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Abstract (249 words)

Introduction: Urgent action is needed to fight the ongoing COVID-19 pandemic by reducing the number of infected people along with the infection contagiousness and severity. Chlorpromazine (CPZ), the prototype of typical antipsychotics from the phenothiazine group, is known to inhibit clathrin-mediated endocytosis and acts as an antiviral, in particular against SARS-CoV-1 and MERS-CoV. The aim of this *in vitro* study was to test CPZ against a SARS-CoV-2 isolate in monkey and human cells.

Material and methods: Monkey VeroE6 cells and human alveolar basal epithelial A549-ACE2 cells were infected with SARS-CoV-2 in presence of different concentrations of CPZ. Supernatants were harvested at day 2 and analysed by RT-qPCR for the presence of SARS-CoV-2 RNA. Cell viability was assessed on non-infected cells.

Results: We evidenced an antiviral activity of CPZ against SARS-CoV-2 in monkey VeroE6 cells with an IC50 of 8.2 µM, a CC50 of 13.5µM and a SI of 1.65. In human A549-ACE2 cells, CPZ was also associated with an anti-SARS-CoV-2 activity, with an IC50 of 11.3 µM, a CC50 of 23.1 µM and a SI of 2.04.

Discussion: Even though the measured SI are low, such IC50 measured *in vitro* may translate to CPZ dosage used in clinical routine because of CPZ high biodistribution in lungs and in saliva. Also, CPZ brain distribution could be of high interest for treating or preventing the neurological and psychiatric forms of COVID-19.

Conclusions: These preclinical findings support clinical investigation of the repurposing of CPZ, a largely used drug with mild side effects, in COVID-19 treatment.

Keywords: chlorpromazine; SARS-CoV-2; COVID-19; repurposing of molecules; human cells

Chemical compounds studied in this article Chlorpromazine (PubChem CID: 2726)

Introduction

From the beginning of the COVID-19 outbreak we observed in Sainte Anne hospital (GHU PARIS Psychiatrie & Neurosciences, Paris, France) a higher prevalence of symptomatic and severe forms of COVID-19 infections among health care professionals (~14%) compared to patients in psychiatric wards (~4%) [1]. This unexpected finding, that patients with more comorbidities and risk factors (overweight, cardiovascular disorders, etc.) seem to be protected against symptomatic and severe forms of COVID-19, drew our interest to decipher putative factors that could mediate this anti-SARS-CoV-2 protection. Because patients in psychiatric wards receive psychotropic medications, we screened the literature for antiviral effects associated with those drugs.

This literature analysis identified chlorpromazine (CPZ), the prototype of phenothiazine-type antipsychotics, as the lead candidate [1]. The reasons why CPZ was identified as the lead candidate among psychotropic drugs are multiple. Firstly, in addition to its antipsychotic activity, CPZ has been used for decades in virology. On the one hand, *in vitro* studies have demonstrated CPZ antiviral properties, for example against influenza [2], hepatitis viruses [3], alphaviruses [4], JC virus [5], Japanese encephalitis virus [6], bronchitis-virus [7], MHV-2 [8], Zika virus [9] or dengue virus [10]. On the other hand, CPZ is the leading drug inhibiting clathrin-mediated endocytosis [11–14] – via translocation of clathrin and AP2 from the cell surface to intracellular endosomes [12] – and therefore is commonly used to determine the pathways of entry of viruses into cells [11–14]. In a recent review article, the authors underlined the therapeutic potential of targeting clathrin-mediated endocytosis – essential for coronavirus cell entry [15] – to tackle SARS-CoV-2 [16].

Secondly, CPZ has been shown to have antiviral activity against coronaviruses in multiple studies [17–20]. It was identified active against both MERS-CoV and SARS-CoV-1 in a screen of 348 FDA-approved drugs, together with three other compounds (chloroquine, loperamide, lopinavir), using different cell lines [17]. Similar results were obtained in a different library screen [18], as well as in another study using primary human monocytes [19]. More recently, CPZ (as well as 16 other compounds) has been shown to have antiviral activity against SARS-CoV-2 on monkey Vero E6 cells [20].

In this context, the aim of the current study was to investigate *in vitro* CPZ antiviral activity against SARS-CoV-2 on monkey Vero E6 cells as well as – for the first time – on human alveolar basal epithelial cells.

Material and methods

2.1 Cell culture and virus isolates

Vero E6 cells (African green monkey kidney epithelial cells, ATCC, CRL-1586) were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal bovine serum (FBS) and 5 units/mL penicillin and 5 µg/mL streptomycin at 37°C with 5% CO2. A549-ACE2 cells (adenocarcinomic human alveolar basal epithelial cells, transduced to express the human Angiotensin-converting enzyme 2 (ACE2), kind gift of Pr. O. Schwartz, Institut Pasteur, Paris France) were maintained in DMEM containing 10% FBS, 5 units/mL penicillin and 5 µg/mL streptomycin and 40 µg/mL blasticidin at 37°C with 5% CO2.

