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## Interferon alfacon 1 inhibits SARS-CoV infection In human bronchial epithelial Calu-3 cells

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### Abstract

The primary targets for SARS-CoV infection are the epithelial cells in the respiratory and intestinal tract. The angiotensin-converting enzyme 2 (ACE-2) has been identified as a functional receptor for SARS-CoV. ACE-2 has been shown to be expressed at the apical domain of polarized Calu-3 cells. In this report, interferon alfacon 1 was examined for inhibitory activities against SARS-CoV on human lung carcinoma epithelial Calu-3 cell line and the other three African green monkey kidney epithelial cell lines. Interferon alfacon 1 demonstrated significant antiviral activity in neutral red uptake assay and virus yield reduction assay. The data might provide an important insight into the mechanism of pathogenesis of SARS-CoV allowing further development of antiviral therapies for treating SARS infections.

### Keywords

angiotensin-converting enzyme 2; ACE-2; Calu-3 cells; IFN-alfacon 1; neutral red uptake assay; SARS-CoV; severe acute respiratory syndrome; virus yield reduction assay

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Severe acute respiratory syndrome (SARS) is the first new infectious disease of this century caused by a novel human corona virus (SARS-CoV) that leads to pulmonary pathological features [1,2]. SARS, which originated from Southern China at the end of 2002, has a high mortality and morbidity. Since then the disease had spread to more than 30 countries. By July 31, 2003, more than 8,000 SARS cases and nearly 800 SARS-related deaths were reported around the world. Studies on the molecular evolution of SARS-CoV suggested that the virus emerged from non-human sources [3]. The disease poses a new threat for respiratory medicine and represents a challenge for antiviral drug development and administration [4,5].

The combination of ribavirin and corticosteroids was the most frequently administered antiviral agent for SARS-CoV [6,7,8,9,10]. However, ribavirin at nontoxic concentrations has little *in vitro* activity against SARS-CoV [11] and has many side effects [8]. An improved clinical outcome was reported among SARS patients receiving early administration with Kaletra plus ribavirin and corticosteroids [12]. We recently reported that ribavirin could enhance the SARS-

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CoV infectivity in BALB/c mice and our data did not support the use of ribavirin for treating SARS patients [13]. Antibodies to the SARS-CoV spike protein have been shown to block entry [14]. Small peptides derived from the heptad repeat (HR) regions of SARS-CoV S protein have been shown to inhibit SARS-CoV infection by the interference of SARS-CoV fusion with target cells [15,16]. SARS main protease, which is essential for the replication cycle of SARS-CoV, has been a key target for developing anti-SARS drugs [17,18]. Antisense RNA and RNA interference (RNAi) technologies have shown potential prospect in treating some severe diseases [19] and has been considered as important candidate medicines in the treatment of SARS infection. However, no clear evidence was demonstrated to support these clinical observations. Therefore, development of new anti-SARS-CoV agents is urgently needed for the treatment of SARS patients.

Interferons are small, natural or synthetic protein and glycoprotein cytokines that are produced by leucocytes, T-lymphocytes, and fibroblasts in response to infection and other biological stimuli. Interferon can activate tumor-specific cytotoxic T-lymphocytes, which play an important role in destroying foreign cells in the body. Interferons attach to special receptors on the surface of cell membranes and induce transcription, which results in an anti-viral state in the target cells. In 1957 researchers discovered that the immune system produced a substance in response to a viral infection that acted as an anti-viral agent [20,21]. They called that substance "interferon." Since then, recombinant DNA technology has provided a larger supply of interferons and has allowed extensive research regarding interferon's therapeutic properties. In a recent study, we evaluated a few compounds approved for therapeutic use in humans and some *in vitro* inhibitors of SARS-CoV 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 inducer, Ampligen (poly I:poly C124), were shown to potently inhibit virus titers in the lungs of infected mice [22]. In this report, we describe a cell-based assay using SARS-CoV infection of human lung epithelial cells (Calu-3) and three African green monkey kidney epithelial cell lines to evaluate a related compound, interferon alfacon 1 (IFN-alfacon 1, consensus interferon, Infergen™) against SARS-CoV.

