

## Ribavirin and interferon- $\beta$ synergistically inhibit SARS-associated coronavirus replication in animal and human cell lines

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### Abstract

Initial *in vitro* investigations demonstrated type I interferons (IFNs: IFN- $\alpha$ , IFN- $\beta$ ) to inhibit replication of SARS coronavirus (SARS-CoV), but found the nucleoside analogue ribavirin ineffective in Vero cells. In this report, ribavirin was shown to inhibit SARS-CoV replication in five different cell types of animal or human origin at therapeutically achievable concentrations. Since clinical anti-SARS-CoV activity of type I interferons or ribavirin is limited, we investigated the combination of IFN- $\beta$  and ribavirin. Determination of the virus yield indicated highly synergistic anti-SARS-CoV action of the combination suggesting the consideration of ribavirin plus IFN- $\beta$  for the treatment of SARS.

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From the end of 2002 a new form of non-typical pneumonia, the severe acute respiratory syndrome (SARS), emerged from the Guangdong province in southern China, spread throughout the world, infected 8420 people in 29 countries, and resulted in more than 900 deaths (<http://www.who.int/csr/sars>). The causative agent was found to be a new member of the coronavirus family, the SARS-coronavirus (SARS-CoV) [1].

Corticosteroids, antibiotics, and antiviral agents were empirically used for the treatment of SARS. The rapid emergence of the epidemic did not permit the conduction of controlled studies documenting the efficacy of therapies. Ribavirin (1- $\beta$ -D-ribofuranosyl-1,2,4-triazole-3-carboxamide), a synthetic guanosine analogue and broad-spectrum inhibitor of RNA and DNA viruses, was frequently used for treatment of SARS patients [2]. However, reports about treatment success are con-

troversial and therapy-limiting side effects have been reported in SARS patients [2].

In first studies, ribavirin did not inhibit SARS-CoV replication in Vero (African green monkey kidney) cells [3,4]. Newer experiments demonstrated anti-SARS-CoV activity of ribavirin in foetal rhesus kidney 4 cells (fRHK-4) in concentrations of about 50  $\mu$ g/ml [5,6] being still above mean plasma levels which are in the range of 24  $\mu$ g/ml after *i.v.* administration of 1000 mg ribavirin [2]. To date, no systematic investigation of the effect of ribavirin on SARS-CoV replication in human cells lines had been performed.

In numerous *in vitro* studies effects of interferons (IFNs) on SARS-CoV replication had been observed [7]. Our investigations performed in Caco2-(human colon carcinoma) cells and Vero cells demonstrated type I IFNs (IFN- $\alpha$ , IFN- $\beta$ ) to have superior anti-SARS-CoV activity compared to that of type II IFN- $\gamma$ . Although IFN- $\beta$ -1b (betaferon) was shown to inhibit SARS-CoV replication more effectively compared to IFN- $\alpha$ -2b

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(intron A) [8], some other IFN- $\alpha$  subtypes and human leukocyte IFN- $\alpha$  were highly effective [5,9]. Preliminary clinical studies showed treatment with synthetic recombinant type I IFN- $\alpha$  (alfacon-1) to be beneficial for SARS patients [10].

Clinical evaluation of the combination of type I IFNs including IFN- $\beta$  with ribavirin in patients with chronic hepatitis C demonstrated the superiority of the combination to either single treatment and resulted in decreased pharmacological active plasma levels for either drug [11]. Here, we tested the anti-SARS-CoV activity of the combination of ribavirin with IFN- $\beta$  in different human and animal cells.

## Materials and methods

**Compounds.** Ribavirin solution (1 mg/ml) was obtained from ICN Pharmaceuticals (Costa, Mesa, CA), while recombinant interferon- $\beta$ -1b (betaferon) was obtained from Schering (Berlin, Germany).

**Cells and viruses.** African green monkey kidney cell lines Vero (CCL-81) and MA-104 (CRL-2378.1), and pig kidney cell line PK-15 (CCL-33) were obtained from ATCC (Manassas, VA, USA). Human colon carcinoma cell lines Caco2 and CL-14 were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ; Braunschweig, Germany).

Vero, MA-104, PK-15, and Caco2 cells were grown at 37 °C in MEM supplemented with 10% fetal bovine serum (FBS) containing 100 IU/ml penicillin and 100  $\mu$ g/ml streptomycin. CL-14 cells were grown in 37 °C in HAM's F12 supplemented with 10% FBS containing 100 IU/ml penicillin and 100  $\mu$ g/ml streptomycin.

Human primary epithelial kidney (HPEK) cells were prepared from human renal tissue after nephrectomy from portions of the kidney (carcinoma free) and immunomagnetically isolated as described before and grown in medium 199 with 10% FBS at 37 °C [12].

