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

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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.

Keywords: parainfluenza; ribavirin; favipiravir; zanamivir; clinical; therapy

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 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.

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.

STUDY DESIGN

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

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.

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 1mM 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.

Culture of HPIV3 clinical strains

Cell monolayers were inoculated with clinical samples and incubated for 96 hours. 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.

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.

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 hours. 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 was determined by qPCR and normalised to the total RNA in the sample.

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, 1mM.

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.

Pre-incubation with zanamivir

High viral titres were pre-incubated with different concentrations of zanamivir or with equivalent volume of diluent (PBS) for 1 hour 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.

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

Binding inhibition and pre-incubation with zanamivir was 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 1mM. For pre-incubation with ZNV only the maximum concentration of 1mM was used.

RESULTS

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 0.3mm^2 to 1.47mm^2, reflect the diverse plaque phenotype of the strains chosen (table1) The significant difference between the plaque area of clinical strains and strain MK9 likely reflects the culture adaptation of the laboratory strain.

The toxicity of all inhibitors was examined in PLC/PRF5 cells and no significant reduction in cell viability was observed within the range of the concentrations used for the experiments (data not shown).

Impact of zanamivir, ribavirin and favipiravir on culture adapted HPIV3

Figure 1: Laboratory adapted HPIV3 strain MK9 is sensitive to ribavirin and favipiravir but not zanamivir as measured by plaque area reduction. Figure shows mean plaque area reduction as a percentage of the plaque area of untreated control +/- SEM for ribavirin (B), favipiravir (C) and zanamivir (D). Experimental design is shown in (A). All plaque areas were measured using Fiji. Curves were fitted using GraphPad Prism version 6.00 with R^2 >0.9. Dashed lines represent the 95% confidence intervals. Each point represents three biological repeats.

To determine more accurately the effective concentration of each inhibitor against the lab adapted HPIV3 strain MK9, the EC_{50} was determined using plaque reduction assay where the inhibitor was present in the overlay. Ribavirin and favipiravir, but not zanamivir were shown to be effective inhibitors of HPIV3 strain MK9 by this method with an EC_{50} of 53.37μM for ribavirin and 137.8μM for favipiravir (figure 1B and C). This is consistent with the mode of action of zanamivir as a neuraminidase inhibitor affecting viral attachment and release(18, 19).

Figure 2: Growth of HPIV3 laboratory strain MK9 is effectively inhibited at 24 hours in the presence of ribavirin and favipiravir but not zanamivir. Experimental design is shown in A. For each inhibitor concentration, the figure shows reduction of infectious units in the supernatant as a percentage of untreated control quantified by plaque titration (panels B, D and F) and the reduction in viral copy number normalized to the total RNA in the sample as a

percentage of untreated control by qPCR (panels C, E and G). Panels I and H summarize the reduction in infectious particle number (I) and viral copy number by qPCR (H) for all inhibitors. All points are averages of three biological replicates +/-SEM. All plaques were counted using Fiji. All curves were fitted using GraphPad Prism version 6 with R^2 >0.9. Dashed lines represent 95% confidence intervals.

Subsequently the effect of each inhibitor on the growth kinetics of HPIV3 was evaluated. Ribavirin (figure 2B and C) and favipiravir (figure 2D and E) were observed to be effective inhibitors of HPIV3 (figure 2A). Due to the mutagenic nature of favipiravir an EC_{90} was not achieved with this inhibitor when measured by the reduction in genome copy number in cells (figure 2E)). Zanamivir appeared to be the least effective of these inhibitors, achieving a maximum of 10% inhibition of released virus and approximately 70% reduction in genome copy number in cells at maximum concentration assayed (1mM). The EC_{50} of zanamivir (200μM) for subsequent work was calculated from the dose response curve fitted to the reduction in genome copy number (figure 2G).

Zanamivir inhibits HPIV3 at the level of virus binding.

Figure 3: Zanamivir inhibits HPIV3 at the level of virus binding. Experimental design is shown in A (binding inhibition) and C (pre-incubation). The figure shows the effect on laboratory strain MK9 when zanamivir is present during inoculation (B) and when pre-incubated with zanamivir (D) to exclude the possibility of direct effects on virus particles. Panel D (pre-incubation) shows

no significant effect on viral replication. In both cases the figure shows the reduction of the number of infectious units as a percentage of untreated control by plaque titration +/- SEM. All plaques were counted using Fiji. All curves were fitted using GraphPad Prism version 6.00 with R^2 >0.9. Dashed lines represent 95% confidence intervals.

The ability of zanamivir to act as a binding inhibitor of HPIV3 and its effect on the viral particle itself was assessed by adding the inhibitor during the inoculation stage and pre-incubating the virus with it respectively. Zanamivir has been shown to inhibit HPIV3 at a higher concentration (EC_{50} of 295μM) when added during the inoculation stage (figure 3). Pre-incubation of HPIV3 with zanamivir has had no effect on the reduction of infectious particle number, confirming that zanamivir has no direct anti-viral activity.

Clinical strains of HPIV3 are susceptible to ribavirin, favipiravir and zanamivir

Figure 4: Clinical strains of HPIV3 are susceptible to ribavirin, favipiravir and zanamivir. Experimental design is shown in (A). For each clinical strain the figure shows the reduction of infectious units in the supernatant by plaque titration as a percentage of the untreated control +/- SEM. 9 clinical strains and strain MK9 were inoculated at low MOI and incubated for 24 hours in triplicate with two concentrations of each inhibitor (EC_{50} and EC_{90} values interpolated, where possible, from dose response curves using reference strain MK9 (figure 2E)). For zanamivir the EC_{50} value was interpolated from the dose response

curve reduction in viral copy number (figure 2G) and the higher concentration was taken to be the maximum used experimentally (1mM) as EC_{90} was not achieved with this inhibitor.