## Materials and methods

Three African green monkey kidney epithelial cell lines, Vero 76, Vero E6, and MA104 (embryonic), were obtained from American Type Culture Collection (ATCC, Manassas, VA) [23]. Vero 76 and MA104 cells were routinely grown in minimal essential medium (MEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Hyclone Laboratories, Logan, UT) and Vero E6 cells were maintained in MEM supplemented with 10% FBS, 1% MEM non-essential amino acids (Sigma-Aldrich Co., St. Louis, MO) and 1 mM of sodium pyruvate (Sigma-Aldrich). Calu-3 cells are a human lung epithelial cell line originating from a human pulmonary adenocarcinoma and, were grown in Dulbecco's modified Eagle's minimal essential medium (DMEM) supplemented with 20% FBS (D-20) [24]. SARS-CoV, strain Urbani (200300592), was obtained from the Centers for Disease Control (CDC, Atlanta, GA). The strain was propagated and titrated in Vero 76 cells. For antiviral assays, the serum was reduced to 2% and gentamicin added to the medium at a final concentration of 50 µg/ml. Stock solutions of IFN-alfacon 1 (InterMune, Inc., Brisbane, CA) were made at a concentration of 15 µg/0.5 ml and stored at -20°C. IFN-alfacon 1 was solubilized in MEM for *in vitro* experiments.

The neutral red (NR) uptake assay for determination of antiviral efficacy and compound cytotoxicity was used for *in vitro* evaluation [13]. The cells were seeded to the 96-well tissue culture plate (Corning Incorporated Costa, NY) in 0.1 ml at  $2 \times 10^5$  cells/ml one day before, and treated with IFN-alfacon 1 at the different concentrations. Then, the cells were infected with SARS-CoV isolate at the multiplicity of infection (m.o.i) from 0.0007 to 0.003. Compound

and virus were added in equal volumes (0.1 ml). The plates were incubated at 37°C for three days until the cells in the virus control wells showed complete viral cytopathic effect (CPE) by light microscopy. Each concentration of compound was assayed for 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.

At three days post virus exposure, the cells were scored for percent CPE or for cytotoxicity by microscopic observation. The values obtained were converted to percents of untreated, uninfected controls. The 50% cell cytotoxic doses ( $CC_{50}$ ) and 50% virus inhibitory doses ( $IC_{50}$ ), representing the putative concentration at which 50% of the monolayers would show compound cytotoxicity or virus cytopathic effect, respectively, 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 NR uptake assay on the same plate. The NR assay was performed using a modified method of Cavanaugh et al. [25] as described by Barnard et al. [13]. Briefly, NR dye was added to each well of the plate, and the plate incubated for approximately 2 h at 37°C in the dark. The NR solution was removed from the wells, rinsed and any remaining dye extracted using ethanol buffered with Sörenson's citrate buffer. The plates were read at 405/540 nanometers (nm) wavelength with a microplate reader (Opsys MR™, Dynex Technologies, Chantilly, VA). 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 is more sensitive assay to confirm the results of the NR uptake assay. Infectious virus yield from the CPE inhibition assay were determined on the supernatant from the test well as previously described [13]. Sample wells at each compound concentration tested were pooled and titered in Vero 76 cells for infectious virus by CPE assay as previously described by Barnard et al. [26]. A 90% reduction in virus yield ( $IC_{90}$ ) was then calculated by linear regression analysis. This represented a one- $\log_{10}$  inhibition in titer when compared to untreated virus controls.

## Results

We first examined the effect of IFN-alfacon 1 on the replication of SARS-CoV in Vero 76 cells at 37°C. IFN-alfacon 1 inhibited virus replication in a dose-dependent manner. IFN-alfacon 1 had an  $IC_{50}$  of 0.14 ng/ml and  $CC_{50}$  of >100 ng/ml as determined by NR assay (Table 1). However, IFN-alfacon 1 did not always work in the Vero 76 cells. The activity against SARS-CoV in Vero 76 cells was sporadic and appeared to be highly variable depending upon the condition (age and health) of the host cells. The monolayers of Vero 76 cells had to be rinsed gently to achieve valid NR assays. Then, IFN-alfacon 1 inhibition of virus replication was further tested in Vero E6 cells and MA104 cells. IFN-alfacon 1 provided a complete protection at the higher doses and a linear dose response at the lower part of the concentration curve.  $IC_{50}$  was 2.62 ng/ml in Vero E6 cells and, 0.12 ng/ml in MA104 cells, respectively (Table 1). IFN-alfacon 1 also inhibited SARS-CoV isolate with SI values ranging from >38 to >833 (Table 1). The cytopathic effect in infected Calu-3 cells was not observed within three days of infection.

