoV pneumonia (0.025%) whereas non-pneumonic SARS infection was almost 20 times higher at 0.48%.


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In comparison, a truly global respiratory virus like influenza, having arisen in this geographical region in the past, spread rather quickly to infect millions of individuals worldwide in 1957 and 1968 (reviewed by Oxford, 2000). Given the remarkable extent of air travel today, even compared with 1968, the SARS virus is not spreading rapidly, at least to date. Although 30 other countries have reported cases, they are predominantly from individuals who have visited SE Asia. Outside the SE Asia epicentre, there have been few secondary cases in individuals in Europe who had been in contact with an index case. The apparent exception, Toronto in Ontario, Canada, may be explained by the close-knit nature of the Chinese ethnic group infected in Canada itself.

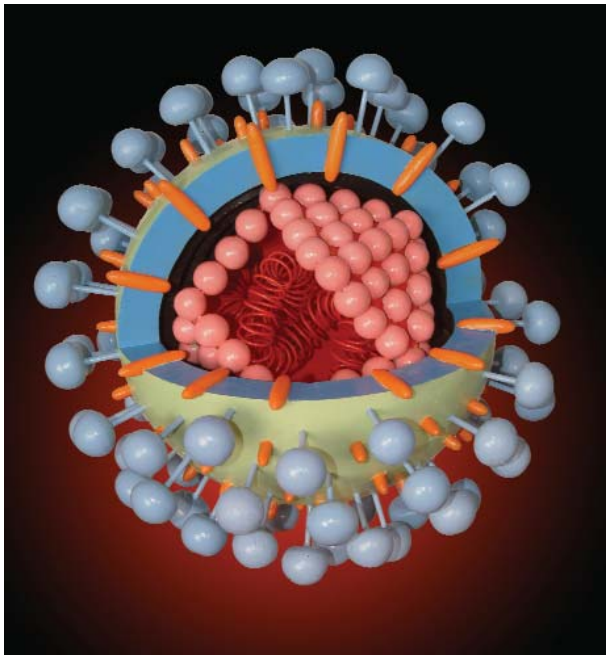
## The positive impact of public health intervention

In terms of mathematical analysis of viral epidemics, the rate of spread of a virus like SARS is directly related to the reproduction number ($R_0$), which is the calculated number of cases generated by one primary case in a susceptible population (Riley *et al.*, 2003; Anderson *et al.,* 2004). Fortunately for SARS, this figure is quite low at around 2.7 compared with >6 for influenza or measles. Key factors of the virology of SARS are the low $R_0$, the lengthy incubation time (7–10 days) and the relative lack of excretion of the virus before the appearance of symptoms. This allows the outbreak to be contained by classic public health measures such as restriction of movement, distancing, rapid hospitalization and quarantine, and tracing of contacts. This has been aided by the rapid development of molecular reverse transcription polymerase chain reaction (RT PCR) diagnostic tests.

## Phylogenetic analysis of the virus

It has been recognized for four decades that there are unknown respiratory viruses, since known viruses only account for 70% of clinical cases. Coronaviruses themselves were first identified, unexpectedly, as a cause of the common cold in volunteers at the Common Cold Unit in Salisbury, UK (Tyrrell & Bynoe, 1965; Bradburne *et al.*, 1967) and in the USA (Hamre & Procnow, 1966). They were identified by electron microscopy as spherical but with a strikingly detached corona of knobbed spikes (Almeida & Tyrrell, 1967), giving an appearance of a globe with a separate halo of small knobs (Figure 1). Over the next four decades, little attention was paid to this human virus family, although it was recognized that the total range of pathogenicity within the wider coronavirus family was very wide, encompassing gastroenteritis in pigs, bronchitis in chickens and liver disease in mice (Ziebuhr & Siddell, 2002; Ziebuhr *et al.*, 2000). There were some early warnings of rare cases of viral pneumonia in humans caused by the two identified human coronaviruses, but only in army camps and other semi-closed communities. Most adults in the world have antiviral antibodies and presumably immunity to the two classic virus serotypes and there is little evidence of antigenic drift or genetic changes over the years.