Subsequently the EC_{50} of all three inhibitors and the EC_{90} of ribavirin and favipiravir, as well as the highest concentration assays (1mM) of zanamivir, were assayed against clinical strains of HPIV3. All clinical strains were shown to be sensitive to the three inhibitors (figure 4) with the majority of the clinical strains typically being at least as susceptible to the drugs as the reference strain (table 2). Although laboratory strain MK9 was shown to be resistant to zanamivir, 5 out of 9 clinical strains at 200μM, all clinical strains at 1mM were shown to be sensitive to this inhibitor.

Clinical strains of HPIV3 are susceptible to zanamivir at the level of virus binding.

Figure 5 Clinical strains are susceptible to zanamivir at the level of virus binding

Experimental design is shown in (A). For each clinical strain the figure shows the reduction in infectious units by plaque titration as a percentage of the untreated control +/- SEM. The lower concentration (EC_{50} value) was interpolated from the dose response curve using reference strain MK9 (figure 3B) the higher concentration was taken to be the maximum used experimentally (1mM). All plaques were counted using Fiji.

In order to investigate further the effect of ZNV on HPIV3 during binding, the above experiment was repeated with the five clinical strains that were significantly susceptible to ZNV at 200μM. All the clinical strains were shown to be as sensitive to ZNV as reference strain MK9 by this method (figure 5). Similarly to the laboratory strain, pre-incubation with ZNV was shown to have no effect on the reduction in infectious particle number of these clinical strains (data not shown).

DISCUSSION

In this study ribavirin, favipiravir and zanamivir have been evaluated as potential inhibitors of HPIV3 in both a laboratory adapted strain and nine distinct minimally passaged clinical strains obtained between the years 2011-2015. The clinical strains selected for this study originated from a diverse population of patients and can therefore be considered representative of the population covered by the PHE diagnostic laboratory Addenbrookes Hospital, Cambridge.

Our results confirmed that ribavirin is an effective inhibitor of HPIV3 *in vitro* both by plaque reduction and by growth inhibition assays (figures 1 and 2). An approximately 4-fold decrease in EC_{50} value against laboratory strain MK9 between the one obtained by plaque titration (53.37μM) (figure 1) and by growth kinetics inhibition (15.14μM) (figure 2) was noted. This discrepancy was likely due to differences in methodology including the stability of the inhibitor in the overlay (7 days vs 24 hour incubation), the timing of data collection and viral spread confined to cell to cell fusion in plaque assays. Clinical strains have been shown to be at least as susceptible to ribavirin as the laboratory strain with a potential lower EC_{50} for clinical strains (figure 4).

These figures are compatible with ribavirin bioavailability studies that demonstrate an average level of 8.19μM, with a potential correlation with haemoglobin drop above 4μM(20) during hepatitis C therapy. In the case of RSV treatment(21, 22), where ribavirin is delivered by aerosol, plasma levels

achieved are significantly less that the EC_{50} observed in this study and range from 0.76μM to 6.8μM, depending on length of delivery(23) . Unfortunately neither study provides data on concentrations in the respiratory tract. As such, although ribavirin remains an effective inhibitor of HPIV3 *in vitro*, further optimization of drug design or combination therapy is required to yield a regimen capable of delivering therapeutically useful concentrations at the site of infection.

Favipiravir is a nucleoside analogue with a broad spectrum of action, and has been shown to be effective against other RNA viruses such as influenza, ebola and laboratory adapted parainfluenza strains *in vitro*(14, 15, 24, 25). Given the similarities in the RNA dependent RNA polymerase, it is a promising inhibitor of HPIV3. Overall we observed that favipiravir is an effective inhibitor of HPIV3 both by plaque reduction and growth kinetics inhibition assay in the current model (figures 1 and 2), with 8 out of 9 clinical strains tested being at least as sensitive to favipiravir as the laboratory strain MK9 (figure 4). In this study an EC_{50} of approximately 138μM was determined for HPIV3 by both plaque reduction and growth kinetics inhibition (figure 2).

As favipiravir is a relatively novel therapeutic drug, very limited *in vivo* data on plasma concentrations achieved in humans is available(15), although a number of *in vivo* studies using small rodent models(14, 26, 27)and well as non-human primates(28) have been conducted. Recently released data from the JIKI trial (Efficacy of favipiravir against ebola trial) quoted trough plasma levels of 293μM on day 2 and 165μM on day 4 of treatment(25). This exceeds the EC_{50} and EC_{90}

values quoted in literature(14, 27) and observed in this study. Although encouraging, this should be interpreted with caution, as no data on favipiravir concentration in respiratory secretions and in the lungs is currently available.

In this study we have observed ZNV to be ineffective against the laboratory strain by two assays (figures 1 and 2), all of the clinical strains demonstrated at least a 50% reduction in infectious particle number in the supernatant at 1mM and 2 out of 9 strains tested demonstrated ~ 50% inhibition at 200μM (figure 4). We have also demonstrated that zanamivir acts as a binding inhibitor of HPIV3 at EC_{50} of 295μM, although an EC_{90} was not achieved below 1mM (figure 3). No difference was observed between the sensitivity to ZNV in its capacity as a binding inhibitor by the laboratory strain MK9 and the clinical strains tested (figure 5).

