Antiviral Research 169 (2019) 104542

Contents lists available at ScienceDirect

Antiviral Research

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

Passaging of an influenza A(H1N1)pdm09 virus in a difluoro sialic acid inhibitor selects for a novel, but unfit I106M neuraminidase mutant

Jennifer L. McKimm-Breschkin[a,b,*], Susan Barrett[a], Charley McKenzie-Kludas[b], Julie McAuley[b], Victor A. Streltsov[a,c], Stephen G. Withers[d]

[a] CSIRO Manufacturing, 343 Royal Parade, Parkville, 3052, Australia
[b] Department of Microbiology and Immunology, The University of Melbourne at The Peter Doherty Institute for Infection and Immunity, 792 Elizabeth Street, Melbourne, 3000, Australia
[c] The Florey Institute of Neuroscience and Mental Health, The University of Melbourne, 30 Royal Parade, Parkville, 3052, Australia
[d] Department of Chemistry, University of British Columbia, 2036 Main Mall, Vancouver, British Columbia, V6T 1Z1, Canada

ARTICLE INFO

Keywords:
Neuraminidase inhibitor
Drug resistance
Difluorosialic acid

ABSTRACT

An influenza A(H1N1)pdm09 and an influenza B virus were passaged in 3-fluoro(eq)-4-guanidino difluoro sialic acid (3Feq4Gu DFSA), an inhibitor of the influenza neuraminidase (NA) to determine whether resistant variants could be selected. 3Feq4Gu DFSA is a mechanism-based inhibitor, forming a covalent link to Y406 in the NA active site. Given its similarity to the natural substrate, sialic acid, we predicted resistant variants would be difficult to select. Yields of both viruses decreased with passaging, so that after 12 passages both viruses were only growing to low titers. Drug concentrations were decreased for another three passages. There was no difference in NA sensitivity in the MUNANA fluorescence-based assay, nor in plaque assays for the passaged virus stocks. All influenza B plaques were still wild type in all assays. There were isolated small diffuse plaques in the P15 pdm09 stock, which after purification had barely detectable NA or hemagglutinin (HA) activity. These had a novel non-active site I106M substitution in the NA gene, but unexpectedly no HA changes. The I106M may impact NA function through steric effects on the movement of the 150 and 430-loops. The I106M viruses had similar replication kinetics in MDCK cells as wild type viruses, but their ability to bind to and infect CHO–K1 cells expressing high levels of cell-bound mucin was compromised. The I106M substitution was unstable, with progeny rapidly reverting to wild type by three different mechanisms. Some had reverted to I106, some had V106, both with wild type NA and HA properties. A third group retained the I106M, but had a compensating R363K substitution, which regained almost wild type NA properties. These viruses now agglutinated chicken red blood cells (CRBCs) but unlike the I/V106, they rebound after elution at 37 °C. There were no mutations in the HA, but each phenotype correlated with the NA sequence. We propose that the activity in the I106M mutant is insufficient to remove carbohydrates from the virion HA and NA, sterically limiting HA access to CRBC receptors, thus resulting in poor HA binding.

1. Introduction

The first antiviral drug, zanamivir (Relenza) targeting the influenza neuraminidase (NA) was licensed 20 years ago. Its design was based on an understanding of how the sialic acid ligand bound in the active site (Varghese et al., 1992). Zanamivir is based on DANA (2,3, dehydro 2 deoxy N-acetyl neuraminic acid), a transition state analog of sialic acid, which was known to be a weak inhibitor of influenza NA activity. It differs from DANA in the single substitution of the 4-hydroxyl with a 4-guanidino side chain, enhancing inhibitory activity by more than 10,000-fold compared to DANA (von Itzstein et al., 1993). Oseltamivir was subsequently designed with further modifications, including

Abbreviations: 3Feq4Gu DFSA, 3-fluoro(eq)-4-guanidino difluoro sialic acid; NA, neuraminidase; HA, hemagglutinin; MUNANA, 4-Methylumbelliferyl *N*-acetyl-α-D-neuraminic acid; CHO–K1 MUC1, Chinese Hamster Ovary -K1 cells expressing MUC1 at the cell surface; cpe, cytopathic effect

* Corresponding author. Department of Microbiology and Immunology, University of Melbourne, The Peter Doherty Institute for Infection and Immunity, 792 Elizabeth Street, Melbourne, 3000, Australia.