SARS-CoV strain FFM 1 [1] and strain 6109 (courtesy of Prof. Wilina Lim, Government Virus Unit, Hong-Kong) were prepared by infecting Vero cell cultures. Supernatants from infected cultures were collected 2 days post-infection and aliquots were stored at -80 °C. Virus titres were determined by 50% tissue culture infective dose (TCID<sub>50</sub>) in confluent cells in 96-well microtitre plates as described [3].

**Antiviral assay.** Confluent cell cultures were infected with SARS-coronavirus strain FFM1 or strain 6109, for 1 h in 96-well microplates. After adsorption period, cells were washed with PBS and incubated in MEM supplemented with 2% FBS. Cytopathogenic effect (CPE) was assessed visually 72 h after infection.

In some experiments virus yield reduction assay was performed as described before [8]. Confluent cell layers grown in 12.5 cm<sup>2</sup> cell culture flasks were infected with SARS-CoV strain FFM 1 or 6109. After 1 h incubation period, cells were washed four times with PBS and incubated (37 °C) in MEM supplemented with 2% FBS. After 72 h cultures and supernatants were freeze-thawed and viral titres were determined by the 50% tissue culture infective dose (TCID<sub>50</sub>) in confluent Vero

cells on 96-well microtitre plates [8]. The inhibitory effects were expressed as effective concentrations of compounds required to inhibit infectious virus titres by 50% (EC<sub>50</sub>), 75% (EC<sub>75</sub>) or 90% (EC<sub>90</sub>) of the control value, respectively.

**Combination studies.** Combination indices (CIs) indicating whether both substances acted synergistically, additively, or antagonistically when used in combination were calculated using CalcuSyn for Windows. The calculation is based on the method of Chou and Talalay [13]. A CI lower 1 indicates synergistic action, a CI higher 1 indicates antagonistic action, and a CI of 1 indicates additive action.

## Results

### Cellular permissiveness to SARS-CoV replication

Cells were inoculated with SARS-CoV strain FFM1 at a MOI of 0.01. Maximum virus titres were  $6.3 \times 10^7$  TCID<sub>50</sub>/ml in Caco2,  $4.2 \times 10^7$  TCID<sub>50</sub>/ml in CL14,  $7.7 \times 10^6$  TCID<sub>50</sub>/ml in HPEK cells,  $7.0 \times 10^6$  in MA104 cells, and  $1.2 \times 10^7$  in PK-15 cells. Infection of Caco2 and CL14 with the SARS-CoV strain 6109 resulted in infectious virus titres of  $1.6 \times 10^7$  and  $1.4 \times 10^7$  TCID<sub>50</sub>/ml, respectively, which is similar to titres determined for strain FFM1. CPE, detected 72 h post-infection with FFM1 or 6109 strain, was characterised by considerable morphological changes such as numerous rounded, enlarged, and detached cells (representative photograph shown in Fig. 1 for Caco2 cells).

### Antiviral activity

As shown before [3,4] ribavirin did not inhibit SARS-CoV-replication and CPE formation in Vero cells at concentrations up to 1000  $\mu$ g/ml. However, ribavirin inhibited SARS-CoV replication in other permissive cell lines tested (Table 1). EC<sub>50</sub>s ranging from 2.2 to 9.4  $\mu$ g/ml, EC<sub>75</sub>s ranging from 5.8 to 14.2  $\mu$ g/ml, and EC<sub>90</sub>s ranging from 15.4 to 29.8  $\mu$ g/ml were found in FFM1-infected cells by determination of virus yield (MOI 0.01). High concentrations of ribavirin (50  $\mu$ g/ml) completely suppressed CPE formation 72 h p.i. in all cell lines infected with FFM1 or 6109 (MOI 0.01). Ribavirin inhibited SARS-CoV strain 6109-replication similarly to strain FFM1 (not shown).

The influence of the infectious dose on the anti-SARS-CoV activity of ribavirin was investigated in Caco2 cells infected at different MOIs by measurement

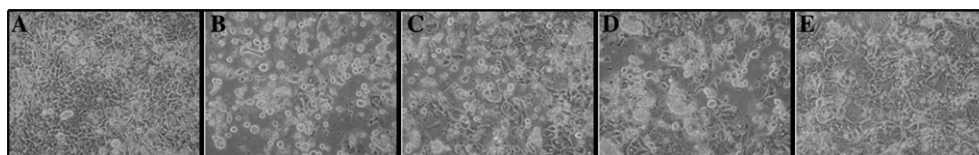


Fig. 1. Morphological appearance of mock-infected cells (A) and SARS-CoV-infected Caco2 cells (strain FFM1) without treatment (B) or treated with ribavirin (6.2  $\mu$ g/ml) (C), IFN- $\beta$  (1250 IU/ml) (D) or a combination of ribavirin (6.2  $\mu$ g/ml) and IFN- $\beta$  (1250 IU/ml) (E).