This is consistent with previous data that indicates that the HN protein of HPIV3 contains two binding sites and is responsible for the binding, fusion triggering and release of the new viral particle(29). In its capacity as a binding inhibitor, ZNV is known to bind to site I with a non-specific distortion of site II(29). The fusion and release processes, on the other hand have been linked to binding site II (29, 30). Moreover a specific mutation (N556D) at binding site II has been linked to culture adaptation and has been shown to confer a 5-fold decrease in neuraminidase activity between a wild type strain and significantly culture adapted one (31). This has been linked to a more robust interaction with the cell receptor(32) and a larger plaque phenotype (table 1) in significantly culture adapted strains(31). It is of note that the reference strain MK9 contains that

mutation and hence the reduced neuraminidase activity whereas the clinical strains used in this study do not. This agrees with the results of this study, where clinical strains have been shown to be more susceptible to ZNV than laboratory strain MK9 by growth inhibition but not at the level of binding.

Although this data is encouraging, the inhibitory concentrations achieved in this study still exceed zanamivir levels in nasal secretions (between 200μM and 300μM) achieved during influenza treatment(33). Nonetheless the observed susceptibility of clinical strains to zanamivir confirms the importance of conducting further studies in this area on clinical strains with minimal culture adaptation.

In this study we have presented an *in vitro* infectivity based model for evaluating HPIV3 susceptibility to potential therapeutic candidates using a tissue culture adapted reference strain MK9 and 9 diverse clinical strains. A necessary limiting factor in methodologies that involve immortalized cell culture is the reliance on viruses that are able to grow in this environment. A markedly larger plaque phenotype is associated with significant culture adaptation as demonstrated by the laboratory strain(31). Within these constraints, and as all clinical samples have been minimally and equally passaged in cell culture, the diversity in plaque size is an indication of diversity of phenotype of the clinical samples used in this study. There is good evidence that heavily laboratory adapted HPIV3 strains are non-representative of the currently circulating clinical strains(30, 31). Despite recent advances in human airway epithelial (HAE) culture systems(31), these are often not suitable when large volume, high titre stocks are required for

412 subsequent downstream analysis. We have found ribavirin and favipiravir, but
413 not zanamivir to be effective inhibitors of both the tissue culture adapted strain
414 and clinical strains of HPIV3. Overall clinical strains were significantly more
415 susceptible to zanamivir. Further work on clinical circulating strains, optimized
416 methods of delivery and targeted clinical trials are required to formulate
417 treatment for this important pathogen.
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COMPETING INTERESTS

None declared

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sample				patient			
Lab ID	Date collected	source	Plaque area mm^2 +/- SEM	sex	age	location	In/out patient
14	Nov-14	URT swab	0.30+/- 0.04	F	1 year	Basildon hospital	in
16	May-14	URT swab	0.33+/- 0.03	M	50 years	Addenbrooke's hospital	in
65	Jun-11	URT swab	1.47+/- 0.11	F	54 years	Addenbrooke's hospital	in
82	Jul-11	URT swab	1.46+/- 0.16	M	2 months	Harlow hospital	in
113	Jul-12	URT swab	0.94+/- 0.05	M	42 years	Addenbrooke's hospital	in
129	Feb-13	Tracheal aspirate	0.92+/- 0.04	F	4 years	Frimley Park hospital	in/ICU
153	Apr-13	NPA	0.83+/- 0.03	F	12 years	Frimley Park hospital	in
180	May-13	NPA	0.57+/- 0.02	F	3 months	Addenbrooke's hospital	out
362	Mar-15	URT swab	0.59+/- 0.06	F	80 years	Essex nursing home	out
LS MK9	n/a	n/a	3.96+/- 0.45	n/a	n/a	n/a	n/a

Table 1: Clinical strains selected for susceptibility testing. Clinical strains were collected between 2011 and 2015, all originated from the upper airway of patients with 4/9 from Addenbrooke's Hospital, Cambridge. Plaque area for the clinical strains averaged at 0.82mm^2 +/- 0.03 (SEM) with a range between 0.3 mm^2 and 1.47mm^2, with strains from 2011 (65 and 82), demonstrating a comparatively large plaque phenotype. Strain MK9 is a laboratory adapted strain obtained from PHE cell culture collections.

		average percentage of untreated control (PFUs/ml) +/- SEM		number of clinical strains with significant deviation from the laboratory strain (FDR 1%)		
drug	concentration	lab strain	clinical strains	less sensitive	more sensitive	no difference
ribavirin	RBVIC50	47.94+/-5.97	21.5+/-2.08	0 (0%)	8 (89%)	1 (11%)
	RBVIC90	9.12+/-0.76	5.15+/-0.78	1 (11%)	4 (44.5%)	4 (44.5%)
favipiravir	FVP IC50	47.95+/-1.37	39.77+/-3.69	1 (11%)	4 (44.5%)	4 (44.5%)
	FVP IC90	13.56+/-0.23	14.03+/-1.73	1 (11%)	3 (33%)	5 (56%)
zanamivir	ZNV 200µM	98.63+/-4.11	75.86+/-3.21	0 (0%)	5 (55.5%)	4 (44.5%)
	ZNV 1mM	90.41+/-4.75	25.94+/-3.72	0 (0%)	9 (100%)	0 (0%)

Table 2: Clinical strain susceptibility to favipiravir, ribavirin and zanamivir

Average EC_{50} and EC_{90} values for each clinical strain and for the laboratory strain MK9 (PHE cultures) determined by plaque titration of supernatant after 24 hour incubation (figure 4) are summarized. A summary of how many clinical strains were more, less or equally susceptible to each inhibitor is included for each inhibitory concentration. All plaques were counted using Fiji.