E-mail addresses: jmbvirology@gmail.com (J.L. McKimm-Breschkin), susan.barrett@csiro.au (S. Barrett), charley.mackenzie-kludas@unimelb.edu.au (C. McKenzie-Kludas), jmcauley@unimelb.edu.au (J. McAuley), vstreltsov@unimelb.edu.au (V.A. Streltsov), withers@chem.ubc.ca (S.G. Withers).

https://doi.org/10.1016/j.antiviral.2019.104542
Received 15 April 2019; Received in revised form 18 June 2019; Accepted 19 June 2019
Available online 22 June 2019

replacement of the sugar ring with a cyclohexene ring, substitution of the glycerol side chain with a pentyl ether group and incorporation of a 4-amino group rather than a guanidine (Kim et al., 1997). However, for the oseltamivir to bind in the active site we initially showed that E276 needs to rotate to accommodate the bulky hydrophobic pentyl ether (Varghese et al., 1998). Based on this steric rearrangement we predicted mutations conferring resistance to oseltamivir were more likely to arise than to zanamivir. This has been confirmed clinically, including the global circulation of an oseltamivir resistant virus in 2007-8 (Dharan et al., 2009; Hurt et al., 2009; Meijer et al., 2009) and isolation of resistant viruses either after oseltamivir treatment, or circulating in untreated patients in the community (Abed and Boivin, 2017; Gubareva et al., 2017; Lackenby et al., 2018; McKimm-Breschkin, 2013). This led to our minimalist hypothesis of drug design that by keeping the inhibitor as close as possible to the natural substrate then it would be more difficult for the target to mutate to become resistant, since it would compromise natural ligand binding (Varghese et al., 1998).

Based on the knowledge of resistance and modifications which enhanced efficacy, we recently described the development of new inhibitors of the influenza NA, based on sialic acid with fluorine groups at the C-2 and C-3 positions (Kim et al., 2013). These difluoro sialic acid (DFSA) inhibitors form a covalent link with Y406 in the active site. We wished to determine how readily resistant variants could be selected by passaging in increasing concentrations of the potent 3-fluoro(eq)-4-guanidino difluoro sialic acid derivative (3Feq4Gu DFSA). Consistent with our minimalist hypothesis resistant NA variants were hard to select, supporting further development of these inhibitors.

2. Materials and methods

Additional details are also provided in the Supplementary Materials and Methods.

2.1. Cells and viruses

The wild type A/Perth/265/2009 (H1N1)pdm09 (pdm A/Perth) was originally obtained as part of the isirv-AVG reference panel from the WHO Collaborating Centre for Reference and Research on Influenza (Melbourne). Prior to passaging, virus was plaque-purified in Madin Darby Canine Kidney (MDCK) cells. The B/Perth/211/2001 (B/Perth) wild type virus has been previously described (Hurt et al., 2006; Oakley et al., 2010).

2.2. Chemicals and inhibitors

Zanamivir was synthesized by GlaxoSmithKline (Stevenage, UK). Oseltamivir carboxylate was prepared by Dr Keith Watson (Walter and Eliza Hall Institute, Australia). 3Feq4Gu DFSA was synthesized as described (Kim et al., 2013). Dilutions of inhibitors for enzyme assays were prepared in water, ranging from 0.001 nM to 1000 nM for zanamivir and 0.1 nM to 100,000 nM for 3Feq4Gu DFSA. For cell culture they were diluted in 2xDMEM/F12 overlay medium. 4-Methylumbelliferyl *N*-acetyl-α-D-neuraminic acid (MUNANA) was obtained from Carbosynth, UK.

2.3. Passaging protocol

As the pdm A/Perth was less sensitive compared to the B/Perth, starting concentrations of 3Feq4Gu DFSA for pasaging were 50 nM for pdm A/Perth and 10 nM for B/Perth. MDCK cells in 24-well plates were infected with serial 10-fold dilutions of passaged virus in concentrations of inhibitor increasing until P6 then constant until P12. Five plaques were picked and amplified without drug at every 3 passages to test sensitivity in an enzyme inhibition assay against zanamivir and 3Feq4Gu DFSA. (Table S1).