**Figure 1.** Archetype model of SARS coronavirus



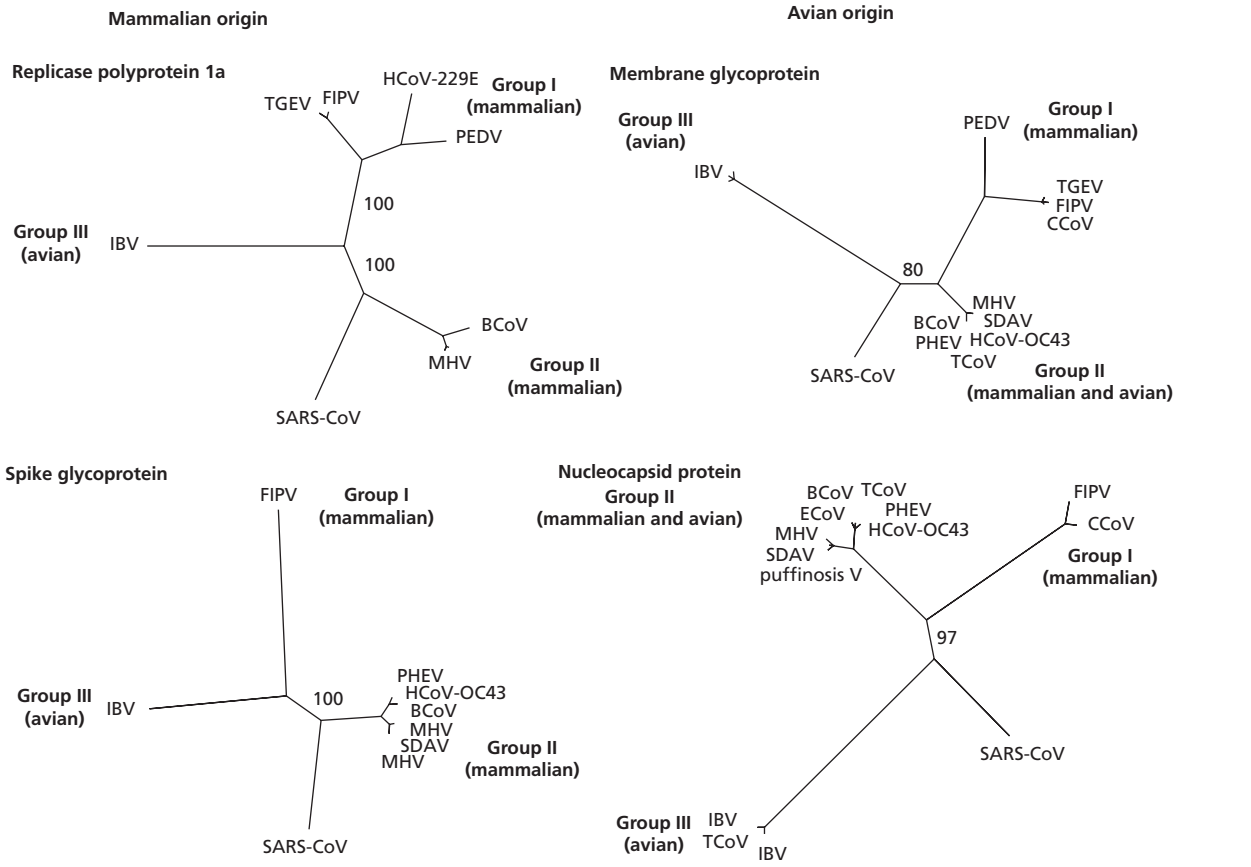
The SARS virus internal structure is illustrated as an icosahedron. The external spike (S) protein covers the surface of the virus. The m protein protudes through the lipid bilayer. Internally the large genome is in the form of ssRNA. See front cover.

The new SARS virus is allocated into a new grouping of its own (Figure 2). The other three groupings are the human coronavirus 229E, feline infectious peritonitis virus (Guan *et al.*, 2004; Yeh *et al.,* 2004; He 2004) and porcine epidemic diarrhoea (group I), bovine coronavirus, mouse hepatitis virus and human coronavirus OC43 (group II) and avian infectious bronchitis (group III). Serologically, human antibodies to the two human viruses 229E and OC43 do not cross-react with the new virus. Nevertheless, certain genes such as the polymerase gene may be a recombinant (Rest & Mindell, 2003).

## *In vitro* and *in vivo* model systems for the new human Urbani SARS coronavirus

The classical techniques of virus cultivation in cell culture, along with electron microscopy, successfully identified the

**Figure 2.** Phylogenetic relationships of SARS-CoV within the family of coronaviruses




ECoV, Equine coronavirus; BCoV, bovine coronavirus; CCoV, canine coronavirus; FIPV, feline infectious peritonitis virus; HCoV-229E, human coronavirus 229E; HCoV-OC43, human coronavirus OC43; IBV, avian infectious bronchitis virus; MHV, mouse hepatitis virus; PEDV, porcine epidemic diarrhoea virus; PHEV, pigeon herpes encephalomyelitis virus, SDAV, sialodacryoadenitis virus (rat coronavirus), TCoV, turkey coronavirus; TGEV, transmissible gastroenteritis virus.

new coronavirus associated with the current SARS outbreak.

