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Is the anti-psychotic, 10-(3-(dimethylamino)propyl)phenothiazine (promazine), a potential drug with which to treat SARS infections? Lack of efficacy of promazine on SARS-CoV replication in a mouse model

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Abstract

Phenothiazine and derivatives were tested for inhibition of SARS-CoV replication. Phenothiazine slightly inhibited SARS-CoV replication in a neutral red (NR) uptake assay. Adding a propylamino group to give promazine reduced virus yields (VYR assay) with an $EC_{90} = 8.3 \pm 2.8$ μM, but without selectivity. Various substitutions in the basic phenothiazine structure did not promote efficacy. Phenazine ethosulfate was the most potent compound by VYR assay ($EC_{90} = 6.1 \pm 4.3$ μM). All compounds were toxic ($IC_{50} = 6.6$–74.5 μM) except for phenoxathiin ($IC_{50} = 858 \pm 208$ μM) and 10-(alpha-diethylamino-propionyl) phenothiazine·HCl ($IC_{50} = 195 \pm 71.2$ μM). Consequently, none were selective inhibitors of SARS-CoV replication (SI values <1–3.3 μM). These data portended the poor efficacy of promazine in a SARS-CoV mouse lung replication model. Intraperitoneal treatment with promazine using a prophylactic (−4 h)/therapeutic regimen of 1, 10, or 50 mg/(kg day) did not reduce virus lung titers at day 3, yet prolonged virus replication to 14 days. Similar therapeutic promazine doses were not efficacious. Thus, promazine did not affect SARS-CoV replication in vitro or in vivo, nor were any other phenothiazines efficacious in reducing virus replication. Therefore, treating SARS infections with compounds like promazine is not warranted.

Keywords: Promazine; Anti-psychotic; SARS-CoV; Coronavirus; Mouse model; Phenothiazines

1. Introduction

Severe acute respiratory syndrome (SARS) emerged in 2002 in the Guandong province of southern China as a new infectious respiratory disease characterized by influenza-like symptoms and signs, but with a very high mortality rate. The initial outbreak of SARS infection rapidly spread through the human population due to international travel, reaching nearly 30 countries by the middle of 2003 (De Clercq, 2006); this episode possibly being a portent of epidemics of the future. The overall mortality rate was 10%, and up to 50% within the elderly population (De Clercq, 2006). The epidemic resulted in about 8000 probable cases of SARS with 800 deaths attributed to the virus infection. There were also four confirmed cases of SARS in Ghangzou, China in late 2003 and early 2004 (Liang et al., 2004; Song et al., 2005). Two subsequent outbreaks were contained rapidly and were due to the escape of the virus from laboratories doing SARS-CoV research (Lim et al., 2006).

Because SARS appeared to be life-threatening and highly contagious, the resources of the science community were quickly marshaled to gain a better understanding of the disease and the etiological agent to facilitate the development of rational therapies for prophylaxis and treatment (Weiss and Navas-Martin, 2005; De Clercq, 2006). Thus, the putative agent was quickly identified as a corona-like virus (Peiris et al.,

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2003) and was soon shown to be the etiological agent for SARS (Fouchier et al., 2003).

Early in the SARS outbreak there was a concerted effort by many laboratories to evaluate clinically approved drugs for efficacy against SARS-CoV to rapidly provide a treatment for SARS infections in humans. For example, several laboratories have evaluated phenothiazine and promazine (clinically approved antipsychotic drugs) and many derivatives of those two classes of compounds for inhibition of SARS-CoV replication (Zhang and Yap, 2004; Barnard et al., 2005; Hsieh et al., 2005). The positive data have prompted recommendations that these types of drugs be considered as therapies for SARS infections or as lead compounds for development of more potent derivatives. Data are now presented that confirm and extend the in vitro anti-SARS-CoV efficacy previously reported, but showing in an in vivo model that clinical use is contraindicated.

2. Materials and methods

2.1. Cells and virus

African green monkey kidney cells (Vero 76) were obtained from the American Type Culture Collection (ATCC; Manassas, VA). The cells were grown in minimal essential medium (MEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Hyclone Laboratories; Logan UT). For antiviral assays, the serum was reduced to 2% and 50 μg/ml gentamicin added to the medium.

SARSCoV, strain Urbani (200300592), was obtained from the Centers for Disease Control (Atlanta, GA), the Frankfurt strain was kindly provided by Jindrich Cinatl (Klinikum der J.W. Goethe Universität, Frankfurt Am Main, Germany), the Toronto-2 strain was supplied by Heinz Feldman (National Microbiology Laboratory, Winnipeg, Manitoba, Canada) and the CHUK-W1 strain was received from Paul KS Chan (Chinese University of Hong Kong, China). All strains were passaged in Vero 76 cells.

All experiments involving infectious SARS-CoV were carried out in BSL-3+ laboratories. All personnel wore complete body protective coverings and HEPA-filtered powered air purifying respirators.

2.2. Compounds

Promazine, phenoxathiin, 10-(1-ethyl-3-propyl-3-pyrrolidinyl-methyl)-phenothiazine·HCL, 10-(1-ethyl-3-pyrrolidinymethyl)-phenothiazine·HCL, 10-(1-methyl-2-pyrrolidinymethyl)-phenothiazine·HCL, chlorprothixene, 2-chloro-10-(3-chloropropanol)-10H-phenothiazine, phenoxazine, phenothiazine, perphenazine, propionylpromazine·HCL, ethopropazine, methotrimeprazine and acetopromazine were obtained from Sigma-Aldrich, Co. (St. Louis, MO); 10-(1-ethyl-3-pyrroli-dinymethyl)-phenothiazine·HCL and trifluopromazine·HCL were from MP Biomedicals (Irvine, CA); trifluoperazine dimaleate was obtained from Calbiochem (La Jolla, CA), and acetophenazine maleate was received from U.S.P.C., Inc. (Rockland, MD). After solubilization in physiological saline (PSS) or dimethylsulphoxide (DMSO) for chlorprothixene, phenoxazine, phenothiazine, all were diluted in PSS to the working concentrations. The reference drug for the in vitro efficacy tests was calpain inhibitor IV (Calbiochem). Mouse interferon-alpha (muIFN-α), the reference drug for the animal studies, was provided by Kurt Berg (Panum Inst., IMMI, The IFN-Lab, Copenhagen, Denmark).