Figure 1

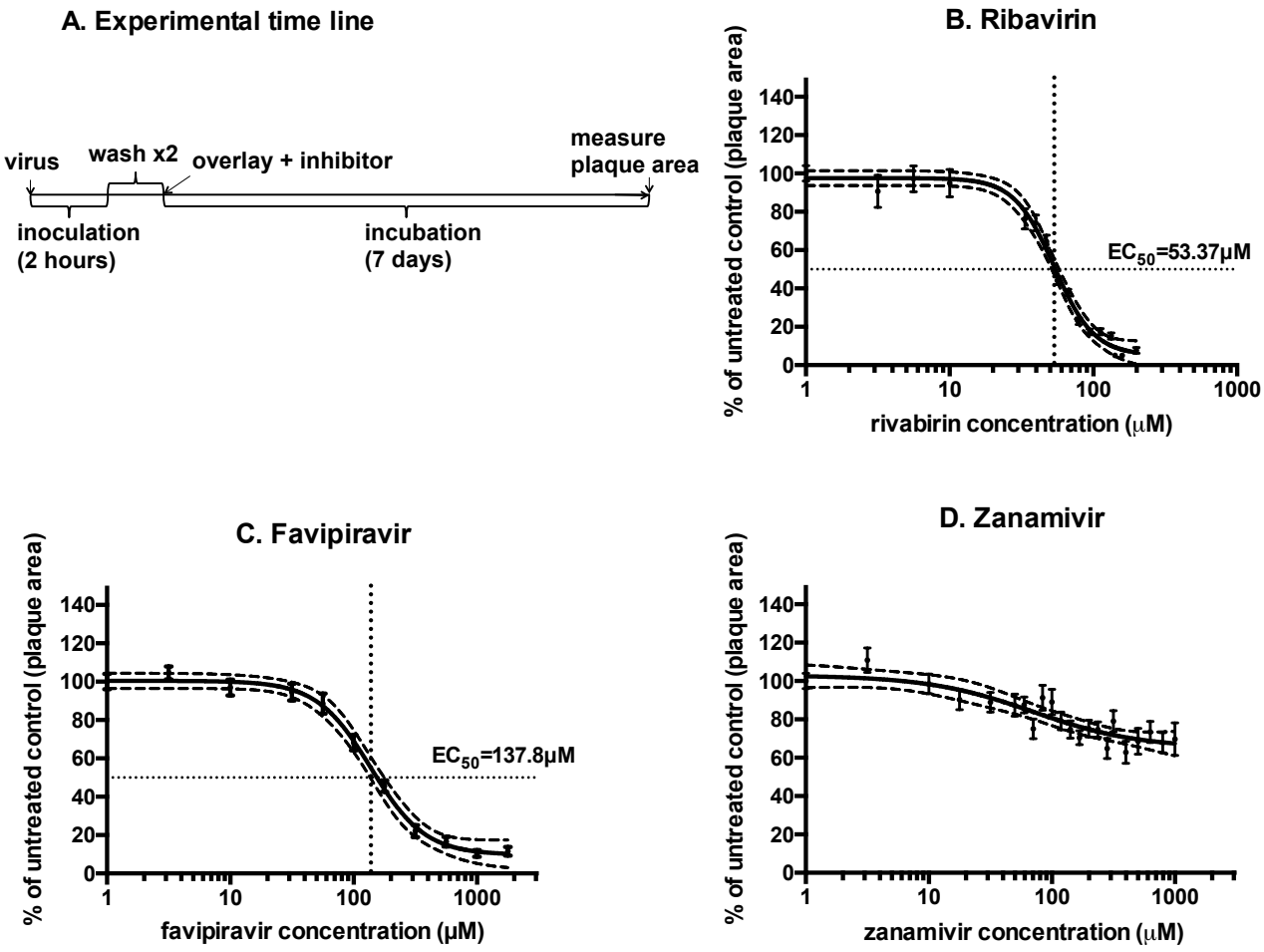

A. Experimental time line
virus
wash x2
overlay + inhibitor
measure plaque area
inoculation (2 hours)
incubation (7 days)
B. Ribavirin
% of untreated control (plaque area)
EC50=53.37µM
rivabirin concentration (µM)
C. Favipiravir
EC50=137.8µM
favipiravir concentration (µM)
D. Zanamivir
zanamivir concentration (µM)