SARS-CoV-2, isolate BetaCoV/France/IDF0372/2020 C2, was supplied by the National Reference Centre for Respiratory Viruses (NRC) hosted at Institut Pasteur (Paris, France) and headed by Pr. S. Van der Werf. The human sample from which this strain was isolated has been provided by Dr. X. Lescure and Pr. Y. Yazdanpanah from the Bichat Hospital, Paris, France. Viral stocks were prepared by propagation in VeroE6 cells in DMEM supplemented with 2% FBS. Viral titres were determined by plaque assay. All experiments involving live SARS-CoV-2 were performed in compliance with Institut Pasteur guidelines for Biosafety Level 3 work. All experiments were performed in at least three biologically independent replicates.

2.2 Antiviral activity assay

Cells were seeded into 96-well plates 24 h prior to the experiment. Two hours prior to infection, cell culture supernatant was replaced with media containing 32µM, 16µM, 8µM, 4µM and 2µM of CPZ, or the equivalent volume of maximum H_2O vehicle used as a control. For the infection, the drug-containing media was removed, and replaced with virus inoculum (MOI of 0.1 PFU/cell for VeroE6 and 1 for A549-ACE2) for 2 hours. The inoculum was then removed and replaced with 100µl fresh media (2% FBS) containing CPZ at the indicated concentrations or H_2O and incubated for 48 hours.

At 48h, cell supernatant was collected and spun for 5 min at 3,000g to remove debris. Toxicity controls were setup in parallel on uninfected cells. RNA was extracted from 50µl aliquots of supernatant using the Nucleospin 96 virus kit (Macherey-Nagel) following the manufacturer's instructions. Detection of viral genomes was performed by RT-qPCR, using the IP4 primer set developed by the NRC at Institut Pasteur (described on the WHO website (https://www.who.int/docs/default-source/coronaviruse/real-time-rt-pcr-assays-for-the-detection-of-sars-cov-2-institut-pasteur-paris.pdf?sfvrsn=3662fcb6_2). RT-qPCR was performed

using the Luna Universal One-Step RT-qPCR Kit (NEB) in an Applied Biosystems QuantStudio 3 thermocycler, using the following cycling conditions: 55°C for 10 min, 95°C for 1 min, and 40 cycles of 95°C for 10 sec, followed by 60°C for 1 min. The quantity of viral genomes is expressed as PFU equivalents, and was calculated by performing a standard curve with RNA derived from a viral stock with a known viral titre.

Cell viability in drug-treated cells was measured using the AlamarBlue reagent (ThermoFisher). At 48 h post treatment, the drug-containing media was removed and replaced with AlamarBlue and incubated for 2h at 37°C. Fluorescence was measured in a Tecan Infinity 2000 plate reader.

Using the same experimental setting, we tested the antiviral activity of remdesivir, as a comparator and a validator of the experiment.

2.3 Data analysis

Antiviral activity was assayed as a percentage of inhibition of SARS-CoV-2, normalized to the quantity of viral genomes under the lowest concentration of CPZ (i.e. 2 µM) for each of the three independent replicates. Percentage cytotoxicity was calculated relative to untreated cells (0% toxicity). Antiviral activity and cytoxicity data were analysed using GraphPad Prism version 8.4.2 for MacOS (GraphPad Software, San Diego, California USA, www.graphpad.com). Non-linear regressions were performed and IC50 (half maximal inhibitory concentration), IC90 (90% maximal inhibitory concentration), CC50 (half maximal cytotoxic concentration) and SI (selectivity index, calculated by dividing the CC50 by the IC50) were calculated from "[Agonist] vs. response -- Variable slope" curves with constraints to remain above 0% and below 100%.

Results

African Green Monkey VeroE6 cells and human alveolar basal epithelial A549-ACE2 cells were infected with SARS-CoV-2 in presence of different concentrations of CPZ. Supernatants were harvested at day 2 and analysed by RT-qPCR for the presence of SARS-CoV-2 RNA. In parallel, cell viability was assessed on non-infected cells.

In monkey VeroE6 cells, we measured an antiviral activity of CPZ against SARS-CoV-2, with an IC50 of 8.2 µM, an IC90 of 15.2 µM, a CC50 of 13.5µM and thus a SI of 1.65 (Figure 1.A).

