



Lack of antiviral activity of darunavir against SARS-CoV-2

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ABSTRACT

Objectives: Given the high need and the absence of specific antivirals for treatment of COVID-19 (the disease caused by severe acute respiratory syndrome-associated coronavirus-2 [SARS-CoV-2]), human immunodeficiency virus (HIV) protease inhibitors are being considered as therapeutic alternatives.

Methods: Prezcoibix/Rezosta is a fixed-dose combination of 800 mg of the HIV protease inhibitor darunavir (DRV) and 150 mg cobicistat, a CYP3A4 inhibitor, which is indicated in combination with other antiretroviral agents for the treatment of HIV infection. There are currently no definitive data on the safety and efficacy of DRV/cobicistat for the treatment of COVID-19. The *in vitro* antiviral activity of darunavir against a clinical isolate from a patient infected with SARS-CoV-2 was assessed.

Results: DRV showed no antiviral activity against SARS-CoV-2 at clinically relevant concentrations ($EC_{50} > 100 \mu\text{M}$). Remdesivir, used as a positive control, demonstrated potent antiviral activity ($EC_{50} = 0.38 \mu\text{M}$).

Conclusions: Overall, the data do not support the use of DRV for the treatment of COVID-19.

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Introduction

In December 2019, the severe acute respiratory syndrome-associated coronavirus-2 (SARS-CoV-2) disease (COVID-19) emerged in Wuhan, Hubei Province, China (Zhu et al., 2020). The virus was subsequently identified as a coronavirus (CoV), in addition to SARS-CoV-1 and Middle East respiratory syndrome CoV (MERS-CoV) that passed from animals to humans where it can cause severe respiratory illness (Lu et al., 2020). As of March 2020, COVID-19 has spread worldwide, with the WHO declaring a global pandemic (Hoehl et al., 2020). Given the extent of the COVID-19 pandemic, there is an urgent need to identify potential treatments for the disease as well as to develop a vaccine.

As no specific antivirals for treatment of COVID-19 are available, one avenue of clinical interest is the use of human immunodeficiency virus (HIV) protease inhibitors (PIs) as a therapeutic intervention. The potential for HIV PIs as a treatment for COVID-19 is mainly based on limited virologic and clinical data on the HIV protease inhibitor lopinavir with low-dose ritonavir (as a pharmaco-enhancer; LPV/r) in patients infected with the severe acute respiratory syndrome, related to a coronavirus (SARS-CoV)

(Chu et al., 2004). After demonstrating the *in vitro* antiviral activity of LPV against SARS-CoV-1, the clinical response of patients with SARS to a combination of LPV/r and ribavirin was examined. Patients treated with LPV/r had lower rates of adverse clinical outcomes at day 21 following the onset of symptoms than historical controls (Chu et al., 2004). However, recent data in hospitalized adults with severe confirmed COVID-19 treated with LPV/r in addition to standard care of ventilation, oxygen, vasopressor support, antibiotics, and renal-replacement therapy showed that there was no significant improvement in time to clinical improvement or mortality at day 28 compared with the standard care (Cao et al., 2020).

The HIV PI darunavir with cobicistat as a pharmaco-enhancer (DRV/c, 800/150 mg given orally once daily with food) in combination with other antiretroviral agents is approved for both treatment-naïve and -experienced patients with HIV-1 infection (Prezcoibix Prescribing Information, 2020; Rezosta Summary of Product Characteristics, 2020). The efficacy and safety profile of boosted-DRV combination therapy is well-established in the HIV setting, based on phase III clinical studies as well as real-world evidence (Tashima et al., 2014; Orkin et al., 2013; Cahn et al., 2011; Navarro and Curran, 2016).

To date, no clear clinical evidence supports the use of DRV (boosted with either ritonavir or cobicistat) in viral diseases other than HIV.