Compared with previously studied human coronaviruses, SARS could be easily cultivated in Vero cells in the laboratory and the virus produces clear cytopathic changes and grows to a high infectious titre.

The SARS virus has been isolated from masked palm civets (*Paguma larvata*) and a raccoon dog (*Nyctereutes procyonoides*) in markets in Guandong Province, China whilst anti-SARS antibodies are found in Chinese ferret badgers (*Melogale moschata*) (Guan *et al.*, 2003; Cyranoski & Abbott, 2003; Ng, 2003). The potential market for palm civets used as food is extensive in SE Asian countries including Vietnam, Cambodia and Laos (Bell *et al.*, 2004). This suggests that following any simple cull of this species in wildlife markets in China and Hong Kong, the numbers would be quickly replenished. It is also possible that the civet is not the end host of SARS but that the civet has, in turn, caught the virus from rats upon which it preys or another more closely related mammal. Obviously, these large wild animals would not be suitable for laboratory investigations.

Fortunately the virus, at least in the laboratory, can also infect domestic cats, ferrets (Martina *et al.*, 2003) and primates (Fouchier *et al.*, 2003). Indeed, the latter study was used to provide the formal proof, the so-called Koch's postulate, that SARS-CoV was the aetiological agent of the disease. Three days post-infection with SARS-CoV, the infected macaques become lethargic. Virus was excreted in the nose and throat between days 2 and 6 post-infection. At necroscopy on day 6, multifocal pulmonary consolidation was noted and virus was recovered from the lung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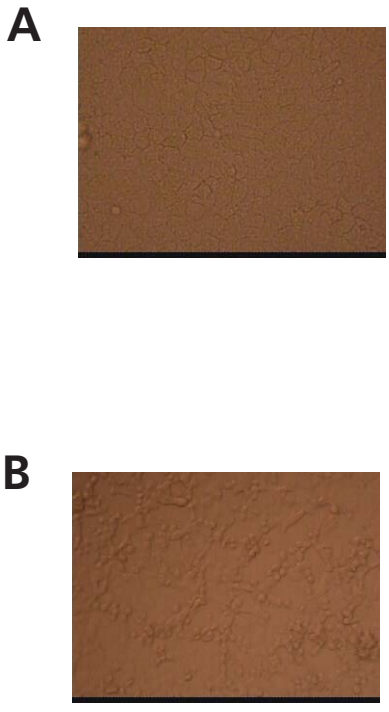
tissue. Diffuse alveolar damage was described with necrosis of the alveoli and bronchiolar epithelia. Syncytia were present in the lumen of both alveolar and bronchioles, similar to those described in SARS patients.

The virus also replicates in laboratory mice (Yang *et al.*, 2004) whereby intranasal infection leads to viral replication in the lungs and turbinates within 1–2 days. There are no clinical signs in unadapted viruses.

## *In vitro* inhibitors of SARS virus

Not unexpectedly, protease inhibitors developed for other viruses had no effects on the treatment of SARS (Chu *et al.,* 2004). The nucleoside analogue ribavirin was the first antiviral to be shown to have SARS inhibitory effect *in vitro*. The drug may be an RNA virus mutagen and could exert this type of activity against a large RNA genome virus such as SARS (Crotty *et al.*, 2000, 2001). The Vero E6 cell system is a comparatively easy *in vitro* cell culture method with which to screen potential anti-SARS drugs. The virus replicates well in these cells and produces clear cytopathic changes and syncytia 2–3 days post-infection (Figure 3). Tan *et al.* (2004) have screened representative antiviral drugs with known clinical activity against a wide range of viruses including HIV, influenza and herpes (Table 1). Both A and B interferons (Cinati *et al.*, 2003a) inhibited replication of SARS-CoV, whilst ribavirin had only a marginal inhibitory effect. As expected, given the known and strictly family-specific antiviral activity of most antiviral drugs synthesized to date, most antiviral drugs had no inhibitory effect against SARS-CoV although they are highly effective against other virus families.

**Figure 3.** Classical cytopathic changes induced by SARS virus replicating in Vero E6 cells




A, control Vero E6 cells; B, Vero E6 cells showing virus-induced cytopathic effect and syncytia 48 h post infection with 1000 $TCID_{50}$/ml of virus.

