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Letter

Recombinant human interferon-α1b inhibits SARS-CoV-2 better than interferon-α2b *in vitro*

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Dear Editor,

The coronavirus disease 2019 (COVID-19) outbreak, has spread across the world (Wu et al., 2020). The causative agent of COVID-19, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), is highly pathogenic and infectious, which become a major public health hazard that has had a devastating social and economic impact worldwide (Li Q.Q. et al., 2020).

Variants of the virus have emerged that behave differently (CDC 2021; Gobeil et al., 2020; Leung et al., 2021). Some of them show increased infectivity (Li Q. et al., 2020; Zhang et al., 2020) and may escape from neutralizing antibodies (Weisblum et al., 2020). Various vaccines have been developed and marketed for COVID-19. However, there is a shortage of specific drugs to treat this novel virus and there is an urgent need for effective broad-spectrum anti-viral drugs to treat COVID-19 and its variants.

Interferons (IFNs) are glycoproteins produced by cells infected with viruses and after other stimuli. IFNs can be divided into three types—type I interferon (IFN-I; mainly IFN-α/β), type II interferon (IFN-II; IFN-γ), and type III interferon (IFN-III; IFN-λ/IL-28/IL-29). IFN-α is a critical cytokine of the immune system that has broad antiviral and immunomodulatory functions, and has been applied to treat many infectious viral diseases (Sadler and Williams, 2008). Recombinant human IFN α1b (rHuIFN-α1b) and rHuIFN-α2b are subtype members of the IFN-α family. Based on mRNA frequency, IFN-α1 should be the predominant IFN subtype in naturally produced IFNs (Hawkins et al., 1984). Studies have confirmed that rHuIFN-α1b is effective in the treatment of hand, foot, and mouth disease, respiratory syncytial virus pneumonia, bronchiolitis in infants, and other infectious viral diseases (Chen et al., 2020; Huang et al., 2016). IFN is effective for treatment of SARS and Middle East respiratory syndrome, diseases caused by other coronavirus (Cinatl et al., 2003; Dahl et al., 2009; de Wilde et al., 2013; Loutfy et al., 2003; Sainz et al., 2004; Sheahan et al., 2020; Zumla et al., 2016). Felgenhauer et al. (2020) and Vanderheiden et al. (2020) have concluded that type I and type III IFN inhibit SARS-CoV-2 infection. Felgenhauer *et al.* tested the effects of IFN-α and IFN-λ against SARS-CoV-2, using two mammalian epithelial cell lines (human Calu-3 and simian Vero E6 cells), and found that both IFNs dose-dependently inhibited SARS-CoV-2. Vanderheiden *et al.* found that pretreatment and post-treatment with type I and III IFNs significantly reduced SARS-CoV-2 replication in human airway epithelial cell cultures, which correlated with the upregulation of antiviral effector genes. Therefore, IFNs application is a potentially effective method to treat COVID-19. Full-length IFN-α induces a strong inflammatory response, while IFN-α1b is truncated and exerts a similar antiviral effect but reduces inflammatory response at the same dose. Moreover, IFN-α1b is an IFN independently designed for Chinese genes (Li et al., 1992). Therefore, IFN-α1b has a better application prospects than full-length IFN-α. Here, we performed a comparison on the safety and viral inhibition effects of IFN-α subtypes (IFN-α1b and IFN-α2b) to provide insight into the clinical exploration of type I IFN.