Figure 2

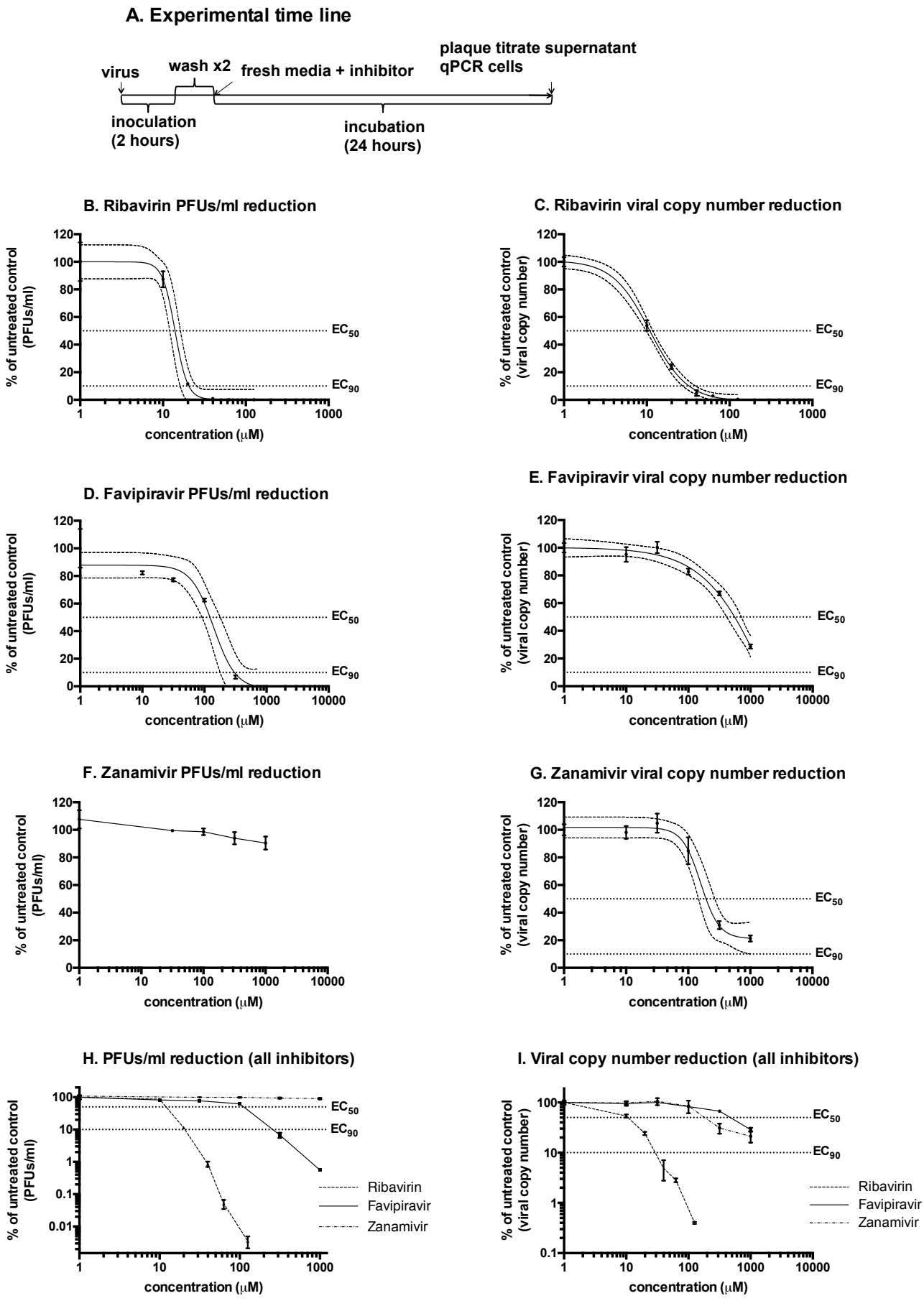

A. Experimental time line
virus
wash x2
fresh media + inhibitor
plaque titrate supernatant
qPCR cells
inoculation
(2 hours)
incubation
(24 hours)
B. Ribavirin PFUs/ml reduction
C. Ribavirin viral copy number reduction
D. Favipiravir PFUs/ml reduction
E. Favipiravir viral copy number reduction
F. Zanamivir PFUs/ml reduction
G. Zanamivir viral copy number reduction
H. PFUs/ml reduction (all inhibitors)
I. Viral copy number reduction (all inhibitors)
% of untreated control (PFUs/ml)
% of untreated control (viral copy number)
concentration (μM)
EC50
EC90
Ribavirin
Favipiravir
Zanamivir