2.4. Neuraminidase inhibition assays IC_{50} kinetics

We used the MUNANA-based fluorescence assay to determine the NA activity, and to determine sensitivity to the inhibitors as previously described (McKimm-Breschkin et al., 2013). The IC_{50} was calculated as the inhibitor concentration decreasing the fluorescent signal by 50% compared to the control. Inhibition was also tested in the IC_{50} kinetics assay which uses two assays with and without preincubation of NA and inhibitor (Barrett et al., 2011) to provide a measure of the relative rates of binding (no preincubation) and dissociation (after preincubation). All samples were assayed in duplicate.

2.5. Hemagglutination assays

We used 1% chicken (CRBC) and turkey (TRBC) red blood cells for hemagglutination assays (HA) in 96-well U-bottomed plates. For HA elution (HAE), plates were then incubated at 37 °C for 16 h. Rebinding was determined by mixing wells and reincubating on ice. We used an anti-HA monoclonal antibody (Clone 178 raised against A/California/07/2009, kindly provided by Dr Ian Barr, WHO CC Melbourne), or 1 μM oseltamivir or 3Feq4Gu DFSA in HA inhibition (HAI) assays.

2.6. Replication kinetics

Multicycle replication kinetics were carried out using the control passaged virus and two serially plaque purified mutant viruses from P15 at a multiplicity of infection (MOI) of 0.01. Duplicate wells were harvested separately at 12 hourly intervals for titration in a plaque assay.

2.7. Slot blot

Serial dilutions of wild type and mutant viruses were bound to nitrocellulose filters in a slot blot apparatus (Schleicher and Schuell) and probed with an anti-nucleoprotein (NP) monoclonal antibody (HB-65, ATCC), or clone178 anti HA antibody, or a broadly reactive anti-NA antibody HCA-2 (kindly provided by the University of Ottawa) (Doyle et al., 2013). Binding was detected with horseradish peroxidase (HRPO) conjugated antibodies and tetramethyl benzidine (TMB) substrate. 1% dextran sulfate was added with the TMB substrate instead of preincubating with the filter as initially described (McKimm-Breschkin, 1990).

2.8. Effect of mucins on infection

Chinese Hamster Ovary-K1 (CHO–K1) control (ATCC) and CHO–K1 $MUC1^+$ epithelial cells, over-expressing the MUC1 cell surface mucin (kindly provided by Prof Michael McGuckin, University of Melbourne), were grown and infected and analyzed for surface HA expression, intracellular NP levels and SAα2-3Gal by flow cytometry.

2.9. Egg infection

As the 36 h replication kinetics samples had comparable titers for the mutants and wild type viruses, they were used to infect 9 day-old embryonated hen's eggs (n = 2). An inoculum of 100 μl of a 10^{-1}, 10^{-2} or 10^{-3} dilution was used for each virus. Allantoic fluids were harvested after 48 h, and individual virus yields were quantified by plaque assay, HA and NA assays.

2.10. Sequencing

Viral RNA was extracted using the RNeasy mini kit (Qiagen, Germany). cDNA conversion was then performed using the Omniscript RT kit (Qiagen, Germany) before amplifying the DNA using a Phusion Flash PCR master mix (Thermo Fisher, Australia) with HA and NA