To test the drug toxicity, Vero cells (ATCC CCL-81) and Calu-3 cells (ATCC HTB-55) were inoculated on 96-well plates (Greiner Bio-One, Kremsmünster, Austria; 10^4 cells/150 μL per well) and incubated at 37 °C in Minimum Essential Medium (Life Technologies, New York, NY, USA) supplemented with 2% fetal bovine serum (Life Technologies) for 24 h in a 5% CO_2 atmosphere. Then cells were treated with five-fold serially-diluted rHuIFN-α1b (25000, 5000, 1000, 200, 40, 8, 1.6, 0.32, 0.064, or 0.0128 IU/mL, 150 μL/well; gifted by Beijing Tri-Prime Gene Pharmaceutical Co. Ltd., Beijing, China) or rHuIFN-α2b (R&D Systems, Minneapolis, MN, USA) for 48 h. The nucleoside analogs, remdesivir (Absin Bioscience Inc, Shanghai, China) and ganciclovir (Sigma-Aldrich,

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St. Louis, MO, USA) were used as effective and ineffective controls, respectively. Remdesivir has been shown to be effective against SARS-CoV-2 *in vitro* in previous studies (Choy et al., 2020), but ganciclovir was not. Cell Counting Kit-8 assay mixture (15 μL; MedChemExpress, Monmouth Junction, NJ, USA) was added to each well to measure cell proliferation and cytotoxicity. Three hours after incubation, the absorbance value at 450 nm was measured using a microplate reader (BioRad iMark, Hercules, Cal, USA). rHuIFN-α1b and rHuIFN-α2b had no significant toxic effect on Vero cells at detected concentrations (Fig. 1A and B). For remdesivir and ganciclovir, the 50% cytotoxic concentration (CC_{50}) was 282.4 μmol/L and >100 μmol/L respectively in Vero cells (Fig. 1C and D). rHuIFN-α1b, rHuIFN-α2b, and ganciclovir also showed no cytotoxicity toward Calu-3 cells, the CC_{50} of remdesivir in Calu-3 cells was 49.5 μmol/L (Fig. 1E–H).

To test the antiviral effect of IFN on SARS-CoV-2, Vero cells and Calu-3 cells were inoculated on 24-well plates (Greiner Bio-One) at 1×10^5/mL/well, and incubated with 5% CO_2 at 37 °C to the logarithmic growth phase. Then, the cell culture medium was discarded, and the cells were infected with SARS-CoV-2 (hCoV-19/Hangzhou/ZJU-05/2020, GISAID, ID: 415709) at a multiplicity of infection of 0.05 (5% CO_2, 35 °C). Three hours after infection, the culture medium containing the virus was removed and the cells were washed twice with phosphate-buffered saline (PBS). After that, 1 mL/well of culture medium containing drugs was added to the pre-infected cells, which were incubated at 35 °C (5% CO_2) for 48 h. All experiments were conducted in triplicate and performed in an approved biosafety level III laboratory (CNAS BL0022, State Key Laboratory for Diagnosis and Treatment of Infectious Diseases, Zhejiang University). Forty-eight hours after infection, the culture supernatants were collected to determine the median tissue culture infective dose ($TCID_{50}$) using the standard $TCID_{50}$ method (Reed and Muench, 1938) and for reverse-transcription quantitative polymerase chain reaction (RT-qPCR) using a One-Step RT-qPCR Kit (Liferiver, Shanghai, China) according to the manufacturer's instructions. Data were analyzed using Prism software (GraphPad Software, San Diego, CA, USA). The inhibition percentage was calculated as: Inhibition%= $(1–2^{-\triangle\triangle Ct}) \times 100\%$. Half-maximal effective concentrations (EC_{50}) were calculated using nonlinear regression.

The viral titer steadily decreased with increasing rHuIFN-α1b and rHuIFN-α2b concentrations in both Vero and Calu-3 cells (Fig. 1A, B and 1E, 1F). rHuIFN-α1b had a prominent anti-SARS-CoV-2 effect in the tested concentration range (EC_{50} = 0.12 IU/mL in Vero cells; EC_{50} = 0.52 IU/mL in Calu-3 cells) (Fig. 1A and E). rHuIFN-α2b also showed an inhibitory effect (EC_{50} = 0.25 IU/mL in Vero cells; EC_{50} = 2.48 IU/mL in Calu-3 cells) (Fig. 1B and F), but it was not as good as that of rHuIFN-α1b. Remdesivir also showed a good anti-viral effect (EC_{50} = 0.67 μmol/mL in Vero cells and 0.81 μmol/mL in Calu-3 cells) (Fig. 1C and G). Ganciclovir showed no antiviral activity in the tested concentration range in either cell types (Fig. 1D and H).