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In this paper, the antiviral activity of DRV against SARS-CoV-2 was investigated in an *in vitro* model at clinically relevant concentrations. When DRV/c is taken at the indicated once-daily dose, the median total trough plasma concentration of DRV was 3.4 μM (1875 ng/mL). (Prezcobix Prescribing Information, 2020). The cell culture assay was shown to be suitable for antiviral assays. Productive viral infection occurs in this model with the number of SARS-CoV RNA molecules increasing continuously after infection, indicating that the virus undergoes several replication cycles (Bojkova et al., 2020). Remdesivir (GS-5734), a nucleotide analog initially developed for Ebola virus disease, has been shown to inhibit SARS-CoV-2 replication *in vitro* with an EC_{50} equal to 0,770 μM (Wang et al., 2020) and was therefore used as a positive control.

Methods

Cell culture and virus preparation

Human colon carcinoma cell line (Caco-2) cells (obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany) were cultured in Minimal Essential Medium (MEM) supplemented with 10% fetal bovine serum (FBS) and containing penicillin (100 IU/mL) and streptomycin (100 $\mu\text{g}/\text{mL}$) in a 5% CO_2 atmosphere at 37 °C. All culture reagents were purchased from Sigma (Hamburg, Germany).

SARS-CoV-2 was isolated from human samples and cultured in Caco-2 cells, as previously described (Hoehl et al., 2020). After one passage in Caco-2 cells, viral stocks were stored at -80 °C prior to use.

Assessment of antiviral activity by inhibition of virus-induced cytopathogenic effect

Confluent layers of Caco-2 cells were cultured at 37 °C in a 5% CO_2 atmosphere for 72 h on 96 multi-well plates (50,000 cells/well). Cells were challenged with SARS-CoV-2 at a multiplicity of infection (MOI) of 0.01. The virus was added together with the compounds under investigation and incubated in MEM supplemented with 1% FBS.

DRV and remdesivir were synthesized at Johnson & Johnson. To assess *in vitro* antiviral activity, DRV and remdesivir, diluted in MEM without FBS, were added in 4-fold dilutions to a concentration range of 0.02 μM to 100 μM . Cells were then incubated for 48 h before the cytopathogenic effect (CPE) was visually scored by two independent laboratory technicians. Evaluation of the CPE was also done using a 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT; Sigma-Aldrich) method according to the manufacturer's instructions. Optical densities were measured at 560/620 nm in a Multiskan Reader MCC/340 Labsystems. Three independent experiments with triplicate measurements were performed. Evaluation of inhibition of CPE using the MTT method was done for two of the three experiments. Data were analyzed by a four-parameter curve-fitting from a dose-response curve using GraphPad Prism (version 7.00) to calculate the EC_{50} (concentration of the compound that inhibited 50% of the infection) based on visual CPE scoring or based on the MTT method.

Assessment of cell viability

To assess the effects of the compounds on Caco-2 cell viability, cell viability was measured in confluent cell layers treated with a range of compound concentrations in the absence of virus using the Rotitest Vital (Roth) according to manufacturer's instructions, as previously described (Bojkova et al., 2020).

All assays were performed three times independently in triplicate. From this, the CC_{50} (cytotoxic concentration of the compound that reduced cell viability to 50%) was calculated from a dose-response curve in GraphPad Prism (version 7.00) using four-parameter curve-fitting.

Selectivity index

The selectivity index for each of the compounds was determined as the ratio of the CC_{50} to the EC_{50} .

Modeling

In general, *in silico* docking can be a useful approach for identifying subsets of molecules for *in vitro* studies and can be used to explain *in vitro* observations on a structural level. The coordinates of the main SARS-CoV-2 protease's crystal structure were retrieved from the PDB database (<https://www.rcsb.org/structure/6lu7>).

Preparing DRV and the protein for *in silico* molecular docking was performed with the software package MOE (Molecular Operating Environment 2019.01; Chemical Computing Group ULC, Montreal, QC, Canada, H3A 2R7, 2019). The force field used was AMBER ETH:10 with default 'quick prep' settings to prepare the protein complex. The original ligand was then removed. General docking settings were then altered to have 50 initial placements.