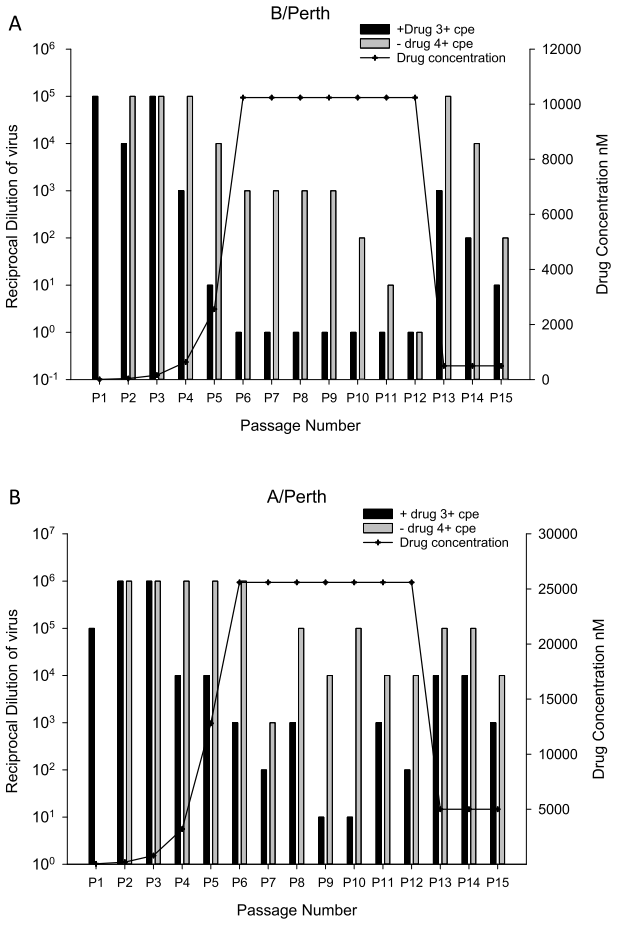

Fig. 1. Passaging of viruses in 3Feq4Gu DFSA.A) Serial $\log_{10}$ dilutions of B/Perth/211/01 and B) A/Perth/265/09 (H1N1)pdm09 viruses were passaged in increasing concentrations of 3Feq4Gu DFSA. Wells were scored for cpe microscopically. Plots show dilutions of virus scored as 3 + in drug (~50–75% cpe) and 4 + cpe (~75–100% cpe) without drug (to demonstrate higher yields) and corresponding drug concentrations. Total virus yields decreased with passaging, and only undiluted samples grew in 3Feq4Gu DFSA by P9 for B/Perth. No resistant viruses were selected by P12, so 3Feq4Gu DFSA concentrations were reduced for P13–P15 for both viruses. (Scale includes 10^{-1} in order to show that the virus was only showing cpe in the undiluted 10^{0} sample).

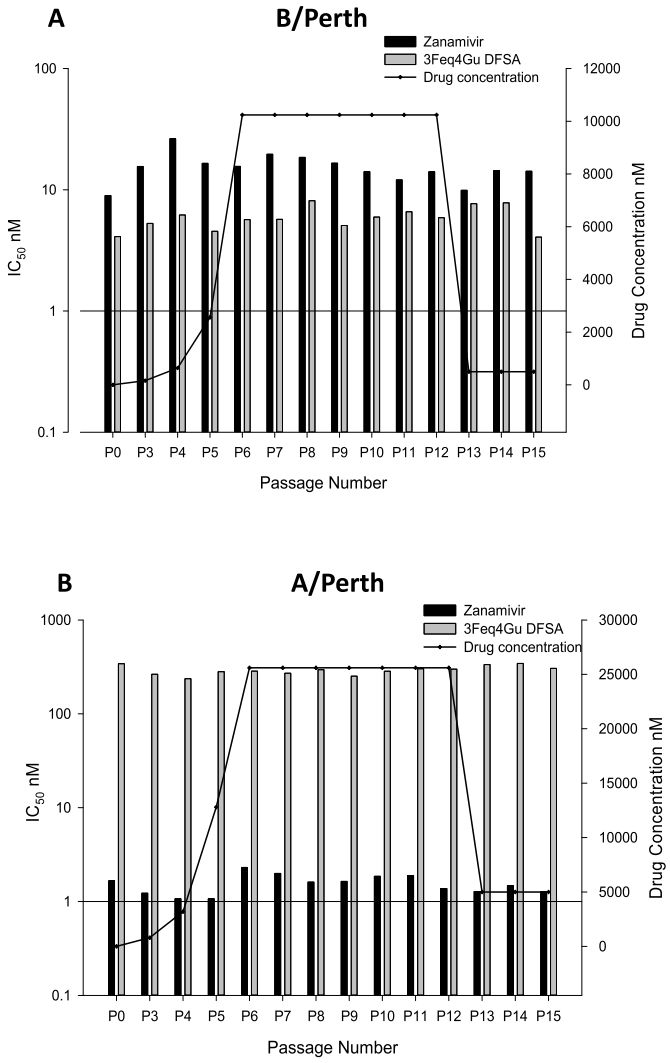

Fig. 2. NA Sensitivity of viruses passaged in 3Feq4Gu DFSA. NA sensitivity of viruses to zanamivir and 3Feq4Gu DFSA was tested in the MUNANA assay after each passage. A) B/Perth/211/01and B) A/Perth/265/09 (H1N1)pdm09. IC_{50} values for both viruses and inhibitors remained constant through all passages. Samples were assayed in duplicate.