At 48 h after infection, the cells were then washed with PBS and fixed in 80% precooled acetone (−20 °C; Sigma-Aldrich, USA) for 20 min. After washing three times with PBS, cells were blocked in 1% bovine serum albumin (BSA) for 30 min and incubated with anti-SARS-CoV-2 Spike Receptor-binding Domain rabbit monoclonal antibody (1:1000 in PBS with 0.1% BSA; Sino Biological Inc, Beijing, China; Cat: 40592-T62) at 4 °C, overnight. Cells were then washed three times with PBS and incubated for 2 h with Alexa Fluor488®-conjugated goat anti-rabbit IgG secondary antibody (1:1500; Abcam, Cambridge, England; Cat No. ab150077) at 20–25 °C in the dark. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI; 2 μmol/L; Abcam). The stained cells were observed by fluorescence microscopy. The proportion of SARS-CoV-2 infected cells decreased with the increasing concentration of rHuIFN-α1b and rHuIFN-α2b in both Vero (Fig. 1I and J) and Calu-3 cells (Supplementary Figs. S1A and S1B), showing a dose–effect correlation. Remdesivir as the effective antiviral drug control, also had prominent effect, but ganciclovir, as the ineffective drug control, did not.

$TCID_{50}$ values were used to further compare the antiviral effect of rHuIFN-α1b and rHuIFN-α2b. The viral titer steadily decreased with increasing rHuIFN-α1b and rHuIFN-α2b concentrations, but rHuIFN-α1b did better in both Vero and Calu-3 cells (Fig. 1K, L). The results for remdesivir and ganciclovir were consistent with RT-qPCR (Fig. 1C, D, G, H) and immunofluorescence (Supplementary Fig. S2). Taken together, these data indicate that rHuIFN-α1b significantly inhibited SAR-CoV-2 proliferation at a low concentration.

Previous studies verified the SARS-CoV-2 inhibition effect of pan-species IFN-α, IFN-β, and IFN-λ *in vitro* (Felgenhauer et al., 2020; Vanderheiden et al., 2020), which suggests that both type I and III IFNs can serve as therapeutic options to treat patients with COVID-19. In our study, IFN-α1b had no significant cytotoxicity but had a prominent anti-SARS-CoV-2 effect at a relatively low concentration in both Vero and Calu-3 cells. IFN-α2b also had a relatively low EC_{50} value, but its antiviral effect was not as outstanding as that of IFN-α1b.

The use of IFN has been reported in several clinical trials of treatment of COVID-19. One of the studies showed that IFN-β1a did not improve clinical status at day 15, nor SARS-CoV-2 clearance (Ader et al., 2021). Another trial showed that IFN-α2b resulted in significant improvement in clinical status in the treatment of moderate COVID-19 disease (Pandit et al., 2021), while IFN-α had no significant antiviral effects in patients with mild-to-moderate COVID-19 (Huang et al., 2020). Although, our study showed that IFN-α1b had a prominent anti-SARS-CoV-2 effect in both Vero and Calu-3 cells, there are significant differences between *in vitro* and *in vivo* studies. The effect of antiviral drugs in patients depends not only on inhibiting viral replication, but also on improving the disease course by repressing the release of inflammatory substances. Nevertheless, based on the results of our *in vitro* study and the results of earlier clinical trials of IFN, IFN-α1b is clearly worthy of further clinical study.