Cinati *et al.* (2003b) described the *in vitro* effects of glycyrrhizin from extracts of liquorice roots on clinical isolates of coronavirus from patients with SARS. He *et al.* (2003) have described the effects of RNA interference on replication of SARS virus. Earlier work had clearly established that α interferons could block colds caused by more weakened members of the coronavirus family (Turner *et al.*, 1986).

Much of the initial screening around the world to date has been on an empirical basis. In an example of this approach, which has served the scientific community well in the past, Barnard *et al.* (2004) screened a class of cystein proteinase inhibitors and also a group of nucleoside analogues using an *in vitro* cytopathic endpoint, identifying two calpain inhibitors and a nucleoside analogue β-D-$N^4$-hydroxycytidine as potential lead compounds.

Haagmans *et al.* (2004) have confirmed the prophylactic effects of pegylated interferon using the macaque model of infection. The study established that SARS-CoV infects type I pneumocytes in experimentally infected cynomolgus macaques and that prophylactic use of interferon reduced viral replication and excretion and also pulmonary damage. There was some indication of efficacy of post-exposure (1 day post-infection) application of the interferon. The interval between infection and symptoms in humans is much larger than in experimentally infected macaques and therefore the interferon would be expected to have even more significant effects. At present, pegylated interferon would be the antiviral of choice for treating healthcare workers, laboratory staff or family members exposed to a case of SARS. Given the recent laboratory-acquired infections in China, Singapore and Taiwan, laboratories worldwide would be recommended to hold a supply of interferon for immediate use following any virus spillage.

## Identification of molecular targets of SARS-CoV for new inhibitors

The typical genome layout of Coronaviridae has been known for several decades but the impetus to select antiviral targets and spend 400 million Euros developing new antiviral drugs for this group of viruses, which

**Table 1.** A screen of available antiviral agents against SARS virus

| Antiviral agents | Highest concentration tested | Inhibition of cytopathic effect ($CIA_{100}$) |
|---|---|---|
| Interferons | | |
| Interferon α– 2a (Roferon) | 100 000 IU/ml | No |
| Interferon α– 2b (Intron A) | 500 000 IU/ml | No |
| Interferon α– nl (Wellferon) | 500 000 IU/ml | Yes |
| Interferon α – n3 (Alferon) | 10 000 IU/ml | Yes |
| Interferon β– 1a (Rebif) | 500 000 IU/ml | No |
| Interferon β– 1b (Betaferon) | 100 000 IU/ml | Yes |
| Nucleoside analogues | | |
| Acyclovir | 1000 μg/ml | No |
| Ganciclovir (Cymevene) | 50 000 μg/ml | No |
| Ribavirin | 10 000 μg/ml | Yes |
| Protease inhibitors | | |
| Indinavir (Crixivan) | 100 μmol/l | No |
| Nelfinavir (Viracept) | 10 000 μmol/l | No |
| Saquinavir (Fortovase) | 10 000 μmol/l | No |
| Reverse transcriptase inhibitors | | |
| Lamivudine (Epivir) | 1000 μmol/l | No |
| Zidovudine (Retrovir) | 1000 μg/ml | No |
| Neuraminidase inhibitors | | |
| Oseltamivir (Tamiflu) | 10 000 μmol/l | No |
| Zanamivir (Relenza) | 1000 μmol/l | No |
| Other | | |
| Amantadine (Symmetrel) | 1000 μg/ml | No |
| Foscarnet (Foscavir) | 8000 μmol/l | No |

From Tan *et al.* (2004).

commonly caused only mild disease in the past, has been absent. However, given the vulnerability of healthcare and laboratory workers to the SARS virus, this selective view has now changed. Obvious molecular targets would be the virus RNA polymerase (Xu *et al.*, 2003), replicase (Campanacci *et al.*, 2003), cystein proteinases (Yang *et al.*, 2003; Chou *et al.*, 2003; Anand *et al.,* 2003), CD13 viral receptor (Kontoyiannis *et al.*, 2003), the mRNA cap-1 methyl transferase (von Grotthus *et al.*, 2003) and NTPase/helicase (Tanner *et al.*, 2003).

