

# Evaluation of immunomodulators, interferons and known *in vitro* SARS-CoV inhibitors for inhibition of SARS-CoV replication in BALB/c mice

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Compounds approved for therapeutic use and *in vitro* inhibitors of severe acute respiratory syndrome coronavirus (SARS-CoV) were evaluated for inhibition in the mouse SARS-CoV replication model. A hybrid interferon, interferon alpha (IFN- $\alpha$ ) B/D, and a mismatched double-stranded (ds) RNA interferon (IFN) inducer, Ampligen® (poly I:poly C<sub>124</sub>), were the only compounds that potently inhibited virus titres in the lungs of infected mice as assessed by CPE titration assays. When mice were dosed intraperitoneally (i.p.) with IFN- $\alpha$  B/D once daily for 3 days beginning 4 h after virus exposure, SARS-CoV replication in the lungs of infected mice was reduced by 1 log<sub>10</sub> at 10,000 and 32,000 IU; at the highest dose of 100,000 IU, virus lung titres were below detectable limits. Ampligen® used i.p. at 10 mg/kg 4 h prior to virus exposure also reduced virus lung titres to below detectable limits. Nelfinavir,  $\beta$ -D-N<sup>4</sup>-hydroxycytidine, calpain inhibitor

VI, 3-deazaneplanocin A and Alferon® (human leukocyte IFN- $\alpha$ -n3) did not significantly reduce lung virus titres in mice. Anti-inflammatory agents, chloroquine, amodiaquin and pentoxifylline, were also inactive *in vivo*, suggesting that although they may be useful in ameliorating the hyperinflammatory response induced by the virus infection, they will not significantly reduce the replication of the virus, the inducer of inflammatory response. Thus, anti-inflammatory agents may only be useful in treating virus lung infections if used in combination with agents that inhibit virus replication. In summary, the data suggest that induction of IFN by mismatched dsRNA or actual treatment with exogenous IFN- $\alpha$  can inhibit SARS-CoV replication in the lungs of mice.

**Keywords:** Ampligen®, chloroquine, interferon- $\alpha$  B/D, nelfinavir, SARS-CoV

## Introduction

Severe acute respiratory syndrome (SARS) is a life-threatening and contagious febrile respiratory illness. Index cases were initially detected in early 2003 in the Guangdong Province in southern China, and were rapidly followed by secondary and tertiary outbreaks primarily in Vietnam, Hong Kong, Singapore, Canada and the United States (Hsueh & Yang, 2003). SARS was known to be extremely life-threatening and thought to be highly contagious, so a better understanding of the disease and the aetiological agent was quickly sought and acquired, which has facilitated rational approaches to the development of therapies for prophylaxis and treatment (De Clerq, 2006; Weiss & Navas-Martin, 2005). The putative agent was quickly identified as a corona-like virus (Peiris *et al.*, 2003; Rota *et al.*, 2003), which was soon shown to be the aetiological agent for SARS, that is, severe acute respiratory syndrome coronavirus (SARS-CoV; Fouchier *et al.*, 2003).

Initially, systemic corticosteroids were used to suppress the production of inflammatory mediators that appeared in response to the viral infection (Ho *et al.*, 2003; Meng *et al.*, 2003). Later, other therapies such as combination treatment with ribavirin and corticosteroids were also attempted (Koren *et al.*, 2003). However, the efficacy of these treatments was not demonstrated in controlled studies (Wenzel & Edmond, 2003), subsequently leading to a major effort to find clinically approved drugs that could inhibit SARS-CoV.

Some laboratories have, therefore, evaluated clinically approved drugs for efficacy against SARS-CoV in an attempt to provide an early treatment for SARS infections in humans. Chloroquine and its derivatives were shown to inhibit viral replication *in vitro* by a variety of assays (Keyaerts *et al.*, 2004; Vincent *et al.*, 2005; Biot *et al.*, 2006). One rationale for testing chloroquine, a traditional anti-malarial (Arav-Boger & Shapiro, 2005), was that it

modestly inhibited HIV *in vivo* (Romanelli *et al.*, 2004). The drug has also been shown to prevent the up-regulation of major histocompatibility complex (MHC) I antigens and cellular abnormalities in coxsackie B4-infected human foetal thymocytes (Brilot *et al.*, 2004). The mechanism of inhibition for HIV may be related to some inhibitory interaction with gp120 (Romanelli *et al.*, 2004) or inhibition of the inflammatory cytokine response to HIV (Rayne *et al.*, 2004). This mechanism may be important in treating SARS-CoV, since the virus induces a severe inflammatory response in the lungs of patients (Yen *et al.*, 2006). Several other FDA-approved drugs, including HIV protease inhibitors (PIs), were found to inhibit SARS-CoV *in vitro* (Chu *et al.*, 2004; Yamamoto *et al.*, 2004). Nelfinavir, an isoquinoline carboxamide-based HIV PI (Yamamoto *et al.*, 2004), potently inhibited SARS-CoV replication in Vero cells, although it was not a very potent inhibitor of the SARS-CoV 3CL(pro) protease, a probable target of inhibition (Liu *et al.*, 2005). Pentoxifylline (PTX), approved for use in several diseases, has been recommended for treating SARS because of its selective anti-inflammatory activity and antiviral properties (Bermejo & Munoz-Fernandez, 2004). Lastly, a non-approved drug,  $\beta$ -D-N<sup>4</sup>-hydroxycytidine (BHC), was found to be one of the more potent nucleoside analogue inhibitors of SARS-CoV tested to date with an EC<sub>90</sub> of 6  $\mu$ M by virus yield reduction assay (Barnard *et al.*, 2004a). Its putative target remains unknown.