specific primers. The samples were gel purified (QIAquick, Qiagen, Germany) and sequencing performed using the BigDye Terminator v3.1 Cyle Sequencing Kit (Applied Biosystems). To identify substitutions associated with altered drug sensitivity, the Bioedit program (Hall, 1999) was used for alignment and translation.

3. Results

3.1. Passaging and selection of variants

The B/Perth virus was very sensitive to the increase in inhibitor concentration, and by passage 5 (P5) in 2.56 μM inhibitor only the 10^{-1} diluted sample grew to 3 + cpe (Fig. 1, Table S1). The inhibitor was kept constant at 10.24 μM from P6, but the yields continued to be very low. By P12 virus was only recovered from the undiluted sample. Similarly for the pdm A/Perth virus, because virus yields dcreased with increasing inhibitor concentration, inhibitor was kept constant from P6 at 25.6 μM (Fig. 1, Table S1). However, yields continued to be low.

After each passage, the drug passaged sample was grown without inhibitor and used to test NA sensitivity to zanamivir and 3Feq4Gu DFSA in the IC_{50} kinetics reaction (with preincubation only). Up to P12 the 60 min IC_{50} values (Fig. 2) and profiles of the IC_{50} kinetics bar graphs were the same as the P0 wild type controls for both viruses (Fig. S1 P9–P12). Additionally, five plaques were picked after every third passage and had similar sensitivity in an enzyme inhibition assay as the P0 viruses.

As we did not have any resistant virus by P12 we passaged each virus for another three passages in lower 3Feq4Gu DFSA, 500 nM for B/Perth and 5 μM for pdm A/Perth (Fig. 1, Table S1). No differences were seen in NA sensitivity to either 3Feq4Gu DFSA or zanamivir from P12 to P15 for either virus (Fig. 2). The P15 viruses were then tested in a plaque reduction assay (PRA) against 3Feq4Gu DFSA and zanamivir, but overall sensitivities appeared to be similar to the wild type. In order to screen for a very low potential mutant population we plaqued serial 10-fold dilutions of the P15 samples under 2.5 nM for B/Perth, and 25 nM 3Feq4Gu DFSA for the pdm A/Perth. The B/Perth plaques were homogeneous at all dilutions and picked plaques showed similar sensitivities in the enzyme inhibition assays.

Interestingly, as well as large plaques, the P15 pdm A/Perth sample contained a minor population of small, diffuse plaques. Due to overlap of plaques we had to serially plaque both types of plaque, to get homogeneously plaquing populations. On amplification all plaques wiped out cells in 2–3 days. However, when we tested the supernatants

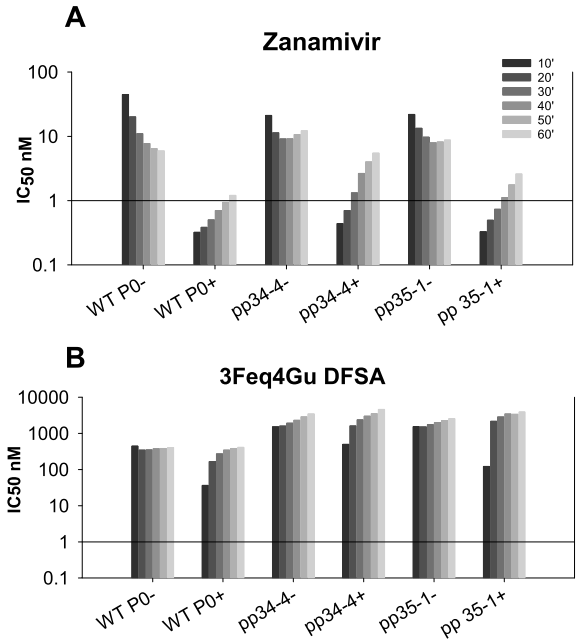

Fig. 3. IC_{50} Kinetics for mixed pdm A/Perth plaque stocks. NA sensitivities of amplified plaques were tested in the IC_{50} kinetics assay, without (−) and with (+) preincubation. A) Zanamivir B) 3Feq4Gu DFSA. Changes in IC_{50} values were different for the pp 34-4 and 35-1 compared to the wild type P0 virus, suggesting a mixed population of wild type and mutant viruses. Samples were assayed in duplicate.