Essentially, coronaviruses translate the large replicase gene from the infectious genomic viral RNA. Virus-coded proteases, both papain-like and 3c-like, cleave the viral polyproteins into 16 end products. The RNA replication and transcription complex is composed of non-structural proteins and mediates the replication of genome RNA as well as the many viral sub-genome RNAs. Since the functional details of most coronavirus replicase gene products is not known, random screening of potential antiviral compound libraries will remain a key stage of drug discovery. The science of autonomously replicating RNAs (replicon RNAs) has been developed for hepatitis C virus (Bartenschlager, 2002) and coronavirus (Hertzig *et al.*, 2004). Stable cell lines containing non-cytopathic selectable replicon RNAs without structural genes can be used safely for drug screening. This replicon system can be used to screen for inhibitors of RNA polymerase, MTPase/helicase, poly(U) specific endonuclease, exoN, S-adenosyl methionine-dependent ribose 2-O methyl transferase, adenosine diphosphase-ribose 1′′-phosphatase and cyclic phosphordiesterase (Hertzig *et al.*, 2004). Obviously, the replicon system will not detect inhibitors of other viral replication stages such as adsorption, penetration or release.

## Immunotherapy and prophylaxis with antibodies and vaccines

High titres of neutralizing antibody to SARS-CoV are present in convalescent sera from previously infected patients, and SARS patients improve clinically following administration of passive antibody. This indicates both that a SARS vaccine could be developed and, more immediately, that human monoclonal antibodies could be used as immediate therapy (Pearson *et al.*, 2003, Li *et al.*, 2003; Sui *et al.*, 2004).

The virion surface projection, the S spike, is a major antigenic determinant of all members of the coronavirus family. Li *et al.* (2003) have shown that the SI domain of the protein contains the viral receptor binding site and interacts with angiotensin-converting enzyme 2 (ACE2) at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the point of cellular entry. Sui *et al.* (2004) characterized a neutralizing human monoclonal antibody that blocks binding of the SARS spike SI protein to the ACE2 receptor molecule. The antibody has a nanomolar affinity that neutralizes infectious virus and virus-induced syncytia in cell cultures. This potent virus neutralizing antibody was selected from a non-immune human antibody library.

Yang *et al.* (2004) described a DNA vaccine which induced SARS neutralizing antibody and which reduced lung titres of virus in infected mice. Plasmids were produced encoding the S protein, one with the native leader sequence retained and also two S carboxy-terminal mutants, one with a truncated cytoplasmic domain and the other with deleted transmembrane and cytoplasmic domains. Both expression vectors induced significant numbers of CD4 T cells and CD8 cellular immunity and neutralizing antibody in mice. Immunized mice were challenged intranasally with infectious SARS virus. An approximate reduction of lung virus titre of 6 $\log_{10}$ $TCID_{50}$ was observed 2 days post-infection in vaccinated compared with control mice. This is a very significant reduction in virus titre. Thus, although theoretical concerns may persist about possible induction of enhancing antibodies, as noted with flaviviruses such as dengue and also certain members of the coronavirus family, these data represent the first step in developing a SARS vaccine.

## Conclusions

When the SARS virus emerged, the first human cases were treated empirically with corticosteroids in an attempt to reduce a virus-induced immunopathology, combined with the broad-spectrum antiviral ribavirin (So *et al.*, 2003, Tsang & Zhang, 2003). However, SARS cases in the USA treated without ribavirin did have a high survival rate. It is acknowledged that ribavirin may exert its antiviral effect via host cell functions but nevertheless, as with hepatitis C, the combination of interferon and ribavirin is worthy of future study.

However, from an immediate clinical viewpoint the most useful drug at present is pegylated interferon and this can be utilized in hospital- and laboratory-acquired infections.

Cellular systems using Vero E6 cells are in place in several laboratories worldwide although it is quite clear that following laboratory-acquired infections in China, Singapore and Taiwan only the highest containment at level 3 can be assured to be safe. The molecular genetics of the SARS-CoV group are known and there are crystallographic data from the viral protease. It can be predicted that inhibitors will be quickly discovered against the virus RNA replicases and protease enzymes, particularly using coronavirus replicon systems for primary screens against key replication enzymes.

Meanwhile, the first experimental DNA vaccine has been shown to have very significant antiviral effects in the mouse model whilst passive human monoclonal antibodies have also been produced.

The investment in the sciences of molecular biology and virology are returning significant public health benefits to the community but the discovery of another human coronavirus (van der Hoek *et al.*, 2004) warns that more human respiratory viruses are yet to be discovered and that the design of antivirals is likely to preoccupy virology and chemistry laboratories throughout the 21st century, as it has done in the last 50 years. SARS virus has not disappeared (Parry 2004). Realistically, the application of viral evolutionary genetics (Holmes & Rambout, 2004) may also help an understanding of the details of viral emergences and be used for screening for new RNA virus pathogens, using degenerate PCR primers in animal populations, even before the viruses cross the species barrier.

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