Figure 3

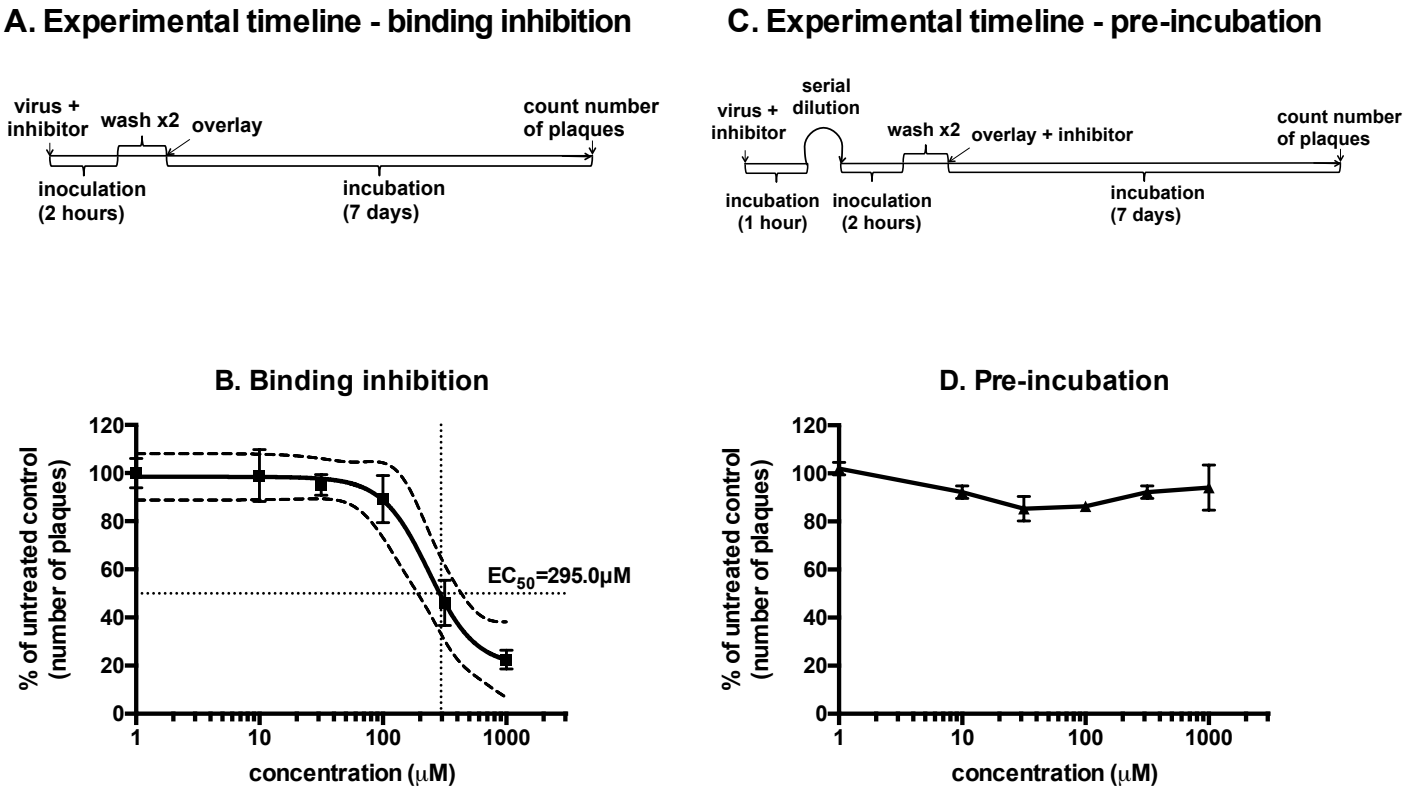

A. Experimental timeline - binding inhibition
virus + inhibitor
wash x2
overlay
count number of plaques
inoculation (2 hours)
incubation (7 days)
C. Experimental timeline - pre-incubation
virus + inhibitor
serial dilution
wash x2
overlay + inhibitor
count number of plaques
incubation (1 hour)
inoculation (2 hours)
incubation (7 days)
B. Binding inhibition
% of untreated control (number of plaques)
EC50=295.0µM
concentration (µM)
D. Pre-incubation
% of untreated control (number of plaques)
concentration (µM)

