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Evaluation of Crimean-Congo hemorrhagic fever virus *in vitro* inhibition by chloroquine and chlorpromazine, two FDA approved molecules

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ABSTRACT

Crimean-Congo hemorrhagic virus (CCHFV) causes hemorrhagic fever with high case mortality rates and is endemic in south-eastern Europe, Africa, and Asia. The limited catalog of specific treatment, highlight the necessity to look for additional therapeutic solutions.

Previous experiments suggested that CCHFV enters the cells via a clathrin dependent pathway. Therefore, we have evaluated the potential anti-CCHFV activity of several molecules targeting this entry possibility. We identified two molecules chloroquine and chlorpromazine. Neutralization and virus yield reduction assays were tested in Vero E6 and Huh7 cells on two different CCHFV strains. Several combinations, including ribavirin, were assayed to test a potential synergistic effect.

The two molecules inhibited CCHFV, and depending on the virus and the cell lines, the 50% inhibitory concentration (IC_{50}) values for chloroquine and chlorpromazine ranged from 28 to 43 and 10.8–15.7 μM, respectively. Time-of-addition studies demonstrated that these molecules had a direct effect on CCHFV infectivity and spread. The antiviral activity of the two molecules was still effective even when added up to 6 h post-infection and up to 24 h. The selectivity index ranging from 3 to 35 lead us to evaluate combinations with ribavirin. Combinations of ribavirin and chloroquine or chlorpromazine were synergistic against CCHFV. Though the low chlorpromazine selectivity index suggests the need for a chemical improvement, our present study highlights chloroquine as the main drug having the potential for drug repurposing.

1. Introduction

Crimean-Congo hemorrhagic virus (CCHFV) is an enveloped, segmented negative-stranded RNA virus belonging to the *Nairovirus* genus of the *Bunyaviridae* family. The virus is endemic in western areas of the former Soviet Union and in southeastern and southwestern Europe; eastern and central Asia (Bente et al., 2013). *Hyalomma* spp. ticks are considered to be the most important vector for human transmission in the epidemiology of CCHF. Human to human transmission was documented after contact with blood and other body fluids. Human infection with CCHFV often results in severe hemorrhagic disease characterized by extensive ecchymosis, visceral bleeding, hepatic dysfunction (Ergönül, 2006) with high case mortality rates. The average mortality rate is often cited at 30% (Bente et al., 2013).

In a CCHFV infection context the current medical management is largely based on the treatment of symptoms. Currently, the only drug used to treat CCHFV infection is the ribavirin. Ribavirin antiviral therapy have to be initiated as soon as possible to be effective (Tasdelen Fisgin et al., 2009). However, there is still a debate on the ribavirin therapeutic beneficial treatment (Soares-Weiser et al., 2010). Other treatments such as type I interferon (Van Eeden et al., 1985); corticosteroid therapy (Dokuzoguz et al., 2013), or the efficient polyclonal anti-CCHFV human immunoglobulin treatment, (Vassilenko et al., 1990), were tested without removing the need of new antiviral molecules.

In the present study, we aimed at characterizing the *in vitro* anti CCHFV efficacy of chloroquine and chlorpromazine.

Chloroquine was used to prevent and treat malaria (Rolain et al., 2007). It was also shown that chloroquine is a broadspectrum molecule displaying an *in vitro* antiviral activity against a range of

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RNA viruses (Rolain et al., 2007). The mechanism of action of chloroquine is multiple, depending on the pathogen, it likely acts via the endosome, lysosome and golgi vesicle pH increase (Savarino et al., 2003). Chlorpromazine a well known anti-psychotic drug showed antiviral activity against adenovirus (Diaconu et al., 2010), Ebola virus (Bhattacharyya et al., 2010) and coronavirus (De Wilde et al., 2014). The mechanism of action of chlorpromazine is known to interfere with the formation of clathrin coated pits inhibiting clathrin-mediated endocytosis (Wang et al., 1993).

In this study, chloroquine and chlorpromazine were found to inhibit CCHFV replication *in vitro*, even if added up to 6 h post infection. Moreover when these two molecules were in combination to ribavirin, a synergistic effect was observed in Vero E6 cells. Further studies on the anti-CCHFV efficacy of these two FDA-approved drugs should be investigated in KO mice models.

2. Materials and methods

2.1. Cells and viruses

Vero E6 (African green monkey kidney, ATCC CRL-1586) and Huh7 (hepatocarcinoma cell line; CelluloNet, Cat N°120, Lyon, France) cells were grown at 37 °C in DMEM (Gibco®, Life technologies™) supplemented with 10% inactivated fetal calf serum (FCSi, Gibco®, Life technologies™), 1% NEAA 100× (Gibco®, Life technologies™) and 1% penicillin–streptomycin (PS) (10,000 U penicillin/ml; 10,000 U streptomycin/ml; Gibco®, Life technologies™).

All work involving infectious viruses was carried out in a biosafety level 4 (BSL-4) facility.

The stocks of 86–07 (IbAr10200, Nigeria) and 87–07 (ArD39554, Mauritania) CCHFV strains were prepared in Vero E6 cells and titrated by plaque formation on Vero E6 cells as previously described (Peyrefitte et al., 2010).

