Inhibition of SARS-CoV-2 Infection by the Cyclophilin Inhibitor Alisporivir (Debio 025)

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ABSTRACT Cyclophilins play a key role in the life cycle of coronaviruses. Alisporivir (Debio 025) is a nonimmunosuppressive analogue of cyclosporine with potent cyclophilin inhibition properties. Alisporivir reduced SARS-CoV-2 RNA production in a dose-dependent manner in Vero E6 cells, with a 50% effective concentration (EC_{50}) of $0.46 \pm 0.04\ \mu$M. Alisporivir inhibited a postentry step of the SARS-CoV-2 life cycle. These results justify rapidly conducting a proof-of-concept phase 2 trial with alisporivir in patients with SARS-CoV-2 infection.

KEYWORDS SARS-CoV-2, alisporivir, antiviral, cyclophilin

In December 2019, an outbreak of pneumonia emerged in the Chinese city of Wuhan. A novel coronavirus was identified as the pathogen causing the disease, named COVID-19 (for coronavirus disease 2019). This new virus was called severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) because of its genetic proximity to SARS-CoV. At the time of writing, over 3.5 million people have been diagnosed with COVID-19 worldwide, while over 250,000 of them have died from complications of the disease.

Currently, there are no vaccines or effective antiviral drugs targeting SARS-CoV-2. A pragmatic approach is to assess whether drugs that are approved for other indications or have reached late clinical developmental stages are effective against SARS-CoV-2 and could be rapidly repurposed for this indication. For instance, chloroquine has been shown to bear potent antiviral properties against SARS-CoV-2 *in vitro*, and several clinical trials are under way to assess its efficacy in patients with COVID-19. The nucleotide analogues remdesivir and favipiravir, as well as the antiretroviral drug lopinavir in combination with ritonavir, are also under clinical investigation.

Cyclophilins are cellular peptidyl-prolyl *cis-trans* isomerases that catalyze the interconversion of the two energetically preferred conformers of the planar peptide bond preceding an internal proline residue. Cyclophilins play a key role in the life cycle of many coronaviruses, including human coronaviruses 229E (HCoV-229E) and NL-63 (HCoV-NL63), feline infectious peritonitis coronavirus (FPIV), SARS-CoV, and Middle East respiratory syndrome coronavirus (MERS-CoV) (1–7). Cyclosporine A (CsA), a potent cyclophilin inhibitor, blocks the replication of various coronaviruses *in vitro*, including HCoV-229E, HCoV-NL63, FPIV, mouse hepatitis virus (MHV), avian infectious bronchitis virus, and SARS-CoV (5, 8–10). However, CsA cannot be used in patients with COVID-19 because of its strong immunosuppressive properties.