in an NA enzyme assay, there was barely detectable NA activity, (1–2% of the wild type), in the small plaques (pp 30-6 and pp 35-6), but high activity from the large plaques. After amplification of two other small plaques there were mixed populations of large and small plaques (pp 35-1 and pp 34-4) with some NA activity. The small plaques had insufficient NA activity to test in an NA inhibition (NAI) assay. However, we tested the mixed population, as mixed wild type and resistant populations often show different kinetics. IC_{50} kinetics showed 2-3-fold higher IC_{50}s, but also a different IC_{50} kinetics profile, suggesting mixed wild type and resistant populations (Fig. 3).

The P15 pdm A/Perth virus only weakly agglutinated CRBC and after agglutinating, eluted rapidly at 37 °C (Table 1). After mixing and reincubating at 4 °C there was minimal rebinding. Neither of the pp 30-6 or 35-6 amplified plaques agglutinated CRBC.

Table 1
NA amino acid sequences and agglutination of CRBCs.

Virus	NA 106[c]	NA 363[c]	HA 4 °C	Rebind 4 °C > 37 °C
P15 WT	I	R	16	< 2
30–6	M	R	< 2	< 2
35–6	M	R	< 2	< 2
Revertants				
30-6 P4 1-2-1[a]	M	K	32	64
30-6 P5 1-1-2[a]	M	K	64	64
35-6 P4 2-1-1[a]	V	R	16	< 2
35-1L 1-1-2[b]	I	R	16	< 2
35-1 amp 2-1[b]-	I	R	32	< 2

Viruses were mixed with CRBCs for 60 min on ice, the HA titer was recorded, then plates were placed at 37 °C to allow elution. After they were fully eluted, wells were remixed and plates were incubated back in the cold again for 60 min.

[a] Mutant viruses were passaged for 4–5 passages without inhibitor, then plaques were picked and serially plaqued to purify to homogeneity.

[b] Revertants plaque purified from initial P15 amplified plaque with mixed plaque phenotypes.

[c] **S**equence numbering is based on the A(H1N1)pdm09 NA sequence.

3.2. Plaque reduction assay

The pp 30-6 and 35-6 virus samples were tested in a plaque reduction assay (PRA) against zanamivir and 3Feq4Gu DFSA. Both were completely resistant to at least 10 μM of both (Fig. 4 shows the 3Feq4Gu DFSA PRA).

3.3. Replication kinetics

Replication kinetics were the same for both the pp 30-6 and 35-6 mutants, and despite the apparent low NA activity, they grew to higher titers at the later time points than the wild type virus (Fig. 5). This may have been due to the destruction of the cell monolayer after 48 h by the wild type virus. However, there was negligible NA activity in any of the pp 30-6 or 35-6 samples. Although the supernatants taken from cultures infected with mutant viruses at 48 h and 57 h had the highest titers ($\sim 5 \times 10^7$ pfu/ml), their HA titer with CRBCs was < 2, while the titer for the wild type was 8–16 HAU. Titers assessed using TRBCs were 1–2 HAU for the mutants in contrast to ~8 HAU for the wild type (Table 1).

3.4. Slot blot

To examine whether the minimal HA and NA activity related to levels of protein expression on the virus, the amounts of NA and HA protein were compared to the amount of NP for each of the mutant viruses (pp 30-6 and 35-6) and the P12 wild type virus in a slot blot assay. Despite lack of agglutination of CRBC by the mutants, levels of HA protein were 2-4-fold higher in the mutants than in the wild type virus. Although we found the HCA-2 was poorly reactive, there were comparable levels of NA protein for the wild type and mutant viruses (Fig. S2).