In human A549-ACE2 cells, CPZ was also associated with an anti-SARS-CoV-2 activity, with an IC50 of 11.3 µM (Figure 1.B) and an IC90 of 14.3 µM. CPZ was associated with a cytotoxic effect in this model at the highest doses assessed, with a CC50 of 23.1 µM and therefore a SI of 2.04 (Figure 1.B).

Remdesivir was associated with an antiviral activity against SARS-CoV-2, with an IC50 of 5 µM in monkey VeroE6 cells, and an IC50 of 0.15 µM in human A549-ACE2 cells.

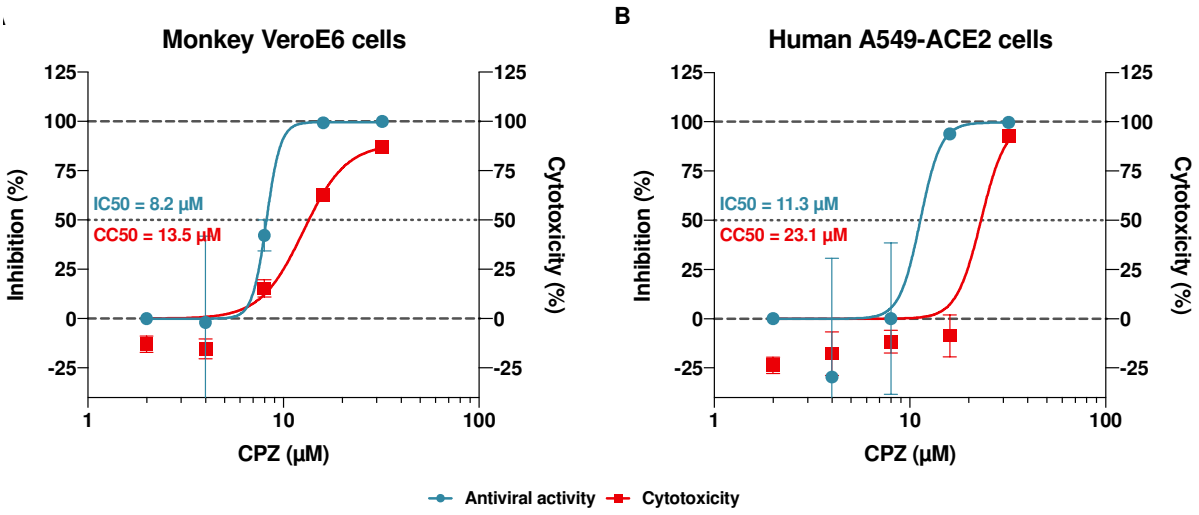

Figure 1. Antiviral activity of CPZ against SARS-CoV-2 in vitro in monkey VeroE6 cells (A) and human A549-ACE2 cells (B). Viral load in supernatants were measured at 48h (left Y axis), and viability under increasing concentrations of the antiviral compound are shown. Error bars denote s.e.m.

Discussion

With more than 6 000 000 infections and 370 000 deaths worldwide in just a few months [21], tools are urgently needed to help against the SARS-CoV-2 pandemic, to diminish disease severity along with contagiousness and to reduce the socio-economic consequences of the pandemic. In this study, we evidenced *in vitro* antiviral activity of CPZ against a SARS-CoV-2 isolate in monkey and human cells, with IC50 of respectively 8.2 and 11.3 µM. These results are in line with previous demonstrations of antiviral properties of CPZ, a well-known inhibitor of clathrin-mediated endocytosis [11–14], against older coronaviruses. Besides, the measured IC50 of remdesivir are consistent with previously published work [22,23], reinforcing the validation of our experimental setup.

Even though the measured SI of CPZ are very low, such IC50 measured *in vitro* may translate to CPZ dosage used in clinical routine. Indeed, one of the main advantages of using CPZ against SARS-CoV-2 could lie in its biodistribution (Figure 2), and mainly and foremost in its pneumophilic properties. In 1968, Forrest et al. quantified the CPZ distribution in selected organs through a post-*mortem* study of 6 patients with schizophrenia and treated by CPZ until their death [24]. Among the 5 patients with available lung measurements, the highest CPZ concentration was found in the lungs. These high pneumophilic properties of CPZ have also been described in preclinical studies, reporting CPZ concentration in the lungs 20 to 200 times higher than in plasma after a single dose of CPZ [24–27]. Besides the lungs, CPZ concentrations have been demonstrated to be very high In the salivary glands, 30-100 times higher than in plasma after a single dose of CPZ [26,28]. In humans, May et al. studied saliva concentration of CPZ in 48 newly admitted patients with schizophrenia, and saliva concentrations were found to be between 1300 to 22000 ng/ml (*i.e.* 4.1 to 69 µM; CPZ molar mass = 318.86 g/mol) 1 to 8 hours after a single dose of CPZ [28]. These high saliva concentrations of CPZ could reduce the contagiousness of COVID-19 [29]. Moreover, because of its lipophilic nature, CPZ cross the blood-brain barrier [30]. CPZ brain distribution, underlying its antipsychotic action and side effects, has been described with brain to plasma concentration ratio of up to 50 in rodents [25–27,31]. In humans, CPZ distribution in the brain was studied in 22 patients with schizophrenia in our hospital in 1979, with brain to plasma ratio ranging from 15 to 25 [32]. This could be of great interest for treating or preventing the neurological and psychiatric forms of COVID-19 [33] with, to date, no available therapeutic options. Indeed, remdesivir, anti-IL6 drug tocilizumab and hydroxychloroquine, three of the most studied drugs in the treatment of COVID-19, do not cross, or cross to a far lesser extent the blood-brain barrier [34–37].