2.3. Neutral red (NR) uptake assay for determination of antiviral efficacy and compound cytotoxicity

Compounds were tested at varying concentrations (four $\log_{10}$ or eight 1/2 $\log_{10}$ dilutions). Virus and compound were added in equal volumes to near-confluent cell monolayers in 96-well tissue culture plates. The multiplicity of infection ranged from 0.001 to 0.004 in order to produce viral cytopathic effects (CPE) for each strain of virus in 100% of the cells in the virus control wells within 3–4 days. The plates were incubated at 37 °C in a 5% CO_2 atmosphere until the cells in the virus control wells showed complete viral CPE as observed by light microscopy. Each concentration of drug was assayed for virus inhibition in triplicate and for cytotoxicity in duplicate. Six wells per plate were set aside as uninfected, untreated cell controls and six wells per plate received virus only and represented controls for virus replication. Calpain inhibitor IV was included as positive control drugs for each set of compounds tested.

After examining the virus-infected controls, by light microscopy for viral CPE, CPE and compound cytotoxicity were quantitated by NR assay. The NR assay was performed using a method of Cavenaugh et al. (1990) modified by Barnard et al. (2004b). Briefly, medium was removed from each well of a plate, 0.034% NR was added to the test medium in each well of the plate, and the plate incubated for 2 h at 37 °C in the dark. The solution was removed from the wells, rinsed and any remaining dye extracted using ethanol buffered with Sörenson's citrate buffer. Absorbances at 540/405 nm were read with a microplate reader (Opsys MR™, Dynex Technologies, Chantilly, VA). Absorbance values were expressed as percents of untreated controls and EC_{50}, IC_{50} and SI values were calculated as described previously (Barnard et al., 1997a).

2.4. Virus yield reduction assay

Some compounds were evaluated in a more sensitive assay to confirm the results of the CPE inhibition/NR uptake assays. Infectious virus yields from each well from a second CPE inhibition assay were determined as previously described (Barnard et al., 2004a). After CPE was determined, each plate was frozen at −80 °C and thawed. Sample wells at each concentration tested were pooled and titered in Vero 76 cells for infectious virus by CPE assay.

A 90% reduction in virus yield (EC90) was then calculated by linear regression analysis. This represented a one $\log_{10}$ inhibition in titer when compared to untreated virus controls.

2.5. Virucidal assay

For compounds showing good antiviral inhibitory activity, a virucidal test was done to exclude the possibility that the

compounds inhibited the virus by physically inactivating or disrupting the virion. The method of Barnard et al. (1997b) was used. Virus and compound were incubated at room temperature for 1 h. Surviving virus was quantified by CPE assay and titers were calculated as described previously by Barnard et al. (1997b). Concentrations of compound tested bracketed the concentration determined to represent the EC_{50} from previous assays; each concentration of test compound was assayed in duplicate.

2.6. Animal studies

2.6.1. Animals

Specific pathogen-free BALB/c female mice (11–16 g, range varied with each experiment) were obtained from Charles River Laboratories (Wilmington, MA). They were quarantined for 1 week prior to use. Mice were fed standard mouse chow and tap water *ad libitum*. Mouse studies approved by the Utah State University Animal Care and Use Committee were carried out in an approved biosafety level 3 facility. Personnel entering the facility wore powered air-purifying respirators (3M HEPA Air-Mate; 3M, Saint Paul, MN). For the infectious disease experiments, mice were housed in bonneted filter-topped cages placed within a HEPA-filtered horizontal laminar flow ventilated animal rack.

2.6.2. Lung virus titer determinations

Each mouse lung was homogenized and varying dilutions assayed for infectious virus in Vero 76 cells. Each lung homogenate was stored at −80 °C until each supernatant fluid was titered by CPE assay.

2.6.3. Experiment design

In one set of experiments, twenty mice per group were treated by intraperitoneal (i.p.) injection with the appropriate dosage of compound or placebo (saline) administered 4 h prior to infection of mice. After the 4 h pretreatment, the mice were sedated with an i.p. injection of 100 mg/kg of Ketamine® and were infected intranasally (i.n.) with 50 μl of clarified virus lysate (2×10^3 50% cell culture infectious doses, strain Urbani). Treatments continued 8, 32, and 56 h after virus exposure. Uninfected animals (three for each dose of compound or placebo) were treated with the same dosages of drug or with placebo to serve as toxicity controls. Fifteen animals from both the placebo-treated and drug-treated, infected groups were sacrificed on day 3 post-virus exposure; the lungs were removed and assayed for virus. The remaining mice were sacrificed on day 7 and the lungs were removed, weighed, and titered for virus. This experiment was repeated using only the 50 and 10 mg/kg doses with additional animals being sacrificed at day 14. Lung samples were taken at days 3, 7, and 14 from sacrificed animals for cytokine analysis and titration of virus in the lung homogenate.

In another set of experiments, promazine was evaluated for therapeutic efficacy. Prior to infection with virus, the mice were sedated with an i.p. injection of 100 mg/kg of Ketamine® and were infected intranasally (i.n.) with 50 μl of clarified virus lysate described above. Ten mice per group were treated i.p. with promazine at 1, 10, or 100 mg/kg or 15 animals were given placebo (saline) administered one time 8 h post-virus exposure or injected 8, 32, and 56 h after virus exposure (qd × 3). Uninfected animals (three for each dose of compound or placebo) were treated with the same dose of drug or with placebo to serve as toxicity controls. Ten animals from both the placebo-treated and seven animals from the drug-treated, infected groups were sacrificed on day 3 post-virus exposure; the lungs were removed and assayed for virus. The remaining mice were sacrificed on day 7 and the lungs were removed, weighed, and titered for virus.

2.6.4. Histopathology

Lung sections were fixed in 10% formalin and then shipped to the Utah State Veterinary Diagnostic Laboratory (Logan, UT) for processing (sectioning and hematoxylin and eosin staining) and descriptive analysis.