Results

Assessment of *in vitro* antiviral activity, cytotoxicity, and selectivity

Remdesivir showed strong antiviral activity against SARS-CoV-2 with an EC_{50} of 0.11 μM based on visual scoring of the inhibition of CPE (Figure 1a). In the same experiments, DRV did not show any inhibition of SARS-CoV-2 induced CPE (Figure 1b; $\text{EC}_{50} > 100$ μM). Similar results were obtained using the MTT method. Remdesivir showed potent antiviral activity with an EC_{50} value of 0.38 μM (Figure 1a), while DRV showed no effect ($\text{EC}_{50} > 100$ μM , Figure 1b). No cytotoxicity of remdesivir or DRV was observed on Caco-2 cells with CC_{50} values > 100 μM . The selectivity index ($\text{CC}_{50}/\text{EC}_{50}$) for DRV could not be calculated (> 100 $\mu\text{M}/> 100$ μM for SARS-CoV-2) due to the lack of antiviral activity. In contrast, remdesivir had a selectivity index of > 900 by visual CPE scoring and > 260 by the MTT method, confirming a strong *in vitro* antiviral effect against SARS-CoV-2.

Modeling

In silico docking of DRV in the crystal structure of the SARS-CoV-2 main protease (3CL protease) identified five docking poses. The docking scores ranged from $S = -8.6$ to -8.2 , showing that DRV can fit into the pocket, but these values are indicative of suboptimal binding to this protein. Visual inspection of each of these poses showed very few interactions of DRV with the active site of the protease, and the catalytic cysteine residue was not directly targeted, unlike the many strong interactions observed for DRV bound to the HIV protease (King et al., 2004).

Discussion

Current efforts to manage the COVID-19 pandemic have primarily focused on improved hygiene, quarantine of infected individuals, social distancing to limit transmission, and development of a vaccine (Kruse, 2020). Despite the expedited efforts to develop a vaccine and collaborative efforts to screen compounds in