3.5. Effect of mucins on infection

While the mutant viruses appeared to be fit in terms of replication in MDCK cells, due to their deficient NA and HA functions, they may however, be compromised in their ability to penetrate a mucus barrier *in vivo*. Since it has been demonstrated that influenza viruses with low NA activity are generally poorly infectious in animal models (Abed et al., 2016; Ann et al., 2016; Tu et al., 2017) we evaluated whether the mutant viruses would be potentially inhibited by a mucus rich environment by infecting cells *in vitro* known to overexpress MUC1. These cells overexpress SAα2-3Gal, a target for both influenza HA and NA (Fig. 6A). Thus the overexpression of MUC1 in our cell culture system serves as a *de facto* model for the ability of the viruses to infect and penetrate the mucus barrier *in vivo*.

CHO–K1 control and CHO–K1 $MUC1^+$ cells were infected with an MOI of 1 PFU/cell and 16 h later the percentage of cells with detectable HA bound to the cell surface and NP produced within the infected cell were assessed by flow cytometry. The mutant viruses with low NA activity (pp 30-6 and pp 35-6) were less able to attach to the cell surface (Fig. 6B), confirming reduced NA activity impedes virus ability to penetrate mucosal layers. Furthermore, the ability of these viruses to infect the MUC1 + cells was limited, as evidenced by the reduced percentage of cells producing viral NP intracellularly (Fig. 6C). Importantly, the wild-type virus had an equivalent percentage of HA^+ and NP^+ cells regardless of MUC1 expression, indicating that this virus was not hindered by the presence of excess sialic acid for virion attachment and entry. These findings indicate that the mutant viruses exhibit a significantly reduced ability to bind to and infect epithelia in a sialic acid rich environment, thus suggesting they would be highly compromised in fitness *in vivo*.

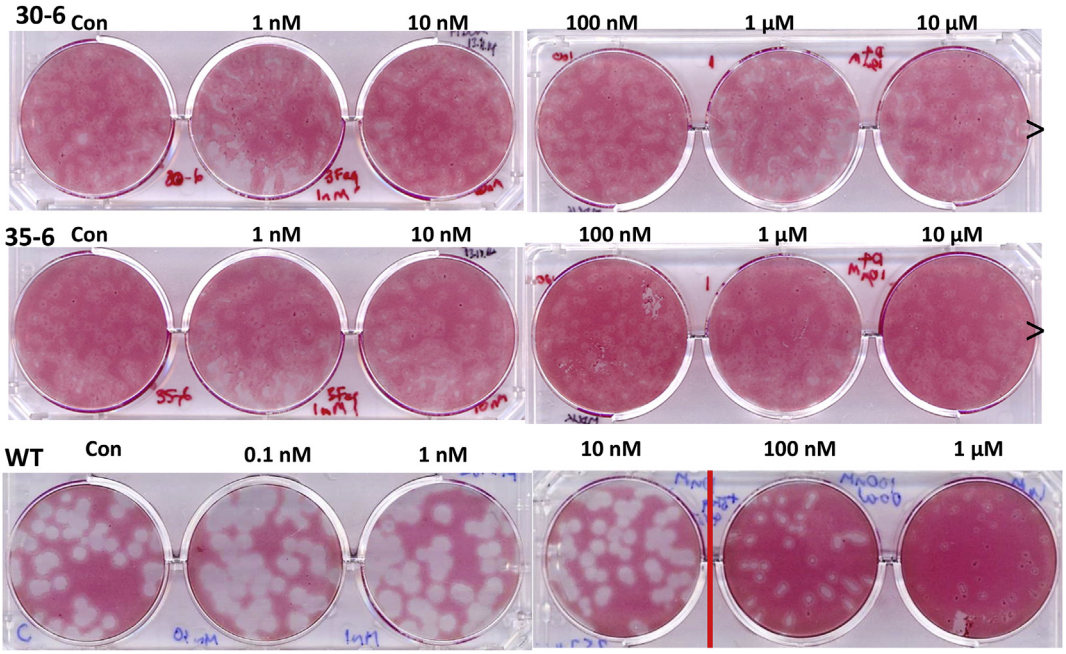

Fig. 4. Plaque reduction assay with 3Feq4Gu DFSA. The wild type, 30-6 and 35-6 low NA viruses were plaqued under $\log_{10}$ dilutions of 3Feq4Gu DFSA. Cells were fixed in formalin and stained with neutral red. While the wild type plaque size was reduced by 10–100 nM inhibitor, no decrease in plaque size or number was observed for any concentration of 3Feq4Gu DFSA for the mutants.