2.6.5. Cytokine analysis

Lung samples were taken at 3, 7, 14 days post-virus exposure and homogenized in MEM with 10% FBS. Samples were held frozen at −80 °C, then thawed and equilibrated to 50 mg/mL. Samples were then tested for IL-1α, IL-1ß, IL-2, IL-3, IL-4, IL-6, IL-9, IL-10, IL-12, MCP-1, TNF-α, MIP-1α, GM-CSF and RANTES using the Q-Plex™ mouse cytokine array screen (Quansys Bioscience, Logan, UT). This cytokine screen is a quantitative ELISA-based test with 14 distinct capture antibodies absorbed to each well of a 96-well plate in a defined array. Cytokines are detected and quantified by relative luminescence of each spot in the array. Values (pg/ml) were calculated using software developed by Quansys based on the standard curve run concurrent with the assay.

2.6.6. Statistical analysis

Differences in mean lung virus titers were evaluated by the analysis of variance. For cytokine level analysis, Wilcoxon pairwise comparisons between test groups were done using "jmp 6.0 Statistical Discovery™ (SAS, Cary, NC).

3. Results

3.1. In vitro antiviral activity of phenazines and phenothiazines

A number of phenazines and phenothiazines were tested for efficacy against SARS-CoV (Urbani strain) replication in Vero 76 cells (Table 1). EC_{50} values ranged from 763 μM (phenoxathiin) to 5.2 μM (phenazine ethosulfate) by NR assay. Most compounds exhibited considerable toxicity with the exception of phenoxathiin and 10-(alpha-diethylaminopropionyl)phenothiazine·HCL. The most potent inhibitor by virus yield reduction assay was phenazine ethosulfate ($EC_{90} = 6.1$ μM). Promazine, a compound reported to inhibit SARS-CoV (Zhang and Yap, 2004; Hsieh et al., 2005) also reduced virus yields with an $EC_{90} = 8.3$ μM. In addition, promazine also inhibited other strains of SARS-CoV at similar concentrations (Table 2) as detected by NR assay or by virus yield reduction assays. Most of the other compounds tested against the other SARS-CoV strains were also inhibitory

Table 1
In vitro SARS-CoV (strain Urbani) inhibitory activity of phenazines and phenothiazines

Compound	Neutral red uptake assay			Virus yield reduction assay		
	EC50 (μM)	IC50 (μM)	SI	EC90 (μM)	IC50 (μM)	SI
Promazine	10.8 ± 8.1	13.5 ± 6.2	1.2	8.3 ± 2.8	18.2 ± 14.6	2.1
10-(1-Ethyl-3-propyl-3-pyrrolidinylmethyl)-phenothiazine·HCl	10.8 ± 6.7	13.7 ± 1.3	1.3	10.3 ± 5.4	20.7 ± 13.7	2.0
Phenoxathiin	763 ± 475	858 ± 208	1.1	752 ± 497	968 ± 65	1.3
Chlorprothixene	9.5 ± 5.7	18.3 ± 3.4	1.9	10.4 ± 4.2	18.3 ± 3.4	1.8
10-(1-Ethyl-3-pyrrolidinymethyl)-phenothiazine·HCl	13.1 ± 8.8	29.5 ± 8.7	2.3	11.0 ± 3.9	29.5 ± 8.7	2.7
10-(Alpha-diethylaminopropionyl)-phenothiazine·HCl	93.5 ± 112.9	195 ± 71.2	2.1	203 ± 136	195 ± 71.2	<1
Acetopromazine	21.0 ± 11.0	37.8 ± 1.8	1.8	21.5 ± 14.6	28.8 ± 11.0	1.3
Methotrimeprazine	19.0 ± 8.1	26.0 ± 10.8	1.4	12.3 ± 1.7	21.0 ± 5.4	1.7
Trifluoperazine dimaleate	7.3 ± 4.7	9.5 ± 3.1	1.3	10.2 ± 4.4	8.0 ± 2.1	<1
Acetophenazine maleate	6.6 ± 3.3	13.2 ± 1.6	2.0	10.0 ± 5.0	13.2 ± 1.6	1.3
10-(1-Methyl-2-pyrrolidinymethyl)-phenothiazine·HCl	11.9 ± 5.6	39.9 ± 0.2	3.3	16.9 ± 9.7	39.9 ± 0.2	2.4
Propionylpromazine·HCl	24.3 ± 15.6	19.3 ± 2.2	<1	[a]13.7 ± 0.6	19.3 ± 2.2	1.4
Ethopropazine	30.5 ± 27.5	61.3 ± 34.7	2.0	[a]42.0 ± 1.0	61.3 ± 34.7	1.5
Trifluopromazine·HCl	13.3 ± 5.8	18.0 ± 5.6	1.4	12.8 ± 1.9	18.0 ± 5.6	1.4
Perphenazine	9.7 ± 1.2	15.8 ± 3.4	1.6	[a]8.4 ± 5.3	15.8 ± 3.4	1.9
2-Chloro-10-(3-chloropropanol)-10H-phenothiazine	23.0 ± 11.0	20.8 ± 8.1	<1	27.0 ± 16.8	20.8 ± 8.1	<1
Phenazine ethosulfate	5.2 ± 2.9	6.6 ± 4.7	1.3	6.1 ± 4.3	6.6 ± 4.7	1.1
Phenothiazine	[a]21.5 ± 8.1	56.5 ± 61.5	2.6	ND[b]	ND	–
Phenoxazine	[a]89.5 ± 14.8	74.5 ± 20.2	<1	ND	ND	–
Calpain Inhibitor IV	[a]2.6 ± 1.4	24.7 ± 7.0	9.4	[a]7.2 ± 0.9	33.0 ± 4.1	3.3

[a] For these compounds, averages were derived three separate experiments, instead from four experiments from which the averages of other compounds in the table were derived.
[b] Not done.

at similar concentrations using either assay with the exception of 10-(1-methyl-2-pyrrolidinymethyl)-phenothiazine-HCl. The antiviral activity shown in Table 1 was not due to virucidal activity, at least for promazine, since virus exposure to promazine for 1 h at 25 °C did not result in virus titer reduction (data not shown). The least toxic compound evaluated was phenoxathiin (IC_{50} = 858 μM). Propionylpromazine·HCL, 2-chloro-10-(3-chloropropanol)-10H-phenothiazine, and phenoxazine were essentially inactive since the concentrations at which cytotoxic effects were detected in cells were less than