Table 1  
Effect of ribavirin on infectious SARS-CoV strain FFM1 titres in different cell types

Cells	Ribavirin ( $\mu\text{g/ml}$ )		
	EC <sub>50</sub>	EC <sub>75</sub>	EC <sub>90</sub>
Monkey			
Vero	>1000	>1000	>1000
MA104	9.4 $\pm$ 4.1 <sup>a</sup>	14.2 $\pm$ 5.4	29.8 $\pm$ 2.2
Porcine			
PK-15	2.2 $\pm$ 0.8	5.8 $\pm$ 1.2	15.4 $\pm$ 4.1
Human			
Caco2	7.3 $\pm$ 3.5	10.6 $\pm$ 4.1	23.3 $\pm$ 9.5
CL14	8.2 $\pm$ 4.2	12.2 $\pm$ 5.2	25.7 $\pm$ 13.7
HPEK	5.2 $\pm$ 2.9	11.0 $\pm$ 3.8	23.9 $\pm$ 12.1

<sup>a</sup> Values are means of three assays  $\pm$  SD.

of virus titre. EC<sub>50</sub>-values were 7.3  $\pm$  3.5  $\mu\text{g/ml}$  for MOI 0.01, 6.1  $\pm$  3.1  $\mu\text{g/ml}$  for MOI 0.1, and 4.7  $\pm$  1.9  $\mu\text{g/ml}$  for MOI 1, showing no significant influence of the infectious dose on ribavirin-caused SARS-CoV replication inhibition.

Combinations of IFN- $\beta$  and ribavirin were tested in a fixed ratio of 200:1 (IU/ml for IFN- $\beta$ ,  $\mu\text{g/ml}$  for ribavirin) starting with 10,000 IU/ml IFN- $\beta$  and 50  $\mu\text{g/ml}$  ribavirin. Starting concentrations were diluted in ratios of 1:2. The combination synergistically inhibited SARS-CoV-induced CPE in Caco2 and CL14 cells (representative photographs shown in Fig. 1) as well as production of infectious virus (Table 2). EC<sub>50</sub>, EC<sub>75</sub>, and EC<sub>95</sub> of ribavirin, IFN- $\beta$  or their combination in infected human intestinal cell lines Caco2 and CL-14 were determined by measurement of infectious virus titre. For example, in Caco2 cells infected with SARS-CoV FFM1 strain (MOI 0.01), ribavirin concentrations inhibiting virus production in combination with IFN- $\beta$  were at

Table 2  
Effect of the combination of ribavirin with interferon- $\beta$  (IFN- $\beta$ ) on infectious virus yields in Caco2 and CL14 cells infected with FFM1 strain

Cell line	EC <sub>50</sub>	EC <sub>75</sub>	EC <sub>90</sub>
<i>Caco2</i>			
Ribavirin ( $\mu\text{g/ml}$ )	4.7 $\pm$ 2.6 <sup>a</sup>	10.6 $\pm$ 3.7	22.4 $\pm$ 5.9
IFN- $\beta$ (IU/ml)	28 $\pm$ 7	433 $\pm$ 90	6686 $\pm$ 850
Combined			
Ribavirin	0.3 $\pm$ 0.12	0.7 $\pm$ 0.27	1.6 $\pm$ 0.51
IFN- $\beta$	0.6 $\pm$ 0.27	1.4 $\pm$ 0.34	3.3 $\pm$ 0.99
CI	0.45 $\pm$ 0.07	0.3 $\pm$ 0.05	0.21 $\pm$ 0.05
<i>CL14</i>			
Ribavirin	5.3 $\pm$ 3.3	12.3 $\pm$ 5.1	28.3 $\pm$ 7.3
IFN- $\beta$	1055 $\pm$ 151	3189 $\pm$ 90	9639 $\pm$ 911
Combined			
Ribavirin	2.4 $\pm$ 0.3	5.2 $\pm$ 1.6	11.4 $\pm$ 4.7
IFN- $\beta$	4.8 $\pm$ 1.7	10.5 $\pm$ 3.1	22.8 $\pm$ 6.2
CI <sup>b</sup>	0.46 $\pm$ 0.06	0.42 $\pm$ 0.06	0.41 $\pm$ 0.04

<sup>a</sup> Mean ( $\pm$ SD) value of three assays.