Figure 4

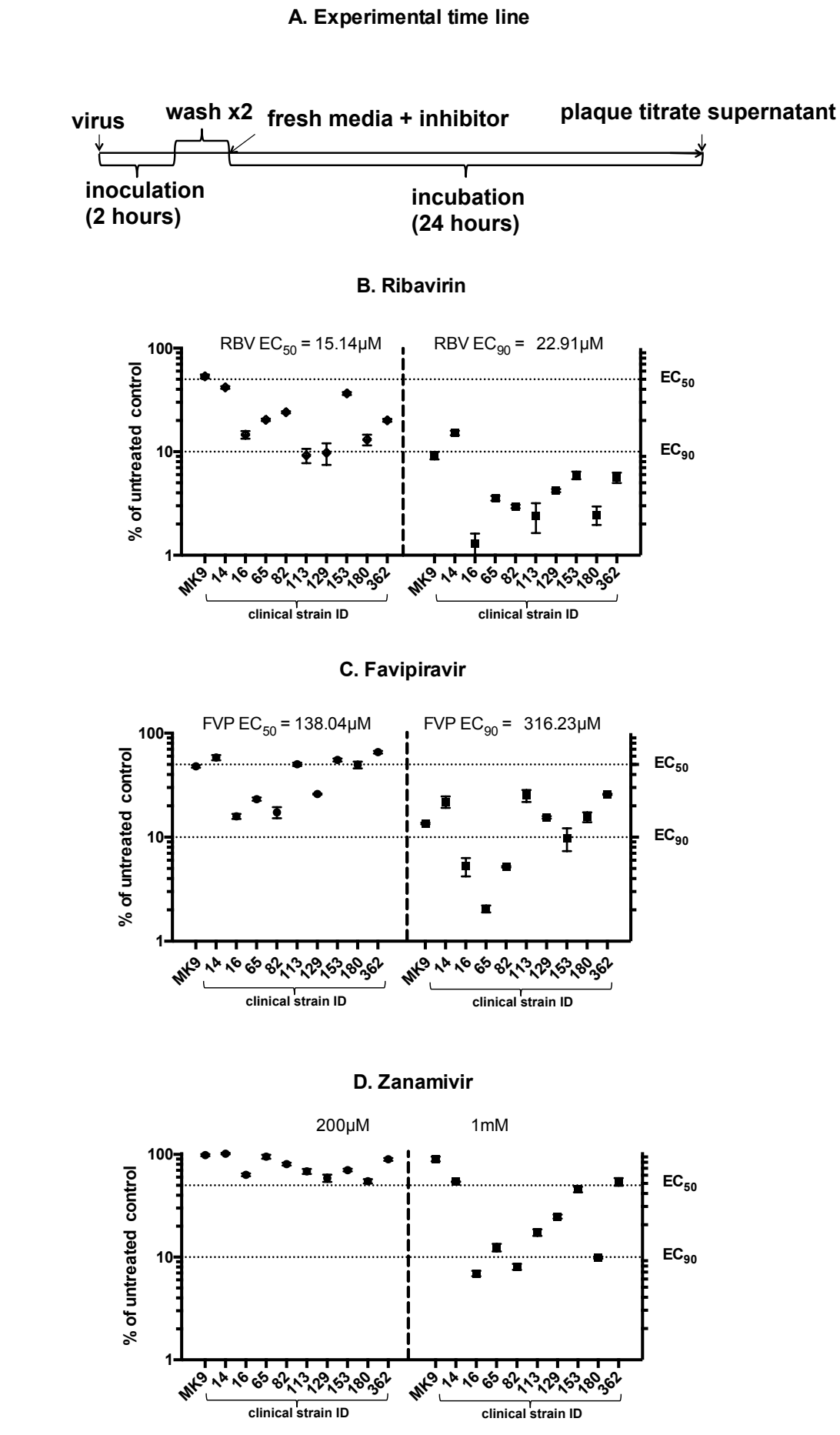
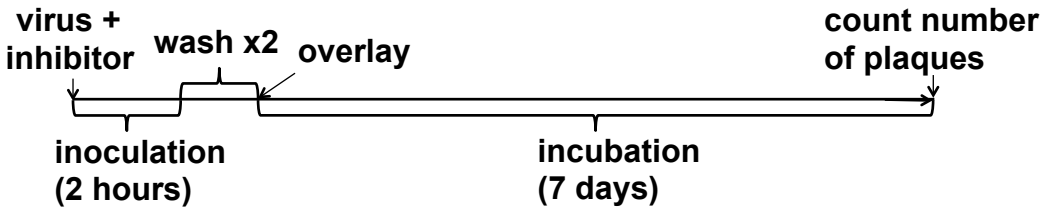
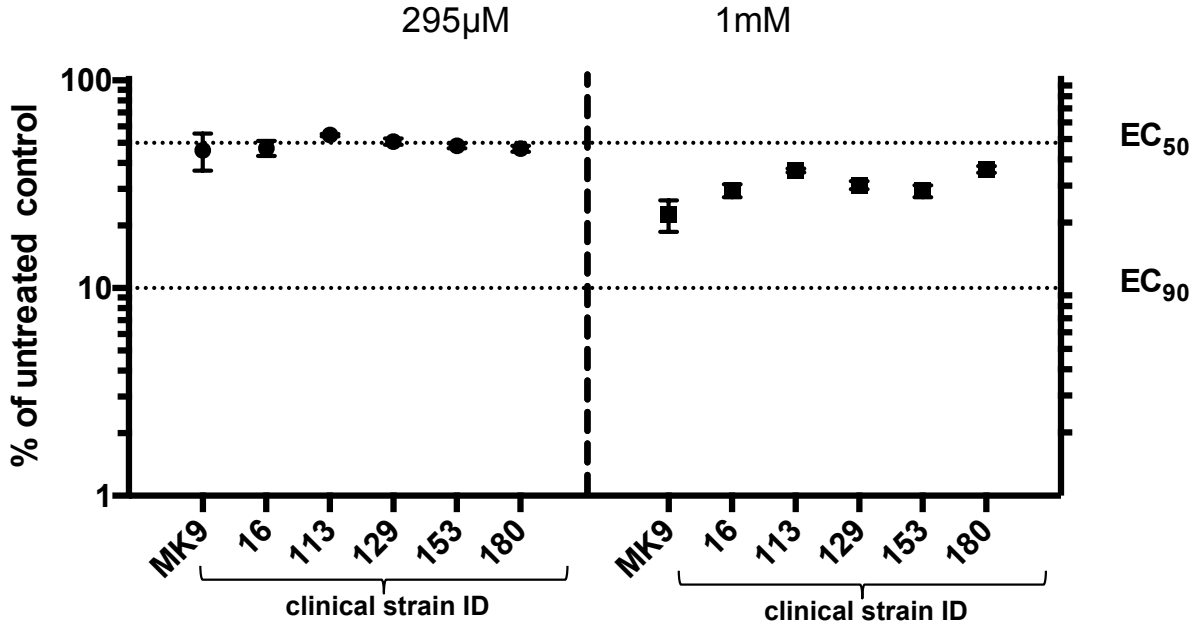

A. Experimental time line
virus
wash x2
fresh media + inhibitor
plaque titrate supernatant
inoculation
(2 hours)
incubation
(24 hours)
B. Ribavirin
RBV EC50 = 15.14µM
RBV EC90 = 22.91µM
% of untreated control
100
10
1
EC50
EC90
MK9 14 16 65 82 113 129 153 180 362
clinical strain ID
C. Favipiravir
FVP EC50 = 138.04µM
FVP EC90 = 316.23µM
D. Zanamivir
200µM
1mM

Figure 5

A. Experimental timeline - binding inhibition



B. Zanamivir - binding inhibition (clinical strains)



SUPPLEMENTARY METHODS

Cells

HPIV3 has been cultured previously in numerous cell lines including, among others: CV-1, 293T, Hep2, MDCK and Vero(1, 2). In this case the PLC/PRF/5 cell line was chosen as it was previously used for tissue culture based diagnosis of respiratory viruses in the laboratory that has supplied the clinical samples for this study. More importantly, it was judged, that as this line was suitable for diagnostics, it would be suitable for isolation of clinical strains.

The cell line was maintained in Dulbecco Modified Eagle Medium (DMEM) high glucose medium supplemented with 10% fetal bovine serum (FBS), penicillin (100 SI units/ml), streptomycin (100µg/ml) and 2mM L-glutamine at 37°C in 5% CO_2.