Since many of the compounds mentioned above had already been approved for therapeutic use for several diseases and/or had been shown to potently inhibit SARS-CoV replication *in vitro*, studies were carried out to determine if the compounds could reduce viral replication in the mouse SARS-CoV replication model (Subbarao *et al.*, 2004; Barnard *et al.*, 2006) as part of the next step in developing these compounds as potential SARS-CoV inhibitors. In addition, other classes of compounds were evaluated *in vivo* for inhibition of virus replication in animals, such as 3-deazaneplanocin A and Ampligen®, immunomodulators and a human hybrid interferon (IFN).

## Materials and methods

### Cells and virus

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

SARS-CoV, strain Urbani (200300592), was obtained from the Centers for Disease Control (CDC, Atlanta,

GA, USA), the Frankfurt strain was kindly provided by Jindrich Cinatl (Klinikum der J.W. Goethe Universitat, 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 (The 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.

### Compounds

N-(4-Fluorophenylsulphonyl)-L-valyl-L-leucinal (calpain inhibitor VI) was obtained from Calbiochem (La Jolla, CA, USA). Nelfinavir (cat # 4621) and lopinavir (cat # 9481) were obtained from the NIH AIDS Reagent and Reference Reagent Program, Division of AIDS, NIAID, NIH (Bethesda, MD, USA) and 3-deazaneplanocin A was provided by Dr Victor Marquez (National Cancer Institute, Bethesda, MD, USA) through the NIAID Antiviral Substances Program. Chloroquine diphosphate was obtained from the NIAID Antiviral Substances Program repository. Pentoxifylline (PTX), chloroquine and amodiaquin were purchased from Sigma (St. Louis, MO, USA). Alferon® was kindly provided by Hemispherx Biopharma, Inc. (New Brunswick, NJ, USA). Ampligen® was also obtained from the NIAID Antiviral Substances Program. BHC was obtained from Pharmasset, Inc. (Princeton, NJ, USA) through the NIAID Antiviral Substances Program. Recombinant human IFN alpha B/D recombinant IFN (rHu-IFN- $\alpha$  B/D) (Wintergerst *et al.*, 1999) was provided by Dr David Gangemi (Clemson University, SC, USA) through the NIAID Antiviral Substances Program. All compounds with the exception of calpain VI and chloroquine were solubilized in MEM for *in vitro* assays and in PSS for *in vivo* experiments. Calpain VI and chloroquine were dissolved in dimethyl sulphoxide (DMSO) and diluted in MEM or PSS to the appropriate concentrations. In one animal experiment, calpain VI was suspended in carboxymethylcellulose at the appropriate doses.

### Cytopathic effect inhibition assay

A modified protocol of Barnard *et al.* (2004a) was used for *in vitro* evaluation of antiviral efficacy of inhibitors of SARS-CoV replication. Compounds were tested at varying concentrations (four log<sub>10</sub> or eight half-log<sub>10</sub> dilutions). Virus and compound were added in equal volumes to near-confluent cell monolayers in 96-well tissue culture plates. The multiplicity of infection (MOI) used ranged from 0.01–0.025 in order to produce complete virus cytopathic

effects (CPE) in 100% of the cells in the virus control wells within 3–4 days. The plates were incubated at 37°C 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 of viral CPE 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. Alferon® (Hemispherx Biopharma, Inc.), a human leukocyte-derived IFN- $\alpha$ -n3, was included as a positive control drug for each set of compounds tested.

Morphological changes resulting from cytotoxicity of a compound or virus CPE were graded on a scale of 0–5, with 5 being defined as the appearance of complete cytotoxicity or CPE involving the entire monolayer, as observed by light microscopy. The values obtained were then converted to percentages of untreated, uninfected controls. The 50% cell cytotoxic concentrations ( $CC_{50}$ ) and 50% virus inhibitory concentrations ( $IC_{50}$ ) were estimated by regression analysis. A selectivity index (SI) was calculated using the formula:  $SI = CC_{50}/IC_{50}$ . The activity in the CPE assay was then verified spectrophotometrically by a neutral red (NR) uptake assay on the same plate (see below).