Remdesivir and ganciclovir are both nucleoside analogs which have antiviral activity through inhibiting viral nucleic acid synthesis. Remdesivir is a broad-spectrum drug against several virus families. It's worth mentioning that the antiviral activity of remdesivir has been demonstrated in all major variants of SARS-CoV-2 *in vitro* (Cao et al., 2020; Choy et al., 2020; Wang et al., 2020; Gilead, 2021a) and clinical improvement was observed in compassionate use (Grein et al., 2020). Gilead declared that remdesivir associated with a reduction in mortality rate in hospitalized patients with COVID-19 across analyses of large retrospective real-world data sets (Gilead, 2021b). Ganciclovir is used for cytomegalovirus infections in immunocompromised patients and superficial ocular herpes simplex infections (Al-Badr and Ajarim, 2018; Seidel et al., 2017). In our research, EC_{50} of remdesivir is significantly low and we got a precise value of CC_{50} (EC_{50} = 0.67 μmol/L, CC_{50} = 282.4 μmol/L, SI = 421.49) which is very close to previous report (Wang et al., 2020). In contrary, ganciclovir, which is also a nucleoside analog, has no antiviral activity to SARS-CoV-2 in our study. This may be due to the low strength of non-specific binding of ganciclovir as a nucleoside analog to polymerase, leading to few anti-virus capabilities.

The limitation of our study is that we have only demonstrated the antiviral effect of IFN at an *in vitro* level, and more studies are needed to explore the pathway and mode of antiviral effect of IFN. Preliminary studies have shown that IFN-α receptors are expressed in respiratory epithelial cells and found that in animal models and cases of bronchiolitis, nebulization of IFN-α1b causes significant symptoms ease (Chen et al., 2020). Thus, in our subsequent research, we may be able to use nebulized IFN-α1b for research to explore the antiviral effects and the side effects of different IFN modes of action. In summary, we performed a comparison of different interferon alpha subtypes (IFN-α1b and IFN-α2b) within live SARS-CoV-2 to provide important insights into the selection of the best type I interferon for clinical application.