The activity of IFN-alfacon 1 was further tested in a virus yield reduction assay. IFN-alfacon 1 reduced virus yields by 90% at 2.25 ng/ml in Vero 76 cells. IFN-alfacon 1 reduced virus yields by 90% at 3.02 ng/ml in Vero E6 cells. IFN-alfacon 1 had an  $IC_{90}$  = 0.33 ng/ml in MA104 cells, which confirmed the activity demonstrated by NR assay (Table 2). SARS-CoV was found to productively infect human bronchial epithelial Calu-3 cells; SARS-CoV-induced CPE was initially observed in medium containing 20% FCS at day 8 and 2% FCS at day 4 [24]. Based

on these observations, IFN-alfacon 1 was evaluated to test the inhibitory effect on plating of SARS-CoV infection from the culture fluids of the infected Calu-3 cells. The supernatants were kept frozen at  $-80^{\circ}\text{C}$  until they were needed for assessing infectious viral titers by a standard TCID<sub>50</sub> assay with permissive Vero 76 cells, as described above. The activity of IFN-alfacon 1 was confirmed by the virus yield reduction assay. IFN-alfacon 1 blocked viral production after incubation for three days in comparison to mock-treated cells and reduced virus yields by 90% at 0.046 ng/ml (Table 2). IFN-alfacon 1 inhibited SARS-CoV infection in Calu-3 with SI value of 2173 (Table 2). We also verified that SARS-CoV can infect human bronchial epithelial Calu-3 cells without causing significant cytopathic effects within 3 days of infection (Table 3).

## Discussion

Interferons have been considered as the first line of defense against viral infections. Interferon type I is divided into interferon-alpha (leukocyte interferon) produced by virus-infected leukocytes and interferon-beta (fibroblast interferon) produced by virus-infected fibroblasts, or virus-infected epithelial cells. Type 1 interferons (alpha/beta) have been shown to be effective for treating patients with hepatitis type C (HCV) [27]. Alpha interferons, which not only block the replication of several viruses but also activate the immune system, have also been shown to be efficacious in treating hairy cell leukemia, malignant melanoma, and Kaposi's sarcoma (an AIDS-related cancer) and interferon alpha was shown to effectively inhibit SARS-CoV replication [28]. IFN-alfacon 1 is a non-naturally occurring, novel synthetic recombinant type 1 interferon alpha developed by comparing the amino acid sequences of several natural interferon-alpha subtypes and assigning the most frequently observed amino acid in each corresponding position to generate a consensus molecule. IFN-alfacon 1 was also approved by the US Food and Drug Administration as the new class of anti-HCV inhibitors. IFN-alfacon 1 is the only IFN tested in human trials. The re-treatment with IFN-alfacon 1 is effective therapy for patients with chronic hepatitis C who have either not responded to previous interferon therapy or relapsed after discontinuation of interferon therapy [29]. Paragas et al. reported that the IFN-alfacon 1 had the anti-SARS-CoV activity only before infection in cell-based model *in vitro* [30]. In this case, IFN-alfacon 1 most likely induces an antiviral state in the target cells, which produce a cellular environment that is not suitable for viral replication. However, IFN-alfacon 1 did not show activity when the cells were treated with IFN-alfacon 1 after infection with SARS-CoV, suggesting that IFN-alfacon 1 might not directly block viral replication in cells that are already infected [30]. Loutfy et al. demonstrated that IFN-alfacon 1 was combined with corticosteroids, in a preliminary pilot study, to assess potential clinical benefit and safety for SARS patients. This data suggested that IFN-alfacon 1 might function as an antiviral therapeutic for the treatment of SARS patients [31].

Epithelial cells are a primary barrier to infection by microorganisms entering the host via body cavities. Epithelial cells are organized in a polarized fashion with two distinct membrane domains, the apical and basolateral domains [32]. The primary targets for SARS-CoV infection are the epithelial cells in the respiratory and intestinal tract. It has been reported that the angiotensin-converting enzyme 2 (ACE-2) is the functional receptor for SARS-CoV [33]. Mossel et al. demonstrated that Vero E6, MA104 and Calu-3 cells expressed human ACE-2 on the apical membrane domain. The infection was initiated successfully from the apical, but not from the basolateral side [23]. Tseng et al. published the first SARS-CoV *in vitro* replication model using monolayers and polarized Calu-3 cells. The receptor for SARS-CoV is localized on and mediates infection through the apical plasma membrane of respiratory epithelial cells and SARS-CoV entry and release may be focused on the apical surface of these cells [24]. Except Calu-3 cells, no human respiratory epithelial cell lines have been shown as yet to support SARS-CoV infection. In addition to searching for vaccines against SARS-CoV, it is important for advancing our knowledge to understand how SARS-CoV interact with the polarized lung

epithelial cells and, to look for other approaches to block viral replication as a means of chemotherapeutic control.