#### NR uptake assay for determination of antiviral efficacy and compound cytotoxicity

This assay was done for each CPE inhibition test plate described above in order to verify the inhibitory activity and the cytotoxicity detected by visual observation. The usual correlation between visual and NR assays in our experience has been greater than 95% (Barnard *et al.*, 1997). The NR assay was performed using a modified method of Cavanaugh *et al.* (1990) as described by Barnard *et al.* (2004b). Briefly, medium was removed from each well of a plate, 0.011% NR was added to each well of the plate and the plate incubated for 2 h at 37°C in the dark. The NR solution was removed from the wells. The wells in each plate were then rinsed and any remaining dye extracted using ethanol buffered with Sörensen's citrate buffer. Absorbances at 540 nm/405 nm were read with a microplate reader (Opsys MR™, Dynex Technologies, Chantilly, VA, USA). Absorbance values were expressed as percents of untreated controls and  $IC_{50}$ ,  $CC_{50}$  and SI values were calculated as described above.

#### Virus yield reduction assay

Some compounds were evaluated by virus yield reduction 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.*, 2006). After CPE was scored as described above, each plate was frozen at –80°C and thawed.

Sample wells at each compound concentration tested were pooled and titred in Vero 76 cells for infectious virus by CPE assay as previously described by Barnard *et al.* (2004a).

A 90% reduction in virus yield ( $IC_{90}$ ) was then calculated by linear regression analysis. This represented a one  $\log_{10}$  inhibition in titre when compared with untreated virus controls. SI values were determined by the formula  $CC_{50}/IC_{90}$ .

#### Animals

Specific pathogen-free BALB/c female mice (11–18 g, range varied with each experiment) were obtained from Charles River Laboratories (Wilmington, MA, USA) and 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, USA). For the infectious disease experiments, mice were housed in bonneted filter-topped cages placed within a HEPA-filtered horizontal laminar flow ventilated animal rack.

#### Preliminary toxicity evaluation

For each compound of unknown mouse toxicity, a dose-range finding experiment was carried out to determine the maximum tolerated concentration. Dosage regimens that were to be used in the actual efficacy studies with the compound were used. Three mice were used per treatment group.

#### Lung virus titre determinations

Each mouse lung was homogenized and the tissue fragments allowed to settle. Varying dilutions of the supernatant fluids were assayed in triplicate for infectious virus in Vero 76 cells by CPE assay, and titres ( $CCID_{50}$  values) were calculated using the Reed–Muench method (Reed & Muench, 1938).

#### Experimental design

If mice were pretreated with compound, they were given the material by intraperitoneal (i.p.) or intranasal (i.n.) exposure 4 h or occasionally 24 h prior to infection. For any i.n. exposure, the mice were sedated with ketamine. After the pretreatment, the mice were infected i.n. with 50  $\mu$ l of clarified virus lysate diluted 1:5 in PSS. In most experiments, animals (10–30 per treatment group) were treated either i.p. or i.n. with an appropriate dose of compound or with placebo subsequent to virus exposure. The frequency of dosing varied with each experiment, but was usually once a day or twice a day for 0–2 days after virus exposure. The placebo was PSS in all experiments except for those

**Table 1.** *In vitro* inhibition of SARS-CoV replication in African green monkey kidney cells by anti-inflammatory drugs, immunomodulators, IFNs and selected antiviral agents

Compound	Visual assay, $\mu\text{M}^*$			NR assay, $\mu\text{M}^*$			Virus yield reduction assay, $\mu\text{M}$
	IC <sub>50</sub>	CC <sub>50</sub>	SI	IC <sub>50</sub>	CC <sub>50</sub>	SI	IC <sub>90</sub>
Alferon®†	61 ±41	>32,000	>525	32 ±0.0	>32,000	>1,000	1,600
β-D-N <sup>4</sup> -hydroxycytidine	0.1 ±0.0	13.5 ±5.0	>135	0.1 ±0.0	12.5 ±3.5	>125	6
Calpain inhibitor VI	6.6 ±6.0	>100	>15	3.0 ±4.0	>100	>33	3
Amodiaquin	2.5 ±0.7	33.0 ±19.8	13	3.5 ±2.1	34.0 ±25.5	4	2
Chloroquine diphosphate	6.0 ±0.7	31.0 ±16.0	5	6.5 ±0.7	22.5 ±10.6	6	7
Chloroquine	2.5 ±0.7	31.5 ±14.8	13	3.0 ±0.0	26.0 ±15.6	9	11
Nelfinavir	3.5 ±3.4	9.5 ±3.5	3	3.5 ±0.7	3.5 ±0.7	1	40
Lopinavir	15	20	1	25	17	1	ND
Pentoxifylline	>100	>100	0	>100	>100	0	ND
3-deazaneplanocin A	>100	>100	0	>100	>100	0	ND
Ampligen®	>100	>100	0	>100	>100	0	ND
IFN-α B/D	451 ±0†	>500	>1	>500	>500	0	ND

\*Values are expressed as mean ± standard deviation from three separate experiments. †All results are expressed in IU. CC<sub>50</sub>, 50% cytotoxic concentration; IC<sub>50</sub>, 50% inhibitory concentration; IC<sub>90</sub>, 90% inhibitory concentration; IFN-α, interferon alpha; ND, not done; SARS-CoV, severe acute respiratory syndrome coronavirus; SI, selectivity index.

involving calpain inhibitor VI, in which case DMSO diluted to 0.5% in PSS or 0.4% carboxymethylcellulose (CMC) was used. See individual tables herein for the dosage schedules used for each compound. Uninfected animals (five at each dose of compound or placebo) were treated with the same concentrations of drug or placebo as the infected mice, using the same dosage schedule, and served as toxicity controls. In most experiments, treatments ceased after day 2 following virus exposure, and animals were sacrificed at day 3 (or sometimes on day 7) after virus exposure. Lungs were removed, weighed and assayed for the presence of virus as described below.