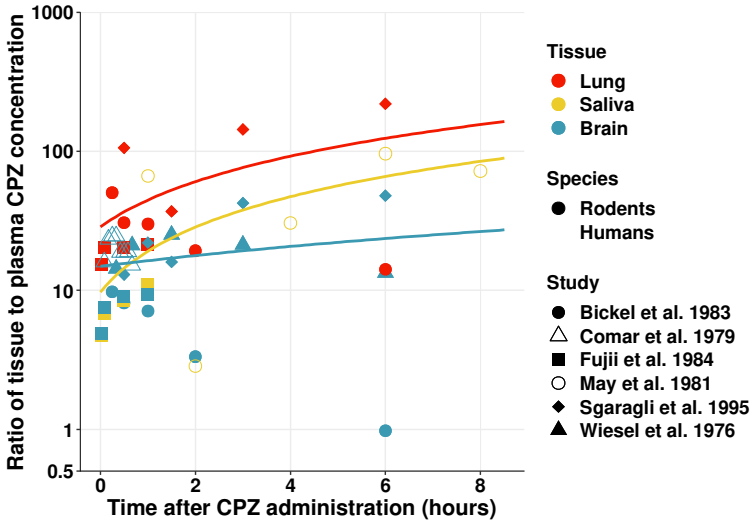

Figure 2. Review of temporal CPZ biodistribution in lung, saliva and brain. Ratio of tissue to plasma CPZ concentrations (log scale) after administration of a single dose of CPZ are represented for lung (red), saliva or salivary glands (yellow) and brain (blue) in rodents (filled) and humans (no-filled). Derived from previous preclinical and clinical studies [25–28,31,32].

Overall, even though the extrapolation from *in vitro* to clinically relevant dosage is not straightforward, the IC50 of 11.3 µM (*i.e.* 3603 ng/ml) measured *in vitro* in human cells may be compatible with CPZ dosage used in clinical routine. Indeed, residual plasma levels of CPZ in patients range from 30 to 300 ng/ml [38], which could correspond to 600 - 60 000 ng/ml in lungs [24–27] and 900 – 30 000 ng/ml in saliva [26,28] according to our review (Figure 2). This extrapolation is supported by our observation of lower prevalence of symptomatic and severe forms of COVID-19 infections in psychiatric patients.

Repurposing CPZ, a molecule already used in clinical practice, could offer both ready-to-use treatment with well-known and very mild side effects. CPZ has been widely used in clinical routine in the treatment of acute and chronic psychoses for decades. This first antipsychotic medication was discovered in 1952 by Jean Delay and Pierre Deniker at Sainte Anne hospital [39]. At this time, CPZ is prescribed for around 70 years and FDA-approved in psychiatry and anaesthesiology, with an excellent tolerance profile. CPZ is also used in refractory nausea and vomiting of pregnancy [40], in advanced cancer [41], and to treat refractory headaches in various neurological conditions [42].

Conclusions

This first *in vitro* study of CPZ antiviral activity against SARS-CoV-2 in monkey and human cells supports that CPZ, a well-known drug with antiviral properties and an excellent tolerance profile, could be tested clinically as an alternative to currently used drugs or combinations of drugs in COVID-19 treatment. This proof of principle for the feasibility of CPZ as anti-SARS-CoV-2 therapeutic is a critical step for future clinical trial (NCT04366739).

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Author contributions

R.G., F.C., E.S.L and M.P. designed the study. E.S.L., M.Pr., L.L. and J.C. performed the experiments. E.S.L., M.Pr., L.L, M.P., A.C. and D.A. analyzed the data. R.G., E.S.L., A.C., M.P. and D.A. wrote the paper with input from F.C., G.F., A.-C.P., M.B., F.V. and F.P.-D. All authors have approved the final article.

Declarations

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Competing Interests: No conflict of interest

Ethical Approval: Not required

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