Inhibition of SARS-CoV-2 infection by the cyclophilin inhibitor

Alisporivir (Debio 025)

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Running title: Cyclophilin inhibition blocks SARS-CoV-2 lifecycle

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Cyclophilins play a key role in the lifecycle of coronaviruses. Alisporivir (Debio 025) is a non-immunosuppressive analogue of cyclosporin A with potent cyclophilin inhibition properties. Alisporivir reduced SARS-CoV-2 RNA production in a dose-dependent manner in VeroE6 cell line, with an EC$_{50}$ of 0.46±0.04 µM. Alisporivir inhibited a post-entry step of the SARS-CoV-2 lifecycle. These results justify that a proof-of-concept Phase 2 trial be rapidly conducted with alisporivir in patients with SARS-CoV-2 infection.
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 with SARS-CoV. On the day of writing this article, 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 in other indications or have reached late clinical developmental stages are effective against SARS-CoV-2 and could be rapidly repurposed in this indication. For instance, chloroquine has been shown to bear potent antiviral properties against SARS-CoV-2 in vitro and several clinical trials are underway to assess its efficacy in patients with COVID-19. The nucleotide analogues remdesivir and favipiravir, and 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 lifecycle 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)\textsuperscript{1-7}. Cyclosporin 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\textsuperscript{5, 8-10}.
However, CsA cannot be used in patients with COVID-19 because of its strong immunosuppressive properties.

Alisporivir (Debio 025) is a non-immunosuppressive 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 replication of HCoV-229E, HCoV-NL63, MHV, SARS-CoV and MERS-CoV at low micromolar concentrations without cytotoxic effect1, 10, 11.

The goal of this study was to assess the antiviral properties of alisporivir against SARS-CoV-2, with the objective to generate 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 VeroE6 cells infected with a clinical isolate of SARS-CoV-2 at a multiplicity of infection (MOI) of 0.02 (Figure 1A). 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 post-infection and quantified by reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR).

Alisporivir reduced SARS-CoV-2 RNA production in a dose-dependent manner: the 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 (Figure 1A). Neither alisporivir, nor chloroquine were cytotoxic at their effective concentrations, with CC_{50} >20 µM and therapeutic indexes >43 and >57, respectively.

We confirmed the anti-SARS-CoV-2 effectiveness of alisporivir by immunofluorescence. VeroE6 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 obtained at 10 µM (Figure 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 lifecycle targeted by alisporivir. Chloroquine, which inhibits endosomal-mediated viral entry, was used as a control. VeroE6 cells were infected at an MOI of 0.4 for 2 h in the presence of 5 µM of 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 of chloroquine, confirming that chloroquine prevents SARS-CoV-2 entry into VeroE6 cells. In contrast, alisporivir did not inhibit SARS-CoV-2 entry into VeroE6 cells (Figure 1C). This result was confirmed by a time-of-addition experiment showing that, in contrast with that of chloroquine, the effect of alisporivir was preserved when the compound was added 3 h post-infection. The antiviral effect of alisporivir was abolished when the compound was added 6 h post-infection (Figure 1D). These results suggest that alisporivir inhibits a post-entry step of the SARS-CoV-2 life cycle.

Taken together, our results demonstrate that the non-immunosuppressive macrocyclic cyclophilin inhibitor alisporivir (Debio 025) bears strong, dose-dependent antiviral properties against SARS-CoV-2 in vitro. Alisporivir inhibits a post-entry step of the SARS-CoV-2 life cycle through mechanisms that remain to be unraveled. These results justify that a proof-of-concept Phase 2 trial be rapidly conducted 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. Preclinical pharmacology data indicate that, after oral administration, alisporivir is widely distributed in the whole body, including the lungs, and that its EC₉₀ against SARS-CoV-2 in VeroE6 cells is clinically achievable in patients. In addition, because alisporivir inhibits all cellular cyclophilins, it also blocks mitochondrial cyclophilin-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.

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Antiviral activity of alisporivir against SARS-CoV-2. The mean±standard deviation of 2 experiments performed in triplicate is shown. (A) VeroE6 cells were infected for 2 h with a SARS-CoV-2 clinical isolate at an MOI=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 line). Cell viability is shown as a dashed line. (B) SARS-CoV-2 infection of VeroE6 cells at an MOI=0.4 assessed by immunofluorescence using anti-dsRNA 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 VeroE6 cells, assessed by immunofluorescence using anti-dsRNA antibodies. (D) Time-of-addition experiments with alisporivir and chloroquine. VeroE6 cells were infected with SARS-CoV-2 at an MOI=0.05 for 3 h; 10 µM alisporivir or 10 µM chloroquine were added at different timepoints and maintained until 20 h post-infection. SARS-CoV-2 RNA was quantified in cell supernatants by RT-qPCR. ALV: alisporivir. CQ: chloroquine.