### Statistical analysis

Differences in mean virus titres were analysed by analysis of variance.

## Results

### *In vitro* activity

A number of classes of compounds previously shown to inhibit SARS-CoV replication *in vitro* were evaluated for inhibition of virus replication in Vero 76 cells. Of the compounds tested, a human α-n3 IFN from leukocytes was the most selective inhibitor of virus replication (Table 1). β-Hydroxycytidine was the most potent inhibitor of the non-IFNs evaluated, with IC<sub>50</sub>=0.1  $\mu\text{M}$ , but it was not as potent in reducing virus yields (IC<sub>90</sub>=6  $\mu\text{M}$ ). Calpain inhibitor VI also inhibited the virus with moderate selectivity with SI

values ranging from 15 to 33. The antimalarial, anti-inflammatory drugs, amodiaquin and the chloroquines were selectively active. Amodiaquin IC<sub>50</sub> values were 2.5–3.5  $\mu\text{M}$  with an IC<sub>90</sub> value of 2  $\mu\text{M}$  and SI values ranging from 4 to 13. The chloroquines, of which amodiaquin is a derivative, also inhibited virus replication at similar concentrations, although all were much more toxic than has been previously reported (Keyaerts *et al.*, 2004; Vincent *et al.*, 2005; Biot *et al.*, 2006). None of the other compounds evaluated were inhibitory, including two HIV PIs previously shown to inhibit SARS-CoV replication (Chu *et al.*, 2004; Yamamoto *et al.*, 2004).

We also evaluated the chloroquines and human α-n3 IFN against various strains of SARS-CoV (Table 2). No significant sensitivity differences to the various drugs were noted among the strains tested, which correlated with the relative homogeneity of the genomic sequences of these viruses.

### *In vivo* activity

Nelfinavir, an HIV PI, was evaluated *in vivo* because of data from other laboratories that demonstrated inhibition of SARS-CoV replication *in vitro* (Chu *et al.*, 2004; Yamamoto *et al.*, 2004). It has the potential to inhibit the two proteases of SARS-CoV that are responsible for virus maturation (Lindner *et al.*, 2005; Liang, 2006), including the SARS-CoU 3Cl (pro) protease.

The results of this experiment are summarized in Table 3. Nelfinavir at both 30 mg/kg and 10 mg/kg reduced viral lung titres by about 1 half-log<sub>10</sub> compared with the control



**Table 2.** *In vitro* inhibition of four strains of SARS-CoV replication in African green monkey kidney cells by various chloroquine derivatives

	Chloroquine, μM			Chloroquine monophosphate, μM			Chloroquine diphosphate, μM			Amodiaquine, μM			Alferon®, IU		
	IC <sub>50</sub>	CC <sub>50</sub>	SI	IC <sub>50</sub>	CC <sub>50</sub>	SI	IC <sub>50</sub>	CC <sub>50</sub>	SI	IC <sub>50</sub>	CC <sub>50</sub>	SI	IC <sub>50</sub>	CC <sub>50</sub>	SI
<b>Urbani</b>															
CPE assay	2	20	10	4	30	8	3	30	10	3	20	7	30	>30,000	>1,000
NR assay	3	20	7	6	20	3	5	10	2	4	30	8	200	>30,000	>150
<b>Toronto 2</b>															
CPE assay	4	20	5	4	20	5	4	20	5	4	20	5	300	>30,000	>100
NR assay	4	13	4	6	30	5	5	20	4	10	20	2	700	>30,000	>43
<b>Frankfurt 1</b>															
CPE assay	1	20	20	6	30	5	4	30	8	6	20	3	200	>30,000	>150
NR assay	3	20	7	6	20	3	8	30	4	3	30	10	1000	>30,000	>30
<b>CHUK-W1</b>															
CPE assay	3	20	7	4	20	5	3	30	10	3	30	10	100	>30,000	>300
NR assay	5	10	2	4	30	8	5	30	6	4	20	5	600	>30,000	>50

CC<sub>50</sub>, 50% cytotoxic concentration; CPE, cytopathic effect; IC<sub>50</sub>, 50% inhibitory concentration; NR, neutral red; SARS-CoV, severe acute respiratory syndrome coronavirus; SI, selectivity index.

animals when administered i.p. for 3 days, twice a day with a 4 h treatment prior to virus exposure, although this inhibition was not statistically significant. Using the same dosing schedule as described above but administering compound by the i.n. route, the virus titres in animals treated with the 30 mg/kg dose were also reduced by about 1 half-log<sub>10</sub>; other dosages were not efficacious. The drug was well tolerated at all doses tested (data not shown), regardless of the route of administration. Thus, the lack of reduction of virus CPD detected *in vitro* in this study correlated with the lack of efficacy in the animal model.