Table 2
Inhibition of various strains of SARS-CoV by selected phenazines and phenothiazines

Compound	Virus strain	Neutral red uptake assay[a]			VYR assay[a]
		EC50 ± S.D. (μM)	IC50 ± S.D. (μM)	SI	EC90 ± S.D. (μM)
Acetophenazine maleate	Urbani	8.1 ± 1.4	14.3 ± 6.4	1.8	12.3 ± 2.1
	Frankfurt-1	12.7 ± 3.1	18.0 ± 0.0	1.4	10.0 ± 5.2
	CHUK-W1	9.6 ± 7.6	15.5 ± 3.1	1.6	11.2 ± 3.2
	Toronto-2	11.5 ± 3.7	13.7 ± 3.8	1.2	13.0 ± 0.0
Chlorprothixene	Urbani	12.0 ± 3.5	16.7 ± 1.5	1.4	10.5 ± 5.2
	Frankfurt-1	11.1 ± 3.4	13.0 ± 2.0	1.2	12.3 ± 0.6
	CHUK-W1	12.6 ± 6.5	18.5 ± 4.9	1.5	12.1 ± 4.2
	Toronto-2	12.9 ± 3.6	15.8 ± 1.5	1.2	13.0 ± 1.0
Promazine	Urbani	14.0 ± 6.1	11.2 ± 5.7	<1	7.8 ± 3.1
	Franfurt-1	21.0 ± 15.4	33.7 ± 20.6	1.6	11.6 ± 6.3
	CHUK-W1	15.6 ± 8.5	34.5 ± 20.4	2.2	16.5 ± 11.1
	Toronto-2	22.6 ± 12.8	34.0 ± 16.7	1.5	20.7 ± 9.5
10-(1-Methyl-2-pyrrolidinymethyl)-phenothiazine·HCl	Urbani	12.9 ± 6.4	39.7 ± 14.0	3.1	28.3 ± 12.9
	Frankfurt-1	24.3 ± 7.4	32.3 ± 22.7	1.3	23.7 ± 6.0
	CHUK-W1	19.0 ± 2.6	48.0 ± 10.5	2.5	31.3 ± 14.2
	Toronto-2	28.5 ± 8.1	42.3 ± 10.9	1.5	41.7 ± 4.0
Calpain inhibitor IV	Urbani	2.6 ± 0.5	24.7 ± 7.0	9.4	7.2 ± 0.9
	Frankfurt-1	4.6 ± 2.1	44.0 ± 11.5	9.5	6.5 ± 3.6
	CHUK-W1	2.6 ± 1.4	30.0 ± 4.1	12.6	6.5 ± 3.6
	Toronto-2	5.4 ± 1.4	45.0 ± 14.2	8.3	9.6 ± 3.1

[a] Averages were derived from three separate experiments.

Table 3
Effects of i.p. promazine treatment[a] on the replication of SARS-CoV (Urbani) in mice

Dosage/injection	Toxicity controls survivor/Total	Virus titer (Log_{10} $CCID_{50}$/g) ± S.D.[b]		Virus titer (Log_{10} $CCID_{50}$/g) ± S.D.
		Day 3	Day 7	Day 14
100 mg/kg	0/3[c]	–	–	–
50 mg/kg	3/3	5.6 ± 1.0	2.2 ± 3.1**	3.4 ± 0.1**
10 mg/kg	3/3	5.6 ± 0.4	3.6 ± 0.2**	3.4 ± 0.0**
1 mg/kg	3/3	5.7 ± 0.3	4.7 ± 0.9**	ND[d]
Placebo	3/3	5.7 ± 0.4	<0.75[e]	<0.75
muIFN-α 100,000 (IU)	3/3	4.7 ± 0.4*	<0.75	<0.75

*$p < 0.05$ compared to the placebo controls. **$p < 0.0001$ compared to the placebo control.
[a] Animals were treated at −4 h, then 8, 32, 56 h after virus exposure. Interferon was administered 12 prior to virus exposure.
[b] Represents the average of two experiments.
[c] All animals in both infected, treated and uninfected, treated groups died on day 3.
[d] Not done.
[e] Represents a titer of <0.75, the limits of detection for this assay.

the concentrations detected that inhibited virus replication. The protease inhibitor, calpain IV (positive drug control), inhibited virus replication as expected (Barnard et al., 2004a).

3.2. Structure activity relationships

Some structure-activity relationships could be established from the data (Fig. 1A and B). Phenothiazine moderately inhibited SARS-CoV replication in Vero cells by neutral red (NR) uptake assay (EC_{50} = 21.5 μM), but the addition of a propylamino group to phenothiazine to give promazine resulted in a twofold increase in inhibition of SARS-CoV replication by NR assay (EC_{50} = 10.8 μM, EC_{90} = 10 μM) (Fig. 1A). In addition, derivatives of phenothiazine with side chains that enhanced solubility or had side chains of electron withdrawing ring structures (trifluoperazine, acetophenazine, perphenazine) were also more inhibitory of SARS-CoV replication (EC_{90} = 8.4–10.2 μM), suggesting that these compounds may act as soluble prodrugs of phenothiazine (Fig. 1B). This effect seemed to be abrogated if methoxy, acetyl or propionyl groups were added to the two position of the basic ring structure to the Substituting N with O in the phenothiazine nucleus to get phenoxathiin resulted in a much less potent and much less toxic compound (phenoxathiin EC_{50} = 763 μM vs. EC_{50} phenothiazine = 21.5 μM). However, substituting the S group in phenothiazine with O resulted in a less potent compound, phenoxazine, which was also less toxic. The nitrogen in the phenothiazine basic ring structure may not be essential for antiviral activity since its removal from the ring, as in for example, chlorprothixene, resulted in inhibition of virus replication similar to that detected with promazine.