<sup>b</sup> Combination index (CI); CI < 1 indicates synergism, CI = 1 indicates additive effect, and CI > 1 indicates antagonism.

least 10-fold lower when compared with cultures receiving single treatment with ribavirin. Inhibitory concentrations of IFN- $\beta$  were 50- to 2000-fold decreased in combination relative to single treatment with IFN- $\beta$ . Calculation of CIs revealed highly synergistic antiviral effects of the drug combination. In FFM1-infected Caco2 cells the CI values were 0.45  $\pm$  0.07 at the EC<sub>50</sub>, 0.3  $\pm$  0.05 at the EC<sub>75</sub>, and 0.21  $\pm$  0.05 at the EC<sub>90</sub>. In FFM1-infected CL14 cells (MOI 0.01) the CI values were 0.46  $\pm$  0.06 at the EC<sub>50</sub>, 0.42  $\pm$  0.06 at the EC<sub>75</sub>, and 0.41  $\pm$  0.04 at the EC<sub>90</sub>.

## Discussion

Ribavirin was initially found not to inhibit SARS-CoV replication at concentrations up to 1000  $\mu\text{g/ml}$  [3,4]. These results had been obtained in Vero cells (or its E6 subclone), in which ribavirin is known to be of low antiviral activity, most probably because of insufficient phosphorylation to its active triphosphorylated form. More recent studies showed that ribavirin inhibits SARS-CoV replication in fRHK-4 cells at concentrations of about 50  $\mu\text{g/ml}$  [5,6]. These findings suggest that multiple cell culture systems should be used to evaluate the activity of antiviral agents against emerging viruses such as SARS-CoV.

In the present study, the effects of ribavirin on SARS-CoV replication were systematically investigated in a panel of SARS-CoV permissive cell lines. Vero cells, human colon carcinoma cell lines Caco2 and CL14, and pig kidney cell line PK-15 had already been described to be permissive to SARS-CoV infection by us and by others [3,8,14]. African green monkey kidney cell line MA-104 and primary epithelial human kidney (HPEK) cells were demonstrated to be permissive to SARS-CoV replication in this report. HPEK cells represent the first normal diploid cell type of human origin permissive to SARS-CoV infection. Ribavirin inhibited SARS-CoV replication in all newly tested cell lines at concentrations at least 100-fold lower when compared with Vero cells.

The clinical value of ribavirin for the treatment of SARS patients is regarded with scepticism. We observed about 90% viral replication inhibition at concentrations up to 24  $\mu\text{g/ml}$  ribavirin which is at reasonable therapeutical plasma levels after an intravenous dose of 1000 mg ribavirin [2]. This may be insufficient to improve clinical symptoms. In concordance, randomised clinical trials showed low dose (400–600 mg/day) ribavirin to be ineffective [15]. On the other hand, the most recently published study reported that ribavirin reduced the viral load in 5 of the 8 patients [16]. Moreover, this study suggested that the peak inflammatory cytokine (IL-6 and IL-8) levels concurred with or after the peak viral load and preceded or

concurrent with the maximum pulmonary infiltrates. Thus, it is probable that viral replication leads to the activation of proinflammatory cytokines that, together with other factors, contribute to disease progression. These clinical findings together with the observation of antiviral activity in different SARS-CoV-infected cell lines encourage the testing of treatment strategies using ribavirin in combination with other antiviral agents such as IFNs to increase inhibitory effects on virus replication and subsequently minimised immunopathological damages.

Type I IFNs had been shown to inhibit SARS-CoV replication *in vitro* most effectively in cells that had been pre-treated before virus inoculation [7]. A pilot clinical report demonstrated the effectiveness of synthetic recombinant IFN- $\alpha$  for the treatment of SARS patients [10]. Overall, these findings suggest that only high doses of IFNs exhibit anti-SARS effects and are mainly effective for prophylaxis during SARS epidemics.

To reduce the drug concentrations needed for SARS-CoV inhibition, the anti-SARS-CoV activity of the combination of ribavirin with type I interferon was tested previously. Tan et al. [9] reported that ribavirin in combination with IFN- $\beta$  did not demonstrate observable synergistic effects whereas Chen et al. [5] showed highly synergistic activity. However, both studies reported solely on CPE formation in Vero cells. Our results demonstrate that both drugs act highly synergistically in combination on formation of CPE as well as on production of infectious virus titres in two human cell lines. Since antiviral drugs were added after virus adsorption, the results show that IFN- $\beta$  in combination with ribavirin may be highly effective not only as prophylactic agent but also for the treatment of already infected SARS patients.

In conclusion, the combination of ribavirin with IFN- $\beta$  inhibits SARS-CoV replication in drastically reduced concentrations compared to either single treatment. This may enable the achievement of therapeutic plasma levels sufficient to suppress SARS-CoV replication during the early phase of SARS, and thus to prevent subsequent immunopathological damages. Moreover, efficient early inhibition of virus replication may reduce virus shedding and consequently, the risk of transmission.

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