BHC was previously shown to be a selective nucleoside analogue inhibitor of SARS-CoV *in vitro* (Barnard *et al.*, 2006). Therefore, the compound was evaluated for efficacy against SARS-CoV replication in the mouse as an i.p. treatment (Table 3). It was readily apparent that BHC, although well-tolerated (data not shown), was not effective in reducing virus lung titres of SARS-CoV-infected mice when administered twice daily for 3 days with a 4 h pretreatment.

Calpain inhibitor VI is a dipeptide consisting of a valine and a leucine with protective groups on each terminus and is a cysteine PI that could potentially inhibit the cysteine protease of the virus, the SARS-CoV main protease 3CL (pro). The compound was a very potent inhibitor of virus replication *in vitro* (Table 1) and was thought to be a good candidate for efficacy testing in the SARS-CoV mouse lung virus replication model. The results of several experiments are summarized in Table 3. In the first experiment, the drug was solubilized at very high concentration in DMSO and diluted to concentrations in PSS, resulting in

turbid suspensions. The material was delivered i.p. twice a day over a period of 3 consecutive days with the first administration of drug 4 h prior to exposure of mice to the virus. This treatment was ineffective in inhibiting lung virus titres. Because of the apparent insolubility of the compound in aqueous solution, even at lower concentrations, a second experiment was done in which the material was again solubilized in DMSO, but CMC was used as the delivery vehicle. The rationale was that perhaps this would deliver the drug in a more biologically absorbable form. The calpain inhibitor at 10 mg/kg did reduce viral lung titres by 0.4 half-log<sub>10</sub> compared with the control animals, however, this was not statistically significant.

Chloroquine has already been approved for therapeutic use for several diseases. Because other groups had shown *in vitro* efficacy (Keyaerts *et al.*, 2004; Vincent *et al.*, 2005; Biot *et al.*, 2006), it warranted further evaluation in the mouse SARS-CoV replication model even though in the present study the material was not found to be active *in vitro* (Table 1). Amodiaquin, a derivative of chloroquine (both are anti-inflammatory agents) was a rather potent inhibitor of SARS-CoV replication in cell culture in the present study (Table 1) and was evaluated in parallel studies with chloroquine. Both compounds were delivered i.p. in one study and i.n. in a second study twice a day for 3 days, beginning 4 h prior to virus exposure. Although the two materials were both well-tolerated (data not shown), they were both ineffective in inhibiting lung virus titres when delivered by the i.p. route. However, i.n. chloroquine lessened viral lung titres by 0.8 half-log<sub>10</sub> at

**Table 3.** Effects of *in vitro* inhibitors of SARS-CoV on SARS-CoV replication in female BALB/c mice

Compound	Mice/group, <i>n</i>	i.p. administration		i.n. administration	
		Dosage, mg/kg	Day 3 virus titre, log <sub>10</sub> CCID <sub>50</sub> /g*	Dosage, mg/kg	Day 3 virus titre, log <sub>10</sub> CCID <sub>50</sub> /g*
Nelfinavir <sup>†</sup>	30	90	5.0 ± 0.0	90	4.8 ± 0.4
		30	4.4 ± 0.4	30	4.2 ± 0.5
		10	4.5 ± 0.4	10	4.4 ± 0.2
		Placebo	5.0 ± 0.5	Placebo	4.6 ± 0.6
β-D-N-hydroxycytidine <sup>‡</sup>	30	20	5.0 ± 0.2	ND	ND
		10	4.8 ± 0.5	ND	ND
		1	5.1 ± 0.1	ND	ND
		Placebo	4.9 ± 0.1	ND	ND
Calpain inhibitor VI <sup>†</sup>	10	100	5.4 ± 0.1	ND	ND
		30	5.2 ± 0.0	ND	ND
		10	5.8 ± 0.4	ND	ND
		1	5.4 ± 0.5	ND	ND
		0.5% DMSO	5.4 ± 0.2	ND	ND
Calpain inhibitor VI <sup>†</sup>	10	10	5.7 ± 0.4	ND	ND
		1	5.8 ± 0.3	ND	ND
		0.5% CMC	6.1 ± 0.6	ND	ND

\*Mean ± standard deviation. Virus was titred in duplicate assays. <sup>†</sup>Twice a day for 3 days beginning 4 h pre-virus exposure. <sup>‡</sup>Once a day for 3 days beginning 4 h pre-virus exposure. CCID<sub>50</sub>, 50% cell culture infectious dose; CMC, carboxymethylcellulose; DMSO, dimethyl sulphoxide; ND, not done; i.p., intraperitoneal; i.n., intranasal; SARS-CoV, severe acute respiratory syndrome coronavirus.

the highest dose used, although this reduction was not statistically significant.