3.3. In vivo antiviral activity

Treatment of mice with promazine at 1, 10 or 50 mg/(kg day) (−4 h, then 8, 32, 56 h after virus exposure) resulted in no significant reduction of viral replication at day 3 (Table 3) in the lungs of mice. The duration of virus replication in the lungs was significantly prolonged by each drug treatment (−4 h, then 8, 32, 56 h after virus exposure); virus was detected in mice treated with 50 mg/kg and 10 mg/kg promazine at day 7 and at day 14. In contrast, in placebo-treated, infected mice, the infection in the lungs was cleared by day 7 as has previously been shown by others (Subbarao et al., 2004; Barnard et al., 2006). Despite the fact that virus persisted to day 14 in promazine-treated mice, histopathogical examination of lungs from treated, infected animals showed no signs of pathology consistent with a virus lung infection or consistent with drug-induced cytotoxicity. Treating mice with 100 mg/(kg day) promazine (−4 h, then 8, 32, 56 h after virus exposure) was lethal to all mice by day 3 (Table 3). For reference, the reported LD_{50} of promazine given to mice i.p. is 140 mg/(kg day) (Yen and Day, 1965). Mice were also treated therapeutically with promazine. Treatment of mice with promazine at 100 mg/(kg day) beginning 8 h after virus exposure was somewhat lethal as well; most animals died between day 3 and day 7 (Table 4). All doses of promazine used therapeutically did not inhibit virus replication in the lungs. Although the 10 mg/kg dose administered once a day either one time or three times beginning 8 h after virus exposure did seem to slightly

Table 4
Effects of therapeutic i.p. promazine treatment on the replication of SARS-CoV (Urbani) in mice

Dosage/injection	Toxicity controls survivor/total	Virus titer (Log_{10} $CCID_{50}$/g) ± S.D.	
		Day 3	Day 7
qd × 3, beginning 8 h after virus exposure			
100 mg/kg	0/3[a]	5.6 ± 0.0	–
10 mg/kg	3/3	5.8 ± 0.4	<0.75[b]
1 mg/kg	3/3	6.0 ± 0.6	<0.75
Placebo-1	3/3	5.5 ± 0.6	<0.75
qd × 1, beginning 8 h after virus exposure			
100	0/3[a]	5.6 ± 0.0	–
10	3/3	6.0 ± 0.6	<0.75
1	3/3	5.4 ± 0.1	<0.75
Placebo-2	3/3	5.3 ± 0.4	<0.75
muIFN-α 100,000 (IU)[c]	3/3	4.7 ± 0.4	<0.75

[a] All animals in both infected, treated and toxicity groups died between days 3 and 7.
[b] Represents a titer of <0.75, the limits of detection for this assay.
[c] Interferon was administered 12 h prior to virus exposure.

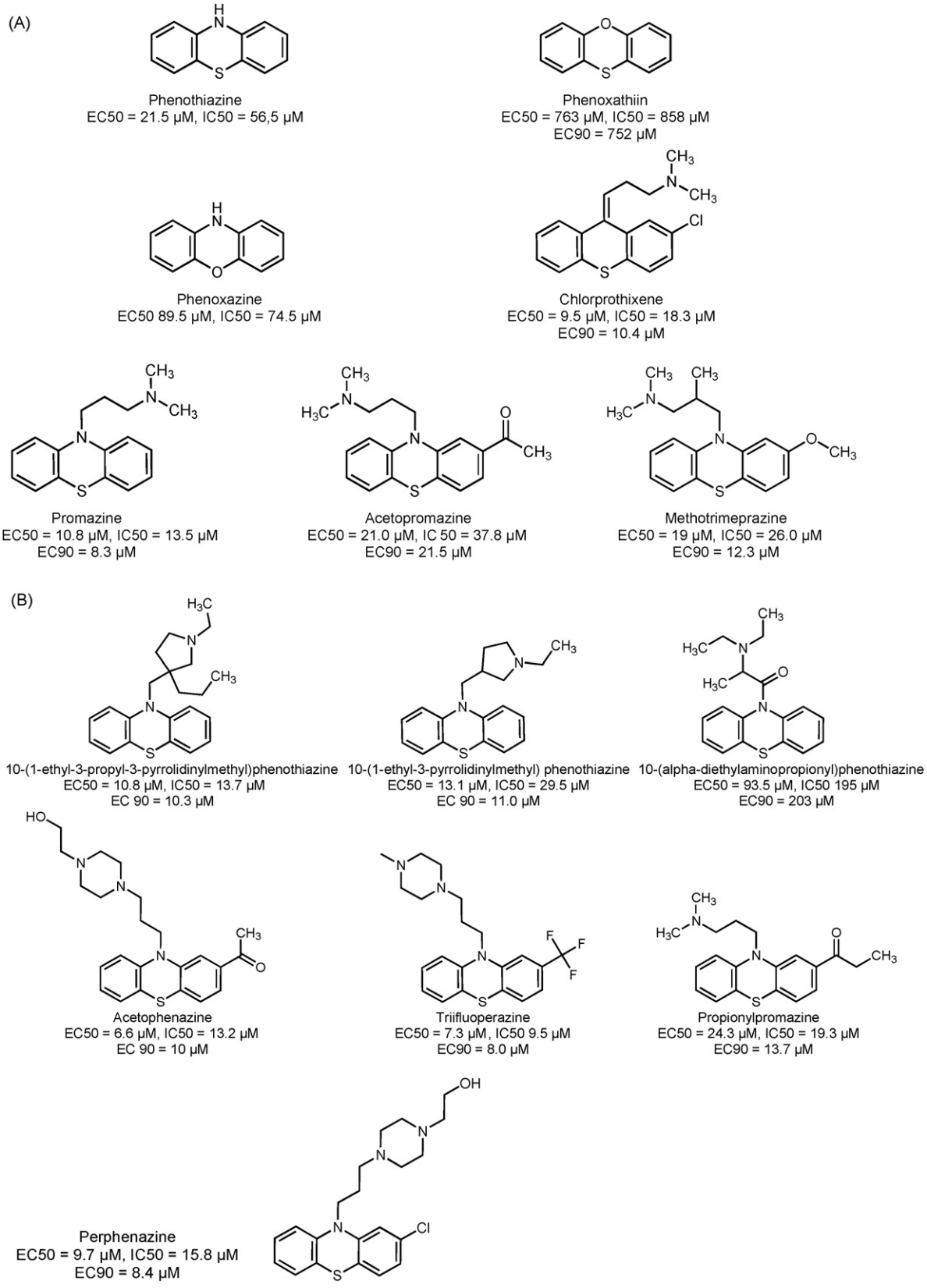

Fig. 1. Structure activity relationships associated with phenoxathiin, phenoxazine and selected phenothiazines: (A) comparison of structure and SARS-CoV inhibitory activity of phenoxathiin, phenoxazine and selected phenothiazines and (B) comparison of structures and anti-SARS-CoV inhibitory activities of phenothiazines with side chains enhancing solubility.