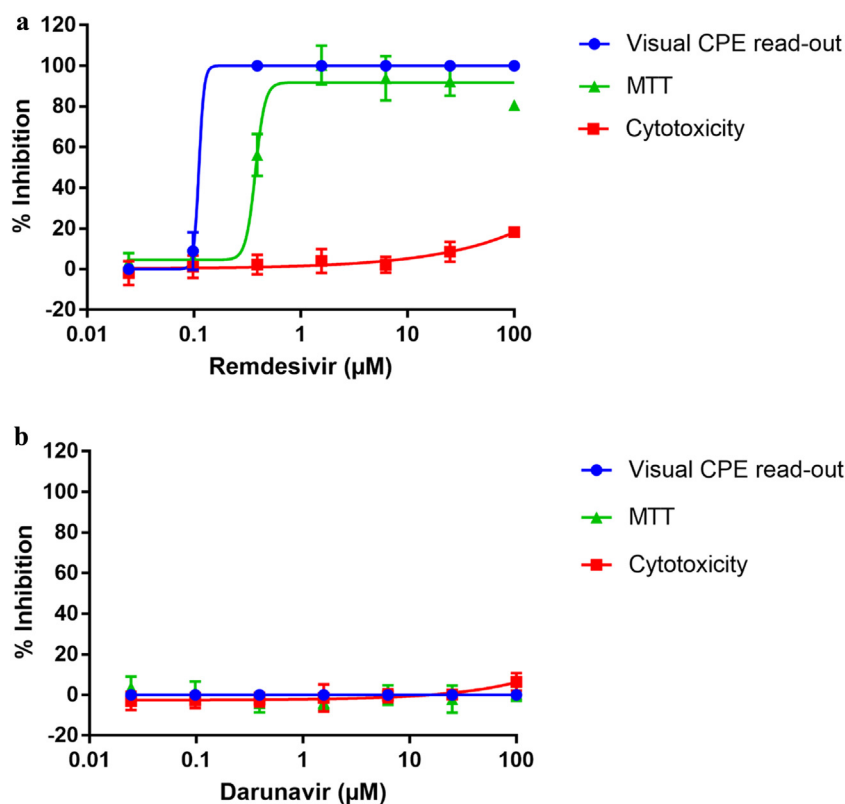


Figure 1. Inhibition of SARS-CoV-2 in Caco-2 cells by cytopathogenic effect assay using a visual read-out (visual CPE read-out) and MTT assay (MTT) following the addition of a) remdesivir, or b) darunavir (DRV). Cytotoxicity data (measured on uninfected cells) are also shown. Mean percent inhibition for each read-out across three independent experiments with triplicate measurements are plotted (2 independent experiments for the MTT assay). The error bars represent the standard deviation. Note: for some points, the error bars are shorter than the height of the symbol.

(a) Visual CPE-read out EC_{50} = 0.11 μ M; MTT EC_{50} = 0.38 μ M; CC_{50} > 100 μ M; Selectivity index = >900 (Visual CPE read-out); >260 (MTT).

(b) Visual CPE read-out EC_{50} > 100 μ M; MTT EC_{50} > 100 μ M; CC_{50} > 100 μ M.

discovery and development across the broader pharmaceutical industry for activity against COVID-19, patients are in immediate need of therapeutic interventions (Bojkova et al., 2020; Baden and Rubin, 2020).

Current data on the therapeutic effect of HIV protease inhibitors in patients with COVID-19 are far from comprehensive. This study demonstrated that DRV showed no *in vitro* antiviral activity against SARS-CoV-2 at clinically relevant concentrations. Furthermore, structural analyses using protease structures are consistent with these data. DRV binds to the active site of the HIV virus' dimeric aspartic protease (King et al., 2004). The crystal structure of this protease is well-elucidated. It has been shown to have an extensive hydrogen-bonding network with DRV, allowing for the potent *in vitro* activity (EC_{50} values = 1.2 to 8.5 nM) of this protease inhibitor against HIV (Prezcobix Prescribing Information, 2020; Rezolsta Summary of Product Characteristics, 2020). In contrast, the SARS-CoV-2 main protease is a cysteine protease (Protein Data Bank-code 6LU7), and while several docking poses have been found for DRV *in silico* models, unlike in HIV, these poses showed little interaction with the SARS-CoV-2 main protease active site. Several publications describe *in silico* docking experiments on the main coronavirus protease that specifically focus on or include DRV (Chang et al., 2020; Haitao and Zihe, 2020; Sang et al., 2020; Wu et al., 2020; Farag et al., 2020). Although these studies suggest DRV as a candidate for further investigation, such promising docking results could not be reproduced in our *in silico* docking studies. Such discrepancies can often result from *in silico* docking, which is primarily a useful approach for identifying subsets of molecules for *in vitro* activity testing. No *in vitro* antiviral activity of DRV against SARS-CoV-2 was found in the experiments reported here.

In this study, remdesivir demonstrated activity against SARS-CoV-2 with an EC_{50} of 0.38 μ M, which is in line with the earlier reported remdesivir EC_{50} of 0.77 μ M, indicating that the *in vitro* antiviral assay used is appropriate to assess antiviral activity against SARS-CoV-2 (Bojkova et al., 2020).

In conclusion, the lack of *in vitro* antiviral activity of DRV against SARS-CoV-2 does not support the use of DRV for the treatment of COVID-19. Hence, DRV (boosted with either ritonavir or cobicistat) should remain solely for the treatment of patients with HIV infection.

Funding source

Funding for this study was provided by Janssen Pharmaceuticals.

Ethical approval

As this was an *in vitro* study, no ethical approval was required.

Data sharing

The data sharing policy of Janssen Pharmaceutical Companies of Johnson & Johnson is available at <https://www.janssen.com/clinical-trials/transparency>. As noted on this site, requests for access to the study data can be submitted through the Yale Open Data Access (YODA) Project site at <http://yoda.yale.edu>.

Conflict of interest

Sandra De Meyer, Christophe Buyck, Ellen Van Damme, Marnix Van Loock, and Brian Woodfall are employees and could be stock

owners of Johnson and Johnson. Sandra Ciesek received research funding from Janssen for this research.

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