PTX, a phosphodiesterase IV inhibitor, is a well-known anti-inflammatory that can reduce the production of proinflammatory cytokines (Bermejo *et al.*, 2003). It has also been shown to inhibit viruses in cell culture including herpes simplex virus, HIV, tick-borne encephalitis virus and rotavirus (Bermejo *et al.*, 2003), and has been in clinical trials for treating certain age groups infected with hepatitis B virus, showing modest beneficial effects (Bermejo *et al.*, 2003). Thus, PTX might be very beneficial for treating a SARS infection by reducing the inflammatory stimulating agent (the virus) and ameliorating the hyperinflammatory response. Because of the potential duality of therapeutic efficacy of PTX, the effects of this compound on SARS-CoV replication *in vitro* and in the mouse were tested, however the compound did not inhibit SARS-CoV replication *in vitro* (Table 1). PTX was administered both i.p. and i.n. in separate experiments for 3 days, beginning 4 h before virus exposure. The compound was not toxic at any dose used. As seen in Table 4, using the i.n. route, the compound was weakly effective in inhibiting virus replication in the lungs, the maximal titre inhibition being 0.3 log<sub>10</sub>. When delivered i.p., the greatest titre inhibition seen was 0.6 log<sub>10</sub>. However, none of these virus titre reductions was statistically different.

3-Deazaneplanocin A has been shown to induce high levels of IFN-α that corresponded to protection of animals from Ebola virus infection (Bray *et al.*, 2002). Since this compound seems to have IFN-inducing properties, it was evaluated for efficacy in the SARS mouse lung replication model because IFNs have been shown to potently inhibit SARS-CoV replication *in vitro* (Sainz *et al.*, 2004; Stroher *et al.*, 2004). The i.p.-delivered 3-deazaneplanocin A was evaluated at various dosages from 0.05 to 10 mg/kg, using once a day and twice a day treatment schedules. As shown in Table 5, treatment with the material administered once or twice a day for 3 days beginning 4 h prior to virus exposure was not inhibitory to virus lung replication.

A known inducer of IFN (Padalko *et al.*, 2004), Ampligen® (a mismatched double-stranded [ds]RNA), was also evaluated for efficacy alone, along with Alferon®, a human α-n3 IFN derived from leukocytes. The latter was evaluated based on the data showing that SARS-CoV replication *in vitro* was potently inhibited by IFN-α (Cinatl *et al.*, 2003; Sainz *et al.*, 2004; Stroher *et al.*, 2004) and from a study by Weck *et al.* (1982) showing that a single high dose of human IFN-α (100,000 IU) could protect mice from death due to encephalomyocarditis virus infection.

Mice were treated once i.p. with Ampligen® at 10 mg/kg or 1.0 mg/kg 4 h prior to exposure to virus. The 10 mg/kg dose effectively reduced virus titres in the lungs to below the detectable limits of the assay (Table 6).

**Table 4.** Effects of i.p. and i.n. treatment with anti-inflammatory agents on SARS-CoV replication in female BALB/c mice

Compound	Mice/group, <i>n</i>	i.p. administration		i.n. administration	
		Treatment, mg/kg	Day 3 virus titre, log <sub>10</sub> CCID <sub>50</sub> /g*	Treatment, mg/kg	Day 3 virus titre, log <sub>10</sub> CCID <sub>50</sub> /g*
Chloroquine <sup>†</sup>	15	50	4.9 ± 0.4	50	4.4 ± 1.2
		10	4.9 ± 0.3	10	5.3 ± 0.5
		1	5.1 ± 0.1	1	5.2 ± 0.1
		Placebo	4.7 ± 0.3	Placebo	5.4 ± 0.5
Amodiaquin <sup>†</sup>	15	75	4.9 ± 0.9	150	5.4 ± 0.1
		37.5	4.7 ± 0.4	75	5.1 ± 0.3
		18.8	4.5 ± 1.2	37.5	5.5 ± 0.1
		9.4	4.6 ± 0.5	10	5.3 ± 0.6
		Placebo	4.6 ± 0.5	Placebo	5.2 ± 0.3
Pentoxifylline <sup>†</sup>	15	100	5.5 ± 0.3	100	6.7 ± 0.4
		32	5.2 ± 0.2	32	6.6 ± 0.4
		10	5.5 ± 0.4	10	6.5 ± 0.5
		Placebo	5.8 ± 1.5	Placebo	6.8 ± 0.2

\*Mean ± standard deviation. Virus was titred in duplicate assays. <sup>†</sup>Twice a day for 3 days beginning 4 h pre-virus exposure. CCID<sub>50</sub>, 50% cell culture infectious dose; i.p., intraperitoneal; i.n., intranasal; ND, not done; SARS-CoV, severe acute respiratory syndrome coronavirus.

Inhibition of virus replication in the lung did not occur at the 1 mg/kg dose. When treatment was begun 24 h prior to virus exposure, no inhibition of virus titres was detected. Alferon<sup>®</sup> given once at 100,000 IU 4 h prior to virus exposure was not efficacious. All doses of each compound were well-tolerated; no dose retarded weight gain (data not shown).