Alisporivir (Debio 025) is a nonimmunosuppressive analogue of CsA that potently inhibits cyclophilins. Alisporivir has been administered to more than 1,800 patients with chronic hepatitis C virus infection in phase 2 and 3 clinical trials, alone or in combination with pegylated interferon alpha and/or ribavirin. *In vitro*, alisporivir inhibits the
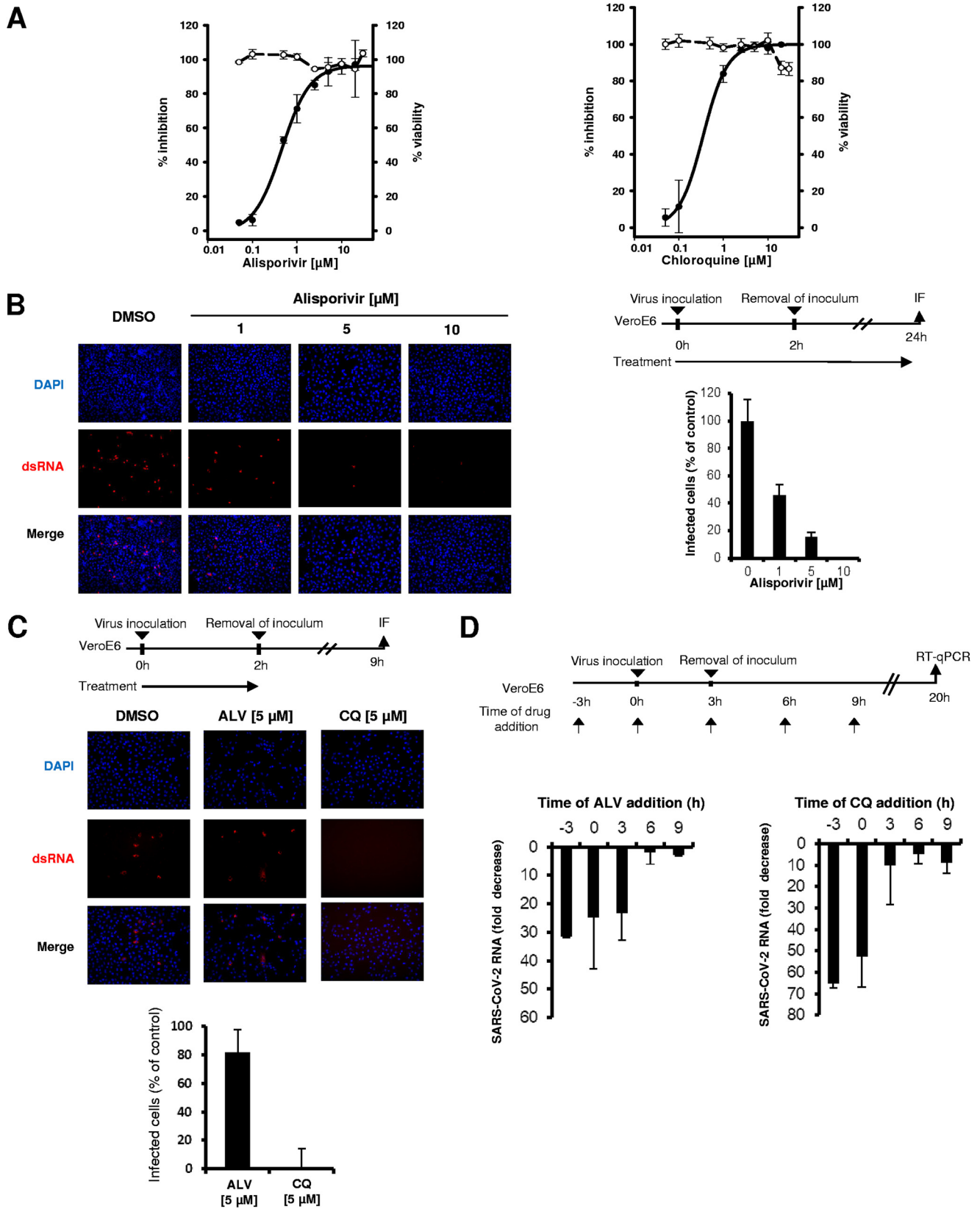
FIG 1 Antiviral activity of alisporivir against SARS-CoV-2. The means ± standard deviations from 2 experiments performed in triplicate are shown. (A) Vero E6 cells were infected for 2 h with a SARS-CoV-2 clinical isolate at an MOI of 0.02 in the presence of increasing concentrations of alisporivir (left) or chloroquine (right). Cells were incubated for 48 h in the presence of the compounds, and SARS-CoV-2 RNA was quantified in cell supernatants by RT-qPCR (solid lines). Cell viability is shown with dashed lines. (B) SARS-CoV-2 infection of Vero E6 cells at an MOI of 0.4 assessed by immunofluorescence using anti-dsRNA

replication of HCoV-229E, HCoV-NL63, MHV, SARS-CoV, and MERS-CoV at low-micromolar concentrations without cytotoxic effect (1, 10, 11).

The goal of this study was to assess the antiviral properties of alisporivir against SARS-CoV-2, with the objective of generating the preclinical proof of concept of antiviral effectiveness required to start a clinical trial in patients with COVID-19.