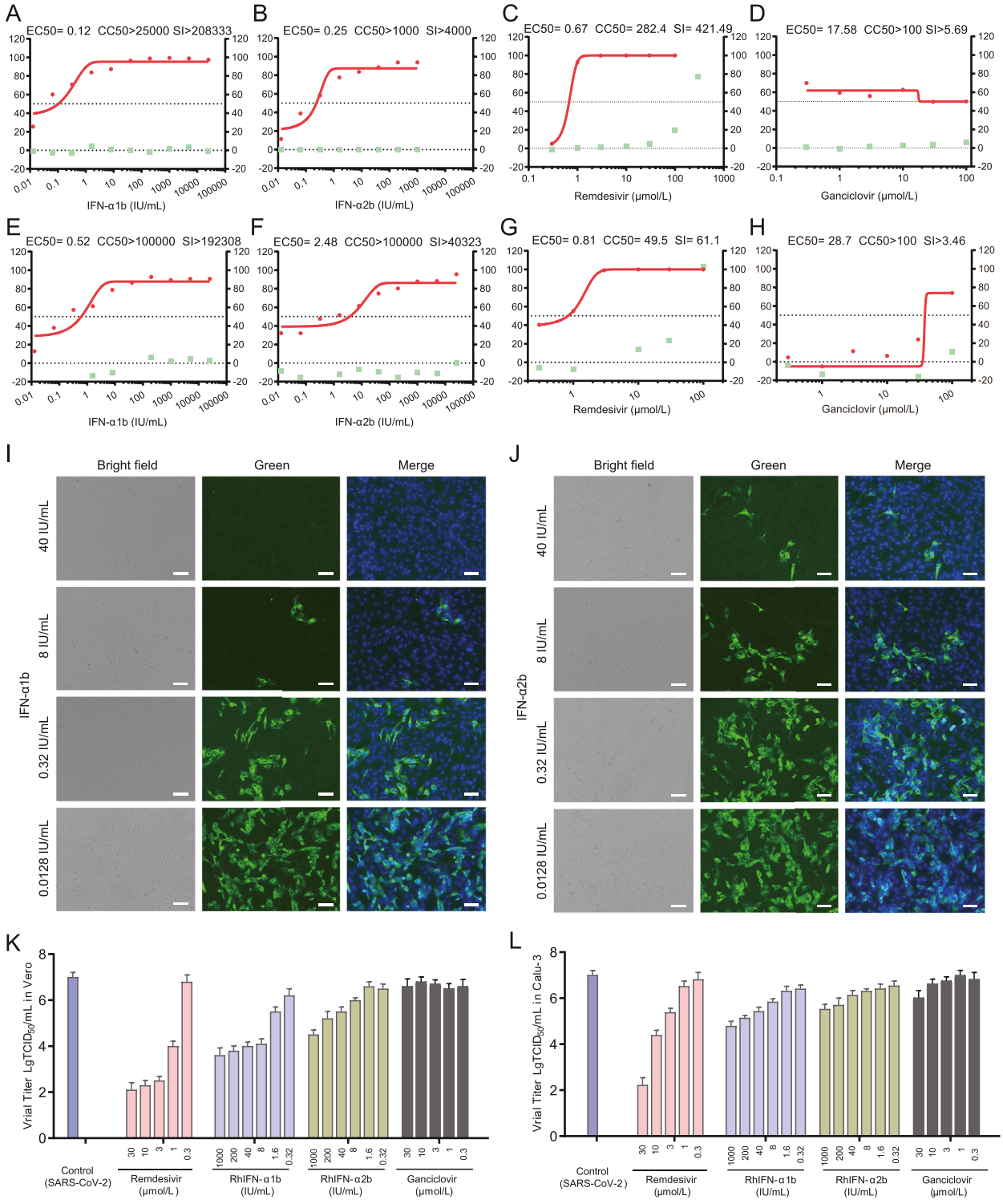

Fig. 1. Anti-viral activity and cytotoxicity of interferon (IFN) *in vitro*. SARS-CoV-2-infected Vero cells and Calu-3 cells (at a multiplicity of infection of 0.05) were treated with different doses of recombinant human IFN-α1b (rHuIFN-α1b), rHuIFN-α2b, remdesivir, and ganciclovir for 48 h. Viral load was measured by reverse-transcription quantitative polymerase chain reaction (RT-qPCR) and the cytotoxicity of drugs toward cells was determined using Cell Counting Kit-8 assays. Inhibition rate of SARS-CoV-2 and cytotoxicity of rHuIFN-α1b, rHuIFN-α2b, remdesivir, and ganciclovir in Vero (**A-D**) and Calu-3 cells (**E-H**). The left and right y-axes of the graph represent the average percentage inhibition of virus reproduction (red) and the drug cytotoxicity (green), respectively. The experiment was performed in triplicate. Half-maximal effective concentration (EC_{50}), half-cytotoxic concentration (CC_{50}), and selectivity index (SI) values are indicated above the graph. **I, J** Immunofluorescence microscopy of viral infection upon treatment of Vero cells with IFN. Cells were fixed in 80% precooled acetone for 30 min. Then, anti-SARS-CoV-2 Spike Receptor-binding Domain (RBD) rabbit monoclonal antibody was used as the primary antibody and Alexa Fluor488®-conjugated goat anti-rabbit IgG as the secondary antibody. Nuclei were stained with DAPI (4′,6-diamidino-2-phenylindole). The stained cells were observed by fluorescence microscopy. The scale bar represents 50 μm. **K, L** Determination of median tissue culture infective dose ($TCID_{50}$) values. Cytopathic effects in infected Vero and Calu-3 cells treated with rHuIFN-α1b, rHuIFN-α2b, remdesivir, or ganciclovir for 48 h were observed under the microscope and used to calculate $TCID_{50}$ values by the Reed-Muench method.

Footnotes

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