Since Alferon<sup>®</sup> was not inhibitory to SARS-CoV replication in the mouse model, a human hybrid IFN shown to be active in animals (Horisberger & de Staritzky, 1987; Sidwell *et al.*, 1994) was then evaluated. Mice were treated with three doses of IFN- $\alpha$  B/D, once a day for 3 days beginning 4 h before virus exposure. At day 3 after virus exposure, all doses of the IFN significantly reduced virus lung titres compared with those from the control animals (Table 7). When animals were treated with 100,000 IU, no detectable infectious virus was recovered from the lungs of those animals. The other doses also resulted in reduced virus lung titres in the mice; there was a 1 log<sub>10</sub> drop in titres when the animals were treated with 32,000 or 10,000 IU. Virus was cleared after day 7 in all animals as is normally found in this SARS-CoV animal model (Barnard *et al.*, 2006). Interestingly, treatment with the two highest doses was somewhat toxic to the treated animals infected with virus, as seen by host weight loss; this was not seen in the uninfected, treated mice.

## Discussion

Of the agents evaluated for efficacy in the SARS-CoV mouse model, only a mismatched dsRNA IFN inducer

(Ampligen<sup>®</sup>) and a hybrid human IFN ( $\alpha$  B/D) significantly reduced virus titres in the lungs of infected animals. The anti-inflammatory agents evaluated, even those with some antiviral activity in cell culture, were not efficacious in this model, suggesting that although they may be useful in ameliorating the hyperinflammatory response, they will not significantly reduce the virus, the inducer of inflammatory response. This suggests that anti-inflammatory agents may only be useful in treating virus-induced lung infections that result in hyperinflammatory responses when used in combination with agents that inhibit virus replication (Barnard *et al.*, 2006).

As predicted by the *in vitro* experiments in this study, agents such as nelfinavir and the marginally selective chloroquine and amodiaquine, which have been suggested by many who have reviewed the field of SARS antiviral therapy (see, for example, Chihrin & Loutfy, 2005; Wu *et al.*, 2006; De Clercq, 2006) to be promising antiviral agents for treating SARS, were not active in the mouse model. The anti-inflammatory agents such as the chloroquines and PTX are therefore presumed not to be likely to be effective alone against a SARS infection.

Alferon<sup>®</sup> did not reduce virus lung titres in the SARS-CoV mouse model most probably because of the well-known species barrier between human IFN- $\alpha$  and the mouse IFN type 1 receptor (Crnic & Segall, 1992). Thus, an alternative to regular human IFN was sought to show efficacy of IFN in mice. A human hybrid IFN shown to be active in animals (Horisberger & de Staritzky, 1987; Sidwell *et al.*, 1994) was then evaluated. This IFN hybrid,

**Table 5.** Effects of i.p. treatment with an immunomodulator, 3-deazaneplanocin A, on SARS-CoV replication in female BALB/c mice

Compound	Mice/ group, <i>n</i>	i.p. administration	
		Treatment, mg/kg	Day 3 virus titre, log <sub>10</sub> CCID <sub>50</sub> /g*
3-Deazaneplanocin A <sup>†</sup> 10	10	10	5.9 ± 0.3
		0.1	5.4 ± 0.3
		Placebo	5.6 ± 0.2
3-Deazaneplanocin A <sup>†</sup> 15	15	1.0	4.7 ± 0.4
		Placebo	4.8 ± 0.3
3-Deazaneplanocin A <sup>†</sup> 30	30	10	4.6 ± 0.3
		1.0	4.0 ± 0.2
		0.1	4.2 ± 0.2
		0.05	3.7 ± 0.2
		Placebo	3.6 ± 0.4

\*Mean ± standard deviation. Virus was titred in duplicate assays. <sup>†</sup>Once a day for 3 days beginning 4 h pre-virus exposure. <sup>‡</sup>Twice a day for 3 days beginning 4 h pre-virus exposure. CCID<sub>50</sub>, 50% cell culture infectious dose; i.p., intraperitoneal; ND, not done; SARS-CoV, severe acute respiratory syndrome coronavirus.

**Table 6.** Effects of i.p. treatment with human IFN and an IFN inducer on SARS-CoV replication in female BALB/c mice

Compound	Mice/ group, <i>n</i>	i.p. administration	
		Treatment, mg/kg	Day 3 virus titre, log <sub>10</sub> CCID <sub>50</sub> /g*
Ampligen <sup>®†</sup>	15	10	<1.2 <sup>††</sup>
		1.0	4.7 ± 0.5
		Placebo	4.5 ± 0.7
Ampligen <sup>®§</sup>	15	32	4.3 ± 0.3
		3.2	4.5 ± 0.5
		Placebo	4.1 ± 0.5
Alferon <sup>®¶</sup>	15	100,000 <sup>§</sup>	4.9 ± 0.4
		Placebo	4.5 ± 0.7

\*Mean ± standard deviation. Virus was titred in duplicate assays.