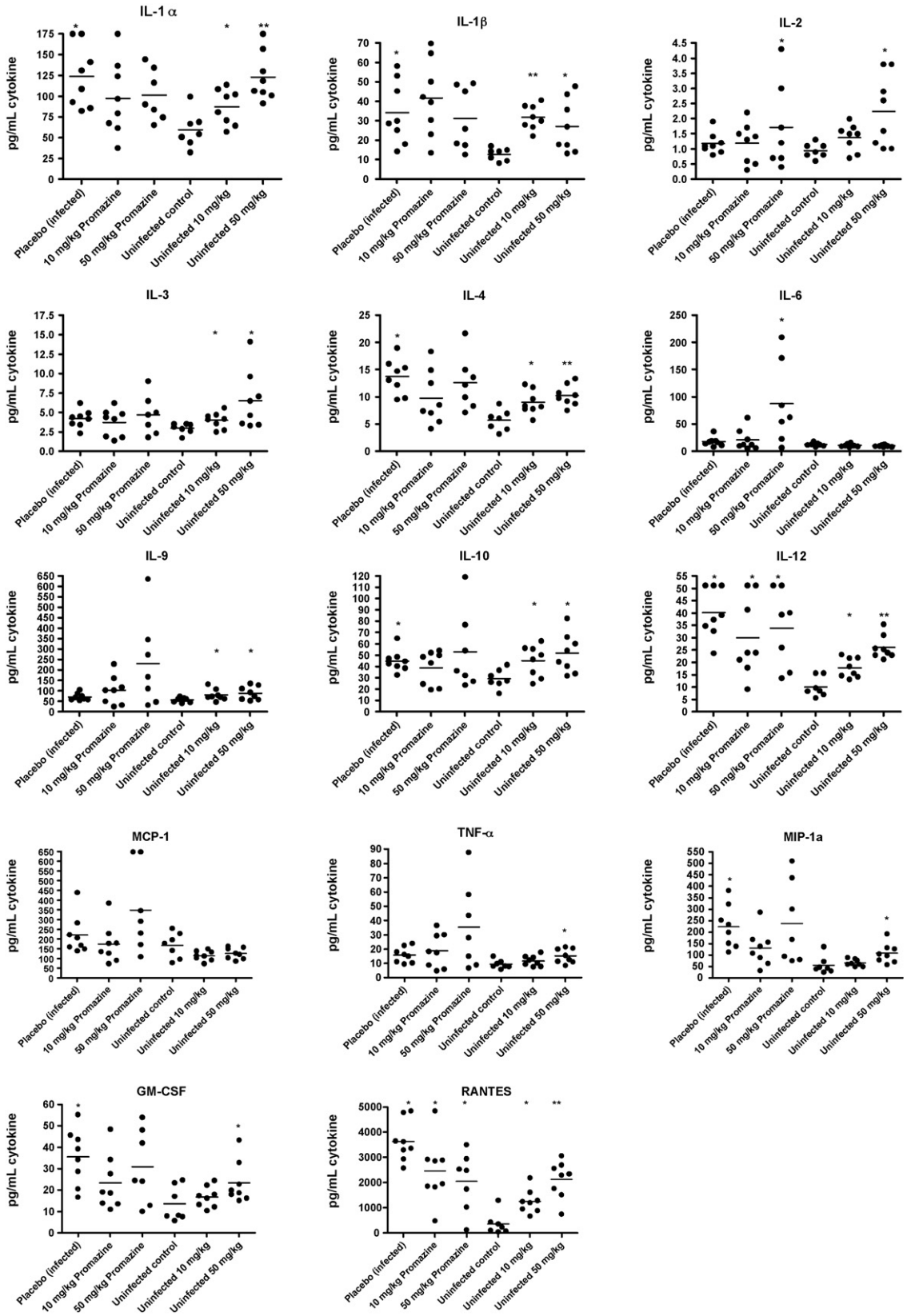

Fig. 2. Cytokine levels detected from the lungs of uninfected and infected mice with or without promazine treatment at day 3 post-virus exposure. Data represents cytokine levels from the lungs of individual mice with a bar indicating the average cytokine levels from each treatment group (*$P < 0.05$, **$P < 0.005$).

increase the amount of virus recovered from lungs at day 3 compared to placebo, neither the 10 or 1 mg/kg doses significantly prolonged virus infection as happened with the more frequent dosing schedule. The 100 mg/kg dose killed all the control animals as well as the infected, treated animals by day 7.

SARS-CoV infection (placebo-treated, uninfected mice) caused a significant increase ($p < 0.05$) in the production of cytokines IL1-α, IL1β, IL-4, IL-10, IL12, MIP-1a, GM-CSF and RANTES when compared to untreated, infected mice (Fig. 2). In uninfected mice, promazine treatment alone caused a significant increases ($P < 0.05$ to $P < 0.005$) in IL1-α, IL1β, IL-2 (50 mg/kg dose only), IL-3, IL-4, IL-9, IL-10, IL-12, TNF-alpha (50 mg/kg dose only), MIP-1 (50 mg/kg dose only), and RANTES, but not in IL-6, MCP-1, or GM-CSF, compared to uninfected, untreated mice. Interestingly, promazine administered to infected mice at 50 mg/kg seemed to promote much higher levels of IL-6 than was detectable in untreated, infected mice or in uninfected mice treated with promazine. In infected animals treated with 50 mg/kg promazine, RANTES titers were significantly lower ($P < 0.05$) than in the lungs of untreated, infected mice. Promazine treatment of infected mice did not significantly affect other cytokines levels in compared to untreated, infected mice. Cytokine levels in lung samples from mice sacrificed 7 or 14 days post-infection were not significantly different when comparing treated, infected mice and untreated, infected mice (data not shown) and by day 14 there were no detectable increases in cytokine levels, regardless of treatment (data not shown).