2.2. Toxicology study

Chloroquine (C6628; Sigma), chlorpromazine (C8138; Sigma) and ribavirin (R9644; Sigma) were dissolved in water and were stored in 100 mM stock solutions at −20 °C until use.

Confluent cell monolayers of Vero E6 and Huh7 cells were incubated in DMEM supplemented with 2.5% FCSi with different concentrations of the molecules, ranging from 0 to 1000 μM of chloroquine and chlorpromazine. Cytotoxic effects were monitored at 4, 24, 48 and 72 h of treatment. Cell viability was measured using the Cell proliferation kit (MTT; Roche) according to the manufacturer's instructions. The 50% cytotoxic concentrations (CC_{50}) of chloroquine and chlorpromazine were then determined. Three independent experimentations were performed in duplicate. The selectivity index (SI) which correspond to the relative effectiveness of the drugs in inhibiting viral replication compared to inducing cell death was then determined.

2.3. Inhibition of virus infection by inhibitor treatment

2.3.1. Plaque reduction assay

For CCHFV studies, Vero E6 or Huh7 cells were seeded in 12 well plates (BD Falcon) at a density of 4×10^5 cells per well. After an overnight incubation at 37 °C, infections with CCHFV (strains 86–07; 87–07) were carried out at a MOI of 0.025. Final molecule concentrations tested ranged from 0 to 250 μM (CQ) and from 0 to 50 μM (CPZ). The 250 μl of CCHFV inoculum was removed after a 1 h incubation at 37 °C then the cells were further incubated at 37 °C after the addition of 2 ml of 3.2% carboxymethylcellulose (CMC, Sigma–Aldrich) in DMEM/2.5% FCSi solution with molecules. At day 3, viral titers were determined as described by Peyrefitte et al. (2010), except for the revelation step in which True Blue (KPL) reagent was used instead of DAB.

To test the antiviral effect of chloroquine and chlorpromazine, three conditions were assayed pre-treatment (1 h before the infection step), concurrent treatment (during the virus infection step) and permanent treatment (before and during the virus infection step). Three independent experimentations were performed in duplicate.

The 50% inhibitory concentration (IC_{50}) and the CC_{50} were calculated with GraphPad Prism 6 software using the non-linear regression model.

2.3.2. Effect of addition time

The antiviral effect of chloroquine and chlorpromazine on CCHFV was performed in 12 well plates. After an overnight incubation at 37 °C, Vero E6 cells (4×10^5 cells seeded per well) were infected using the 86–07 CCHFV strain at a MOI of 0.005. Final molecule concentrations were tested at 50 μM for chloroquine and 10 μM for chlorpromazine. The molecules were added after the 1 h viral adsorption step at 37 °C (t0) and then after 1, 3, 6 or 24 h post-infection (p.i.). Supernatants were harvested at 0, 1, 3, 6, 24 and 48 h p.i., then virus titers were determined by plaque reduction assay as described in 2.3.1 Section. Four independent experimentations were performed in triplicate.

2.3.3. Analysis of the anti-CCHFV effect of molecule combinations

To test if chloroquine or chlorpromazine can be combined with ribavirin, their potential synergistic effect was determined by the isobologram and combination-index methods, derived from the median-effect principle of Chou and Talalay (1984) using the CompuSyn software. Data obtained from the virus growth inhibitory experiments were used to perform these analysis. Vero E6 cells were seeded in 12 well plates (BD Falcon) at a density of 4×10^5 cells per well. After an overnight incubation at 37 °C, cells were infected using CCHFV (strains 86–07) at a MOI of 0.025. Molecules combinations were based on the comparison of their IC_{50}. The molecule combinations were performed at a constant ratio. A 5:8 chloroquine/ribavirin and a 2:5 chlorpromazine/ribavirin combination ratio were applied with molecules concentrations ranging from 10 to 160 μM (CQ), 6.25–100 μM (ribavirin), and 2.5–40 μM (CPZ). The combinations were added concomitantly to the CCHFV infection step and maintained during all the experience. The viral inoculum was removed after 1 h at 37 °C and the cells were further incubated at 37 °C after the addition of 2 ml of 3.2% carboxymethylcellulose (CMC, Sigma Aldrich) in DMEM/2.5% FCSi solution. At 72 h p.i., viral titers were determined as described by Peyrefitte et al. (2010), except for the revelation step in which True Blue (KPL) reagent was used instead of DAB.

The combinations were tested and compared to three theoretical fractional virus inhibition values (Fa), i.e., 0.5 corresponding to a 50% theoretical inhibition, 0.75 (a 75% theoretical inhibition) and 0.9 (a 90% theoretical inhibition). Three independent experimentations were performed in duplicate.

The isobologram method is a graphical representation of the pharmacologic interaction among the molecules. When the combination given values were shown to be under the theoretical additive effects line (represented in the graph by the three segments linking the IC_{50}, the IC_{75} and the IC_{90} of the two molecules) they were considered synergic molecules whereas values above the threshold were considered antagonistic molecules.