Journal of Clinical Virology 102 (2018) 19–26

Contents lists available at ScienceDirect

Journal of Clinical Virology

journal homepage: www.elsevier.com/locate/jcv

# *In vitro* sensitivity of human parainfluenza 3 clinical isolates to ribavirin, favipiravir and zanamivir

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ARTICLE INFO

*Keywords:*
Parainfluenza
Ribavirin
Favipiravir
Zanamivir
Clinical
Therapy

ABSTRACT

*Background:* Human parainfluenza type 3 (HPIV3) is an important respiratory pathogen. Although a number of potential therapeutic candidates exist, there is currently no licensed therapy or vaccine. Ribavirin (RBV), favipiravir (FVP) and zanamivir (ZNV) are inhibitors with proven activity against influenza and with potential inhibitory activity against HPIV3 laboratory adapted strains *in vitro*.

*Objectives:* To evaluate RBV, FVP and ZNV as inhibitors of minimally passaged UK clinical strains of HPIV3 as well as a laboratory adapted strain MK9 *in vitro*.

*Study design:* The inhibitory action of RBV, FVP and ZNV was evaluated against nine minimally passaged clinical strains and a laboratory adapted strain MK9 using plaque reduction and growth curve inhibition in a cell culture model.

*Results:* Clinical isolates were found to be at least as susceptible as the laboratory adapted strains to RBV and FVP and significantly more susceptible to ZNV. However the inhibitory concentrations achieved by ZNV against clinical strains remain prohibitively high *in vivo*.

*Conclusions:* RBV, FVP and ZNV were found to be effective inhibitors of HPIV3 *in vitro*. The lack of efficacy of RBV *in vivo* may be due to inability to reach required therapeutic levels. FVP, on the other hand, is a good potential therapeutic agent against HPIV3. Further studies using wild type clinical strains, as well as better formulation and delivery mechanisms may improve the utility of these three inhibitors.

## 1. Background

Human parainfluenza viruses (HPIV) are a prominent cause of both upper (URTI) and lower (LRTI) respiratory tract infection with a broad spectrum of presentation [1–4]. HPIV3 is recognised as a cause of serious morbidity and mortality in the immunocompromised, in particular among haematopoietic stem cell transplant (HSCT) patients [3,5,6]. Immunity to HPIV3 is incomplete and re-infections occur throughout life. Currently there is no vaccine and no approved treatment for HPIV3, indicating a clear and urgent need for a potential therapeutic candidate.

Ribavirin is a nucleoside analogue with broad anti-viral activity *in vitro* [7,8]. It has been successfully used for treatment of hepatitis C and is licensed for treatment of respiratory syncytial virus (RSV), another member of the *Paramyxoviridae,* in young children. Although originally seen as a promising therapeutic candidate for treatment of HPIV3 in HSCT [9,10] a recent meta analysis has shown that ribavirin had little or no effect on morbidity and mortality in patients with proven lower respiratory tract infection (LRTI) caused by HPIV3 [6]. This lack of therapeutic efficacy in patients necessitates a detailed evaluation of its inhibitory effect on clinical strains.

Favipiravir (T-705), a nucleoside analogue like ribavirin, is a selective and potent inhibitor of RNA dependent RNA polymerase activity and has been shown to be anti-viral by inducing lethal mutagenesis [11–13]. *In vitro* it has demonstrated activity against a broad range of RNA viruses including *Paramyxoviridae* [12,14,15] including laboratory adapted strains of HPIV3.

Zanamivir is a neuraminidase inhibitor commonly prescribed for the treatment of influenza. The structure of the HPIV3 haemagluttinin neuraminidase binding pocket shows sufficient homology with that of the influenza neuraminidase, to suggest a potential high affinity for zanamivir [16]. *In vitro* studies on tissue culture adapted strains have generally concluded that zanamivir has the potential to act as an inhibitor of HPIV3 albeit at therapeutically unachievable 50% maximum effective concentrations ($EC_{50}$) values [17]. To date the potential of zanamivir as a therapeutic candidate for HPIV3 has yet to be evaluated

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https://doi.org/10.1016/j.jcv.2018.02.009
Received 1 November 2017; Received in revised form 16 January 2018; Accepted 13 February 2018

systematically for clinical strains.

Overall, ribavirin, favipiravir and zanamivir have been evaluated *in vitro* against tissue culture adapted strains of HPIV3 and found to be effective to varying degrees.

## 2. Objectives

In this study we present an infectivity based *in vitro* model for the evaluation of potential therapeutic candidates for HPIV3 based on a tissue culture adapted reference strain and a panel of minimally passaged clinical strains. This represents a significant departure from previous *in vitro* models that have focused on significantly laboratory adapted strains.

## 3. Study design

For further details including molecular and plaque assay methods please see supplementary methods.