We developed this simple assay system to screen one compound in Calu-3 cells, which had been infected with SARS-CoV isolate, by virus yield reduction assay. Calu-3 cells were treated with IFN- $\alpha$ 1 and, inoculated with SARS-CoV isolate, the titers of infectious viral particles in the supernatants harvested after infection for three days. We verified that SARS-CoV can be propagated in Calu-3 cells in 3 days (Table 3). The human lung Calu-3 cell line provides a useful model for evaluating efficacy and mechanisms of anti-SARS compounds such as interferon. IFN- $\alpha$ 1 was shown to be more active against SARS-CoV when tested in human lung epithelial Calu-3 cells than in African green monkey epithelial cells (Table 2). In our two assay systems, IFN- $\alpha$ 1 demonstrated significant antiviral activity in neutral red uptake assay and virus yield reduction assay. Our data might provide us an important insight into the mechanism of pathogenesis of SARS-CoV and these properties might be therapeutically advantageous.

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**Table 1**Effect of interferon alfacon 1 on SARS-CoV replication in different cell lines *in vitro*

| Cell lines <sup>a</sup> | Neutral red (NR) uptake assay (ng/ml) |                               |                 |
|-------------------------|---------------------------------------|-------------------------------|-----------------|
|                         | IC <sub>50</sub> <sup>b</sup>         | CC <sub>50</sub> <sup>c</sup> | SI <sup>d</sup> |
| Vero 76                 | 0.14 ± 0.07 <sup>e</sup>              | >100                          | >714            |
| Vero E6                 | 2.62 ± 2.06                           | >100                          | >38             |
| MA104                   | 0.12 ± 0.06                           | >100                          | >833            |
| Calu-3                  | ND <sup>f</sup>                       | >100                          | ND              |

<sup>a</sup>Vero 76, Vero E6 and MA104 cells were seeded to the 96-well tissue culture plate in 0.1 ml at  $2 \times 10^5$  cells/ml. Calu-3 cells were seeded in 0.2 ml at  $4 \times 10^5$  cells/ml.

<sup>b</sup>IC<sub>50</sub>: 50% inhibitory concentration.

<sup>c</sup>CC<sub>50</sub>: 50% cytotoxic concentration.

<sup>d</sup>SI: selectivity index.

<sup>e</sup>Values are expressed as mean ± standard deviation.

<sup>f</sup>Could not be determined, due to lack of virus-induced CPE in this cell line.

**Table 2**Effect of interferon alfacon 1 on SARS-CoV replication in different cell lines *in vitro*

| Cell lines | Virus yield reduction assay<br>IC <sub>90</sub> (ng/ml) <sup>a</sup> | SI <sup>b</sup> |
|------------|--|-----------------|
| Vero 76    | 2.25 ± 3.67  | 44.4            |
| Vero E6    | 3.02 ± 2.30 <sup>c</sup>   | 33.3            |
| MA104      | 0.33 ± 0.36  | 303             |
| Calu-3     | 0.046 ± 0.011  | 2173            |

<sup>a</sup>IC<sub>90</sub>: 90% inhibitory concentration.

<sup>b</sup>SI: selective index (CC<sub>50</sub>/IC<sub>90</sub>), based on CC<sub>50</sub> > 100 ng/ml.

<sup>c</sup>Values are expressed as mean ± standard deviation.

**Table 3**

Cytotoxicity of interferon alfacon 1 and virus yield after infection with Urbani isolate in different un-treated cell lines *in vitro*

| Cell lines | Cytotoxicity <sup>a</sup><br>(%) | Virus yield <sup>b</sup><br>(log <sub>10</sub> CCID <sub>50</sub> per 0.18 ml) |
|------------|----------------------------------|--|
| Vero 76    | 1.7 ± 2.9 <sup>c</sup>           | 4.5 ± 0.39   |
| Vero E6    | 24.5 ± 21.5                      | 4.5 ± 0.075  |
| MA104      | 18.9 ± 21.3                      | 5.2 ± 0.36   |
| Calu-3     | 33.9 ± 8.9                       | 3.8 ± 0.76   |

<sup>a</sup>Cytotoxicity of IFN-alfacon 1 at 100 ng/ml.

<sup>b</sup>Average virus control titers.

<sup>c</sup>Values are expressed as mean ± standard deviation.