4. Discussion

In some reviews of antiviral therapy for SARS-CoV (Chan et al., 2003; Cinatl et al., 2005), it has been suggested that promazine warrants further investigation as a potential treatment for SARS-CoV infections based on the studies of Zhang and Yap, 2004; Barnard et al., 2005; Hsieh et al., 2005). However in the studies presented here, in which the in vitro assays were repeated four times using independent experiments to confirm all EC_{50} and IC_{50} values unlike the data reported in other studies, we were unable to show any convincing antiviral data for promazine or related compounds nor were we able to show efficacy of promazine in an animal model. In fact, it is likely that the cytotoxic properties of the compounds accounted for any slight in vitro inhibition of virus detected in the current studies.

However, an alternative explanation for the slight inhibitory activity of the phenothiazines might be the inhibition of the SARS-CoV main protease. Three phenazines were predicted to be inhibitors of the SARS-CoV main protease through docking predictions and virtual screening. When these compounds were actually evaluated in enzymatic assays, the three phenothiazines inhibited the enzyme activity by 11–15% (Liu et al., 2005). In addition, promazine, a phenothiazine, might also inhibit the SARS-CoV main protease. Zhang and Yap (2004) in a computational study have shown that promazine can bind to amino acid residues in the Domain I of the protease. However, the binding affinity would probably not be sufficient to cause effective inhibition of the enzyme.

The observation that promazine promotes the induction of certain proinflammatory cytokines in schizophrenics may actually account for the prolongation of virus infection in mice observed in the current study when mice were pretreated with promazine. Treatment of schizophrenics with promazine seemed to induce some proinflammatory cytokines such as IL-6 and TNF-α, which in turn stimulated highly reactive oxygen species (ROS) (Kaminska et al., 2003). Induction of proinflammatory cytokines also occurs in many viral respiratory infections including SARS (Xu and Gao, 2004; Barnard et al., 2006). Glass et al. (2004) found that SARS-CoV infection in mice was characterized by a proinflammatory cytokine storm, including the induction of IL-6 and TNF-α. We have found that ribavirin exacerbates the SARS-CoV-induced cytokine storm leading to a prolongation of viral replication in the lungs of infected animals (Barnard et al., 2006). In addition, promazine at 50 mg/(kg day) seemed to significantly decrease RANTES expression, an important chemokine promoting neutrophil migration to the SARS-CoV infection site (Yen et al., 2006). Promazine, by suppressing RANTES levels might have allowed virus to persist because of a lack of a good robust, neutrophil response to the initial infection. Thus, the low-dose promazine treatments (50 mg/(kg day)) used in the current study may have altered the SARS-CoV-perturbed cytokine levels resulting in a prolonged infection and suppression of key chemokines necessary for clearance of virus after day 3, leading to a persistent virus infection. However, the exacerbation of the infection may be dependent on the frequency and the timing of treatment administration; less frequent dosing without a pretreatment before virus exposure resulted in no prolongation of virus infection and in no reduction of virus lung titers; RANTES expression was unaffected at lower doses promazine.

In summary, a number of phenothiazines very weakly inhibited the replication of four strains of SARS-CoV in Vero cells, although this activity was probably due to the cytotoxicity of the compounds tested. When one of the phenothiazines, promazine, was evaluated in a SARS-CoV replication model in mice, toxicity was manifested at the highest dose of promazine treatment administered i.p. (100 mg/(kg day)), resulting in death of all animals treated regardless of dosing schedule or regimen. This observation validated the significant toxicity detected in vitro with many of the phenothiazines evaluated in vitro. Even though a two-fold lower dose of promazine was much less toxic in mice, virus lung titers were not reduced. Lower doses of promazine (50 and 10 mg/(kg day)), when administered frequently and just prior to virus exposure, significantly prolonged the infection. Given the almost total lack of inhibition of virus replication in mice except at one dose, the narrow concentration window between lethality and survival and the potential for prolongation of virus infection in animals, promazine and its prodrugs should not be considered potential therapies for SARS infections.

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References

Barnard, D.L., Hill, C.L., Gage, T.L., Matheson, J.L., Huffman, J.H., Sidwell, R.W., Otto, M.J., Schinazi, R.F., 1997a. Potent inhibition of respiratory syncytial virus by polyoxometalates of several structural classes. Antiviral Res. 34, 27–37.

Barnard, D.L., Bischofberger, N., Kim, C.U., Huffman, J.H., Sidwell, R.W., Dougherty, J.P., Lew, W., Williams, M.A., Yang, W., 1997b. Acyclic phosphonomethylether nucleoside inhibitors of respiratory viruses. Antiviral Chem. Chemother. 8, 223–233.

Barnard, D.L., Hubbard, V.D., Burton, J., Smee, D.F., Morrey, J.D., Otto, M.J., Sidwell, R.W., 2004a. Inhibition of severe acute respiratory syndrome–associated coronavirus (SARSCoV) by calpain inhibitors and ß-D-N^4-hydroxycytidine. Antiviral Chem. Chemother. 15, 15–22.

Barnard, D.L., Hubbard, V.D., Smee, D.F., Sidwell, R.W., Watson, K.G.W., Tucker, S.P.T., Reece, P.A.R., 2004b. In vitro activity of expanded-spectrum pyridazinyl oxime ethers related to pirodavir: novel capsid-binding inhibitors with potent anti-picornavirus activity. Antimicrob. Agents Chemother. 48, 1766–1772.

Barnard, D.L., Day, C.W., Wootton, W.M., Sidwell, R.W., 2005. Derivatives of phenothiazine anti-psychotics inhibit severe acute respiratory syndrome–associated coronavirus (SARS-CoV) replication in vitro. In: American Society for Virology Meetings, State College, PA, June 18–22 (Abstract # W9-9).

Barnard, D.L., Day, C.W., Bailey, K., Heiner, M., Montgomery, R., Lauridsen, L., Winslow, S., Hoopes, J., Li, J.K-.-K., Lee, J., Carson, D.A., Cottam, H.B., Sidwell, R.W., 2006. Enhancement of the infectivity of SARS-CoV in BALB/c mice by IMP dehydrogenase inhibitors, including ribavirin. Antiviral Res. 71, 53–63.

Cavenaugh Jr., P.R., Moskwa, P.S., Donish, W.H., Pera, P.J., Richardson, D., Andrese, A.P., 1990. A semi-automated NR red based chemosensitivity assay for drug screening. Invest. New Drugs 8, 347–354.