<sup>†</sup>Title of ≤1.2 was the limit of detection for this assay. <sup>††</sup>Significantly different from other treatment groups and placebo control, *P*<0.001. <sup>§</sup>Expressed in IU. <sup>¶</sup>Four hour pre-virus exposure. <sup>‡</sup>Twenty-four hour pre-virus exposure. CCID<sub>50</sub>, 50% cell culture infectious dose; IFN, interferon; i.p., intraperitoneal; SARS-CoV, severe acute respiratory syndrome coronavirus.

**Table 7.** Effects of i.p. IFN-α B/D treatment (once a day for 3 days, beginning 4 h before virus exposure) on the replication of SARS-CoV (Urbani) in female 11–14 g BALB/c mice

	Mice/ group, <i>n</i>	Day 3			Day 7		
		Treatment, IU/injection	Virus titre, log <sub>10</sub> CCID <sub>50</sub> /g*	Body weight change, g*	Treatment, IU/injection	Virus titre, log <sub>10</sub> CCID <sub>50</sub> /g	Body weight change, g
Virus-infected mice	20	100,000	<0.8 <sup>†‡</sup>	-1.1 ± 1.5 <sup>‡</sup>	100,000	0	-0.8 ± 2.1 <sup>§</sup>
		32,000	3.4 ± 1.3 <sup>§</sup>	-1.1 ± 0.5 <sup>‡</sup>	32,000	0	-1.0 ± 1.3 <sup>§</sup>
		10,000	3.4 ± 1.1 <sup>§</sup>	-1.1 ± 0.9 <sup>‡</sup>	10,000	0	0.7 ± 1.5
		Placebo	4.5 ± 0.7	0.7 ± 0.6	Placebo	0	0.7 ± 1.8
Uninfected mice	5	100,000	0	1.2 ± 0.5	100,000	0	1.9 ± 0.4
		32,000	0	1.2 ± 0.5	32,000	0	1.0 ± 1.4
		10,000	0	1.6 ± 0.7	10,000	0	2.6 ± 1.8
		Placebo	0	0.7 ± 0.4	Placebo	0	1.3 ± 0.4

\*Mean ± standard deviation. Virus was titred in duplicate assays. <sup>†</sup>Title representing the limits of detection for this assay. <sup>‡</sup>Significantly different from other treatment groups and placebo control, *P*<0.001. <sup>§</sup>Significantly different from placebo control, *P*<0.01. CCID<sub>50</sub>, 50% cell culture infectious dose; IFN-α, interferon alpha; i.p., intraperitoneal; SARS-CoV, severe acute respiratory syndrome coronavirus.

α B/D, consists of amino acids 1 to 60 from HuIFN-α B and amino acids 61 to 166 from HuIFN-α D (Wintergerst *et al.*, 1999). The profile of cross-species activity of the IFN-α B/D hybrid has been compared with that of HuIFN-α F, and of the parents HuIFN-α B and -α D. When both IFN-α B and -α D were active in a cell species, the hybrid IFN had comparable or better activity than the more active parental IFN (Horisberger & de Staritzky, 1987).

The hybrid shared a broad cross-species activity with IFN-α D (Horisberger & de Staritzky, 1987). When evaluated against Punta Toro virus *in vivo*, a very similar preparation, α A/D, significantly reduced mean day to death and improved liver scores compared with infected untreated controls (Sidwell *et al.*, 1994). Thus, it was postulated that IFN-α B/D could be used to treat SARS-CoV infection in mice. The results of the current study demonstrated that



low-dose human hybrid IFN- $\alpha$  B/D could reduce SARS-CoV replication in the lungs of infected mice; at the highest dose of 100,000 IU, detectable infectious virus was inhibited to below detectable limits. This suggests that human IFN- $\alpha$  should be considered for use in treating human infections. It is of concern that infected mice lost significant amounts of weight when treated with IFN. It is likely that IFN may contribute to the tissue degradative effects of tumour necrosis factor (TNF)- $\alpha$ . For example, IFN- $\alpha$  has been shown to enhance TNF- $\alpha$  promotion of apoptosis (Nakashima *et al.*, 2005). Since TNF- $\alpha$  is already induced by SARS-CoV infection, which has polarized the mouse immune system to a Th-2 inflammatory response (Glass *et al.*, 2004; Barnard *et al.*, 2006), addition of IFN may add to the observed cachexia associated with TNF- $\alpha$  treatment (Chiffolleau *et al.*, 2003) by also promoting apoptosis. Thus, the question needs to be asked as to whether a weight loss would be detrimental to patient recovery from SARS-CoV infection when treated with an IFN.

The data also suggest that the poly I:C analogue, Ampligen®, may be useful in treating infections when administered at the appropriate time. Studies need to be carried out to determine if the compound could be used therapeutically. Otherwise, the utility of such an agent may be limited to prophylactic treatment of potentially exposed contacts of diagnosed patients.

The data suggest that induction of IFN by mismatched dsRNAs or actual treatment with IFN- $\alpha$  can inhibit SARS-CoV replication in the mouse. These types of agents should be studied further as possible drugs for treating SARS infections, perhaps in combination with anti-inflammatory agents.

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