Culture of HPIV3 clinical strains

Cell monolayers were set up at 70% confluence in T25 flasks and each monolayer was inoculated with 20µl clinical sample in 200µl maintenance medium (high glucose DMEM with 1% fetal bovine serum (FBS) and 2mM L-glutamine) supplemented with penicillin 100U/ml, streptomycin 100µg/ml, gentamicin 50µg/ml, ceftazidime 50µg/ml, vancomycin 50µg/ml and fungizone (amphotericin B) 5µg/ml to minimize bacterial and fungal out growth. Infections were carried out at 37°C for 2 hours. The inoculum was then removed, the cells washed twice in PBS, covered with maintenance medium as above and incubated for 4 days at 33°C in 5% CO_2. Supernatant samples of 50µl were collected on day zero and day four. Viral growth was evaluated by quantifying viral copy number

in the supernatant samples by qPCR (see protocol below).

Subsequently an aliquot from each stock was tested on the diagnostic respiratory panel (PHE laboratory, Addenbrookes) including the following common viruses: influenza A and B, RSV, enterovirus, rhinovirus, HMPV, adenovirus and HPIV1, 2, 3 and 4.

Statistical analysis

All statistical analysis was carried out in GraphPad Prism version 6.00 for Mac OS X, GraphPad Software, La Jolla California USA, www.graphpad.com. Linear regressions from the standard curve for qPCR were fitted using the linear regression model. Dose response curves for drug inhibition assays were fitted using the 4 parameter logistic (4PL) fit. Curves with $R^2 > 0.9$ and a $p<0.05$ for replicates test for lack of fit were accepted as adequate models.

qPCR

Total RNA from samples was extracted using the GenElute Mammalian Total RNA Miniprep kit (Sigma) according to the manufacturer's guidelines. This was amplified on the ViiA7 Real Time PCR system (Applied Biosystems) using a qPCR protocol obtained and modified from the standard operating procedure (SOP) for HPIV3 typing used by the PHE diagnostic laboratory, Addenbrookes Hospital, Cambridge. The primers and the taqman probe used were: forward 5'-GCTCCTTTYATCTGTATCCTCAGAGATCC-3', reverse 5'-TGATCTTCCCGTCACATACTGTTGCATG-3', probe 5'-FAM-ATAGTTGCCTGGTGCGAA-TAMRA-3'. The cycling conditions used were: hold at 50°C for 30min, hold at 95°C for 2 min, followed by 45 cycles while acquiring

fluorescence data through 95°C for 15s and 60°C for 60s. An amplicon from the diagnostic assay positive control was obtained and cloned by TA cloning using the PureYield™ Plasmid Midiprep System (Promega). The sequence of the amplicon aligned to 138bp of the nucleocapsid gene of HPIV3 (nucleotides 981-1118). Ten fold serial dilutions of the plasmid were subsequently used to establish a standard curve. Linear regression of the standard curve for genome copy number quantification was fitted using GraphPad Prism version 6.00.

Plaque assay

Monolayers at 80-90% confluence (approximately 0.96 – 1.08 x 10^6 cells/well) were set up in 6 well plates and infected with serial dilutions of virus stock (500µl/well). Infections were carried out in maintenance medium (high glucose DMEM supplemented with 1% fetal bovine serum (FBS), penicillin (100 SI units/ml), streptomycin (100µg/ml) and 2mM L-glutamine) at 37°C for 2 hours. The inoculum was then removed and the monolayers washed twice in PBS. A 1% agarose overlay with 50% maintenance medium was applied to the infected monolayer. The plates were then incubated inverted at 33°C in 5% CO_2 for 7 days. Subsequently they were fixed with 2% formaldehyde in PBS, the agarose plugs were removed and the monolayers were washed three times in PBS prior to immunostaining. Each titration was performed in triplicate.

The infected cells were stained with a mixture of three rabbit polyclonal anti-F HPIV3 antibodies at 1:5000 dilution in PBS containing 5% FBS at room temperature for 1 hour (500µl/well). The antibodies were raised against the following epitopes NQESNENTDPRTERF (amino acids 96-110), NRVDQNDKPYVLTNK (amino acids 525-539), and KEWIRRSNQKLDSIG (amino

acids 471-485) of the F protein. The cells were then washed three times with PBS, leaving each wash on for 5 min, and subsequently incubated with an anti-rabbit HRP conjugated secondary antibody at 1:1000 in PBS containing 5% FBS at room temperature for 1 hour (500µl/well). The cells were washed 5 times in PBS and reacted with True Blue Peroxidase substrate (SeraCare) (500µl/well) for 20min at room temperature. The plaques were scanned and subsequently identified and measured using the Fiji analyze particles module(3).

1. Palmer SG, Porotto M, Palermo LM, Cunha LF, Greengard O, Moscona A. 2012. Adaptation of human parainfluenza virus to airway epithelium reveals fusion properties required for growth in host tissue. MBio 3:e00137-12-.
2. Jochmans D, Van Nieuwkoop S, Smits SL, Neyts J, Fouchier RAM, Van Den Hoogen BG. 2016. Antiviral activity of favipiravir (T-705) against a broad range of paramyxoviruses in vitro and against human metapneumovirus in hamsters. Antimicrob Agents Chemother 60:4620–4629.
3. Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, Preibisch S, Rueden C, Saalfeld S, Schmid B, Tinevez J-Y, White DJ, Hartenstein V, Eliceiri K, Tomancak P, Cardona A. 2012. Fiji: an open-source platform for biological-image analysis. Nat Methods 9:676–682.