### 3.1. Cells, virus and inhibitors

The PLC/PRF/5 human Alexander hepatoma cell line and the culture adapted HPIV3 strain (MK9) were obtained from Public Health England (PHE) cultures. Clinical strains were sourced from HPIV3 positive respiratory patient samples collected between 2011 and 2015 by the PHE diagnostic laboratory Addenbrooke's Hospital, Cambridge. Samples were anonymised and data pertaining to patient demographics was collected where possible.

Ribavirin (RBV) and zanamivir (ZNV) were obtained from Sigma and favipiravir (FVP) from Atomax.

### 3.2. Cell viability assay

Cells in 96 well plates were either mock inoculated or inoculated with serial dilutions of each inhibitor (ribavirin, favipiravir or zanamivir) starting with a concentration of 1 mM in eight biological repeats. Plates were then incubated at 33 °C for 7 days and assayed with CellTiter-Blue® Cell Viability Assay (Promega) as per the manufacturer's instructions.

### 3.3. Culture of HPIV3 clinical strains

Cell monolayers were inoculated with clinical samples and incubated for 96 h. Viral growth was evaluated by quantifying viral copy number in the supernatant samples by qPCR on day zero and day four (see supplementary material). All samples demonstrating an increase of $10^3$ or more in viral RNA were passaged again to prepare working stocks.

Subsequently an aliquot from each stock was tested on the diagnostic respiratory panel (PHE laboratory, Addenbrookes). Samples shown to be co-infected with other respiratory viruses were rejected. Nine strains with diverse plaque phenotype collected between 2011 and 2015 were subsequently selected for susceptibility work.

### 3.4. Plaque reduction assay

Cell monolayers were either mock inoculated or inoculated with the MK9 reference strain stock dilutions required to produce 20–100 plaques in each well. Inhibitors at required concentrations, or an equivalent volume of diluent were added to the overlay and the monolayers were incubated for 7 days, fixed and immunostained and plaque area was measured.

### 3.5. Growth inhibition

Cell monolayers were either mock inoculated or inoculated with laboratory strain (MK9) virus stock in triplicate. The inoculum was then removed and the monolayers were washed, covered with maintenance medium containing the inhibitors at required concentrations, or the equivalent volume of diluent, and incubated for 24 h. Following the incubation period both the supernatant and the cells were harvested. Subsequently the concentration of released virus in the supernatant was determined by plaque titration. Viral RNA levels in infected cells were determined by qPCR and normalised to the total RNA in the sample.

### 3.6. Growth inhibition (clinical strains)

Growth kinetic inhibition experiments were carried out on clinical strains as above. Two concentrations of each inhibitor, corresponding to the 50% maximal effective concentration ($EC_{50}$) and 90% maximal effective concentration ($EC_{90}$) values, as interpolated from the dose response curve of infectious particle reduction in the supernatant using reference strain MK9, were used. The $EC_{50}$ value for zanamivir was inferred from the dose response curve of the reduction of viral copy number by qPCR and the higher concentration was taken as the maximum concentration assayed, 1 mM.

### 3.7. Binding inhibition with zanamivir

Cell monolayers were inoculated with laboratory strain (MK9) in triplicate with the required viral dilutions in maintenance medium with or without various concentrations of zanamivir. The inoculum was then removed, the cells were washed, covered with agarose overlay and incubated, fixed and immunostained.

### 3.8. Pre-incubation with zanamivir

High viral titres were pre-incubated with different concentrations of zanamivir or with equivalent volume of diluent (PBS) for 1 h at 37 °C. Mock controls with UV inactivated virus with zanamivir, and zanamivir on its own were included. Post incubation, the remaining infectivity in the sample was determined by plaque assay. Each sample was diluted at least by a factor of $10^4$, ensuring that any residual inhibitor effect was negligible.

### 3.9. Binding inhibition and pre-incubation with zanamivir (clinical strains)

Binding inhibition and pre-incubation with zanamivir were carried out on five clinical strains that were shown to be significantly more susceptible to $EC_{50}$ ZNV by growth inhibition (see above). For binding inhibition two concentrations of the inhibitor were used. The lower concentration corresponded to the $EC_{50}$ interpolated from the dose response curve of binding inhibition using laboratory strain MK9, and the higher was the maximum concentration used; 1 mM. For pre-incubation with ZNV only the maximum concentration of 1 mM was used.

## 4. Results

### 4.1. Isolation and cell culture growth of HPIV3 clinical isolates

Residual clinical samples were collected between 2011 and 2015 from the PHE diagnostic laboratory, Addenbrooke's hospital, Cambridge. 43 out of 407 samples were successfully grown at passage 1. Of these 3 samples were identified as co-infected with another respiratory pathogen and were rejected for subsequent studies. The other 40 samples underwent an additional passage to produce working viral stocks. Nine clinical strains collected from different years from diverse patient demographics and plaque phenotype were chosen for further susceptibility testing (Table 1).

Plaque area measurements, ranging from 0.3 $mm^2$ to 1.47 $mm^2$, reflect the diverse plaque phenotype of the strains chosen (Table 1) The significant difference between the plaque area of clinical strains and