Chan, H.L., Tsui, S.K., Sung, J.J., 2003. Coronavirus in severe acute respiratory syndrome (SARS). Trends Mol. Med. 9, 323–325.

Cinatl Jr., J., Michaelis, M., Hoever, G., Preiser, W., Doerr, H.W., 2005. Development of antiviral therapy for severe acute respiratory syndrome. Antiviral Res. 66, 81–97.

De Clercq, E., 2006. Potential antivirals and antiviral strategies against SARS coronavirus infections. Expert Rev. Anti. Infect. Ther. 4, 291–302.

Fouchier, R.A., Kuiken, T., Schutten, M., van Amerongen, G., van Doornum, G.J., van den Hoogen, B.G., Peiris, M., Lim, W., Stöhr, K., Osterhaus, A.D., 2003. Aetiology: Koch's postulates fulfilled for SARS virus. Nature 423, 240.

Glass, W.G., Subbarao, K., Murphy, B., Murphy, P.M., 2004. Mechanisms of host defense following severe acute respiratory syndrome-coronavirus (SARS-CoV) pulmonary infection of mice. J. Immunol. 173, 4030–4039.

Hsieh, H.P., Wu, Y.-S., Lu, P.-H., Chen, C.-M., Chao, Y.-C., Jan, J.-T., Lo, H.-R., Ma, S.-H., Chao, Y.-S., Hsu, T.-A., 2005. Promazine analogues as potential anti SARS-CoV drugs. In: Proceedings of the 229th ACS National Meeting, (MEDI-335).

Kaminska, T., Szuster-Ciesielska, A., Wysocka, A., Marmurowska-Michalowska, H., Dubas-Slemp, H., Kandefer-Szerszen, M., 2003. Serum cytokine level and production of reactive oxygen species (ROS) by blood neutrophils from a schizophrenic patient with hypersensitivity to neuroleptics. Med. Sci. Monit. 9, CS71–CS75.

Liang, G., Chen, Q., Xu, J., Liu, Y., Lim, W., Peiris, J.S., Anderson, L.J., Ruan, L., Li, H., Kan, B., Di, B., Cheng, P., Chan, K.H., Erdman, D.D., Gu, S., Yan, X., Liang, W., Zhou, D., Haynes, L., Duan, S., Zhang, X., Zheng, H., Gao, Y., Tong, S., Li, D., Fang, L., Qin, P., Xu, W., 2004. Laboratory diagnosis of four recent sporadic cases of community-acquired SARS, Guangdong Province, China. Emerg. Infect. Dis. 10, 1774–1781.

Lim, W., Ng, K.C., Tsang, D.N., 2006. Laboratory containment of SARS virus. Ann. Acad. Med. Singapore 35, 354–360.

Liu, Z., Huang, C., Fan, K., Wei, P., Chen, H., Liu, S., Pei, J., Shi, L., Li, B., Yang, K., Liu, Y., Lai, L., 2005. Virtual screening of novel noncovalent inhibitors for SARS-CoV 3C-like proteinase. J. Chem. Inf. Model. 45, 10–17.

Peiris, J.S., Lai, S.T., Poon, L.L., Guan, Y., Yam, L.Y., Lim, W., Nicholls, J., Yee, W.K., Yan, W.W., Cheung, M.T., Cheng, V.C., Chan, K.H., Tsang, D.N., Yung, R.W., Ng, T.K., Yuen, K.Y., 2003. Coronavirus as a possible cause of severe acute respiratory syndrome. Lancet 361, 1319–1325.

Song, H.D., Tu, C.C., Zhang, G.W., Wang, S.Y., Zheng, K., Lei, L.C., Chen, Q.X., Gao, Y.W., Zhou, H.Q., Xiang, H., Zheng, H.J., Chern, S.W., Cheng, F., Pan, C.M., Xuan, H., Chen, S.J., Luo, H.M., Zhou, D.H., Liu, Y.F., He, J.F., Qin, P.Z., Li, L.H., Ren, Y.Q., Liang, W.J., Yu, Y.D., Anderson, L., Wang, M., Xu, R.H., Wu, X.W., Zheng, H.Y., Chen, J.D., Liang, G., Gao, Y., Liao, M., Fang, L., Jiang, L.Y., Li, H., Chen, F., Di, B., He, L.J., Lin, J.Y., Tong, S., Kong, X., Du, L., Hao, P., Tang, H., Bernini, A., Yu, X.J., Spiga, O., Guo, Z.M., Pan, H.Y., He, W.Z., Manuguerra, J.C., Fontanet, A., Danchin, A., Niccolai, N., Li, Y.X., Wu, C.I., Zhao, G.P., 2005. Cross-host evolution of severe acute respiratory syndrome coronavirus in palm civet and human. Proc. Natl. Acad. Sci. U.S.A. 102, 2430–2435.

Subbarao, K., McAuliffe, J., Vogel, L., Fahle, G., Fischer, S., Tatti, K., Packard, M., Shieh, W.J., Zaki, S., Murphy, B., 2004. Prior infection and passive transfer of neutralizing antibody prevent replication of severe acute respiratory syndrome coronavirus in the respiratory tract of mice. J. Virol. 78, 3572–3577.

Weiss, S.R., Navas-Martin, S., 2005. Coronavirus pathogenesis and the emerging pathogen severe acute respiratory syndrome coronavirus. Microbiol. Mol. Biol. Rev. 69, 635–664.

Xu, X., Gao, X., 2004. Immunological responses against SARS-coronavirus infection in humans. Cell Mol. Immunol. 1, 119–122.

Yen, H.C., Day, C.A., 1965. Evaluation of anti-tremor drugs in tremor-induced rodents. Arch. Int. Pharmacodyn. Ther. 155, 69–83.

Yen, Y.T., Liao, F., Hsiao, C.H., Kao, C.L., Chen, Y.C., Wu-Hsieh, B.A., 2006. Modeling the early events of severe acute respiratory syndrome coronavirus infection in vitro. J. Virol. 80, 2684–2693.

Zhang, X.W., Yap, Y.L., 2004. Old drugs as lead compounds for a new disease? Binding analysis of SARS coronavirus main proteinase with HIV, psychotic and parasite drugs. Bioorg. Med. Chem. 12, 2517–2521.