The antiviral effectiveness of increasing concentrations of alisporivir was measured in Vero E6 cells infected with a clinical isolate of SARS-CoV-2 at a multiplicity of infection (MOI) of 0.02 (Fig. 1A). Dimethyl sulfoxide (DMSO) was used as a negative control, while chloroquine was used as a positive control of antiviral inhibition. The compounds were added at the beginning of infection, and viral RNA was extracted from supernatants at 48 h postinfection and quantified by reverse transcriptase quantitative PCR (RT-qPCR). Alisporivir reduced SARS-CoV-2 RNA production in a dose-dependent manner: the 50% effective concentration (EC_{50}) was 0.46 ± 0.04 μM, and the EC_{90} was 3.10 ± 1.40 μM. The maximum viral RNA reduction was 2 $\log_{10}$ at 5 μM. For comparison, the EC_{50} of chloroquine was 0.35 ± 0.02 μM (Fig. 1A). Neither alisporivir nor chloroquine was cytotoxic at the effective concentration, with 50% cytotoxic concentrations (CC_{50}s) of >20 μM and therapeutic indexes of >43 and >57, respectively.

We confirmed the anti-SARS-CoV-2 effectiveness of alisporivir by immunofluorescence. Vero E6 cells were infected at an MOI of 0.4 for 2 h in the presence of increasing concentrations of alisporivir. After virus removal, infected cells were incubated for 24 h in the presence of alisporivir and immunostained with an anti-double-stranded-RNA (dsRNA) antibody. Alisporivir reduced the number of SARS-CoV-2-infected cells in a dose-dependent manner, and complete inhibition was attained at 10 μM (Fig. 1B). Chloroquine also inhibited SARS-CoV-2 in this assay (data not shown).

The next experiment was aimed at identifying the step of the SARS-CoV-2 life cycle targeted by alisporivir. Chloroquine, which inhibits endosome-mediated viral entry, was used as a control. Vero E6 cells were infected at an MOI of 0.4 for 2 h in the presence of 5 μM alisporivir or chloroquine. After virus removal, cells were incubated for 7 h in the absence of the compounds, fixed, and immunostained with the anti-dsRNA antibody. No infected cells were detected in the presence of 5 μM chloroquine, confirming that chloroquine prevents SARS-CoV-2 entry into Vero E6 cells. In contrast, alisporivir did not inhibit SARS-CoV-2 entry into Vero E6 cells (Fig. 1C). This result was confirmed by a time-of-addition experiment showing that, in contrast to that of chloroquine, the effect of alisporivir was preserved when the compound was added 3 h postinfection. The antiviral effect of alisporivir was abolished when the compound was added 6 h postinfection (Fig. 1D). These results suggest that alisporivir inhibits a postentry step of the SARS-CoV-2 life cycle.

Taken together, our results demonstrate that the nonimmunosuppressive macrocyclic cyclophilin inhibitor alisporivir (Debio 025) exhibits strong, dose-dependent antiviral properties against SARS-CoV-2 *in vitro*. Alisporivir inhibits a postentry step of the SARS-CoV-2 life cycle through mechanisms that remain to be unraveled. These results justify rapidly conducting a proof-of-concept phase 2 trial to assess the antiviral properties and the effect of alisporivir on COVID-19 clinical outcomes in infected patients.

Alisporivir has been shown to be well tolerated when administered as a monotherapy (12). Preclinical pharmacology data indicate that, after oral administration, alisporivir is widely distributed in the whole body, including the lungs, and that its EC_{90} against SARS-CoV-2 in Vero E6 cells is clinically achievable in patients. In addition, because alisporivir inhibits all cellular cyclophilins, it also blocks mitochondrial cyclo-

FIG 1 Legend (Continued)
antibodies in the presence of increasing concentrations of alisporivir. Infected cells were quantified using ImageJ software. (C) Effect of 5 μM alisporivir and 5 μM chloroquine on SARS-CoV-2 entry into Vero E6 cells, assessed by immunofluorescence using anti-dsRNA antibodies. (D) Time-of-addition experiments with alisporivir and chloroquine. Vero E6 cells were infected with SARS-CoV-2 at an MOI of 0.05 for 3 h; 10 μM alisporivir or 10 μM chloroquine was added at different time points and maintained until 20 h postinfection. SARS-CoV-2 RNA was quantified in cell supernatants by RT-qPCR. ALV, alisporivir; CQ, chloroquine.

philin D, a key regulator of mitochondrial permeability transition pore (mPTP) opening, a mechanism involved in triggering cell death. Therefore, besides its antiviral properties, alisporivir may also be effective in preventing lung tissue damage. A phase 2, proof-of-concept trial with alisporivir in patients with COVID-19 is planned to start very soon.

ACKNOWLEDGMENTS

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