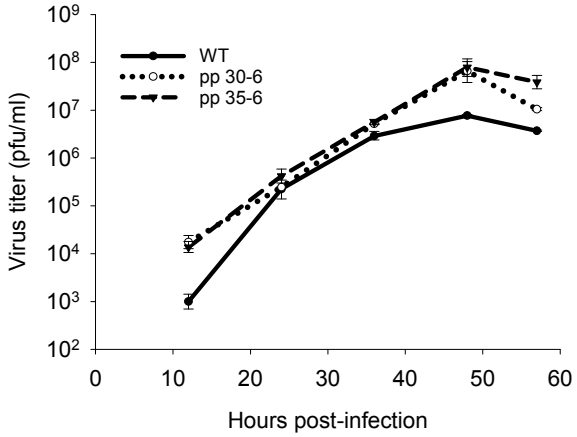

Fig. 5. Replication Kinetics for wild type and mutant viruses. MDCK cells were infected with an MOI of 0.01 and supernatants were harvested separately from duplicate wells every 12 h. Yields were determined by plaque assay and showed the low NA mutants grew as well as the wild type parent virus. Although yields at 48 h were higher for the mutants, this may be due to 100% cpe in the wild type infected cells, and loss of infectivity. Bars are SD.

3.6. Reversion passaging

Interestingly, we observed a single large plaque in one of the 48 h mutant samples from the replication kinetics experiment. We picked and amplified this plaque, which had detectable NA activity suggesting reversion to wild type. As many highly drug resistant viruses are not stable to further passaging and are therefore unlikely to be of clinical concern (Zurcher et al., 2006), we tested the stability of the small plaque phenotypes pp 30-6 and 35-6 by serial passaging without drug. Both had detectable NA and HA activity by P2–P3 and both small and some larger plaques were observed. To examine for reversion, several larger plaques from P4 and P5 were picked, but had to be serially plaqued to obtain homogeneously plaquing populations. Plaque progeny were directly screened in a plaque assay with or without 5 nM zanamivir in the overlay, which significantly inhibits the plaque size of a sensitive virus. After 72 h in the absence of zanamivir, some plaques were large, comparable to wild type and others were medium sized differing from the original small diffuse resistant plaques. Under 5 nM zanamivir the larger plaques all showed significant size inhibition. However the medium ones were only marginally inhibited, suggesting some reduced sensitivity (Table S2). In NAI assays there were also some subtle differences in the IC_{50} kinetics profiles of the medium plaques (Fig. 7). The 60 min IC_{50}s for zanamivir were around 2-3-fold higher than for the wild type (3 nM vs 6–9 nM). While the IC_{50}s for oseltamivir were similar, the kinetics profiles for the reaction without preincubation were subtly different to the wild type. We also observed differences in agglutination of CRBCs. Some like the wild type parent agglutinated, although weakly, eluting within 60 min, even at 4 °C. These did not reagglutinate after 37 °C elution. In contrast other plaques agglutinated well and then rebound well after elution (Table 1).

3.7. Egg infection

To evaluate viral fitness and potential for reversion *in vivo,* we also infected eggs with the mutant and wild type viruses. Viruses from all the pp 30-6 infected eggs grew to comparable titers as the wild type virus. In contrast, the yields for the pp 35-6 infected eggs were 2-3-fold lower. In the plaque assay both small diffuse mutant-like plaques and larger revertant-type plaques were visible from all the mutant infected eggs. All viruses harvested from the mutant and wild type inoculated eggs had detectable NA and HA activities, indicating significant reversion of the mutant viruses, consistent with the presence of some larger plaques. The ratios of HA or NA to pfu were similar for all samples from wild type and mutant infected eggs, except interestingly for the two eggs infected with the 10^{-3} dilution of the 35-6 virus. Despite having a similar infectious titer, the HA and NA titers were 10-fold lower than the other samples, indicating a higher proportion of mutant viruses remained (Fig. S3).

3.8. Sequencing

We sequenced the NA and HA genes from the control P12 and final P15, the low NA mutants and revertants (Table S2). All sequence

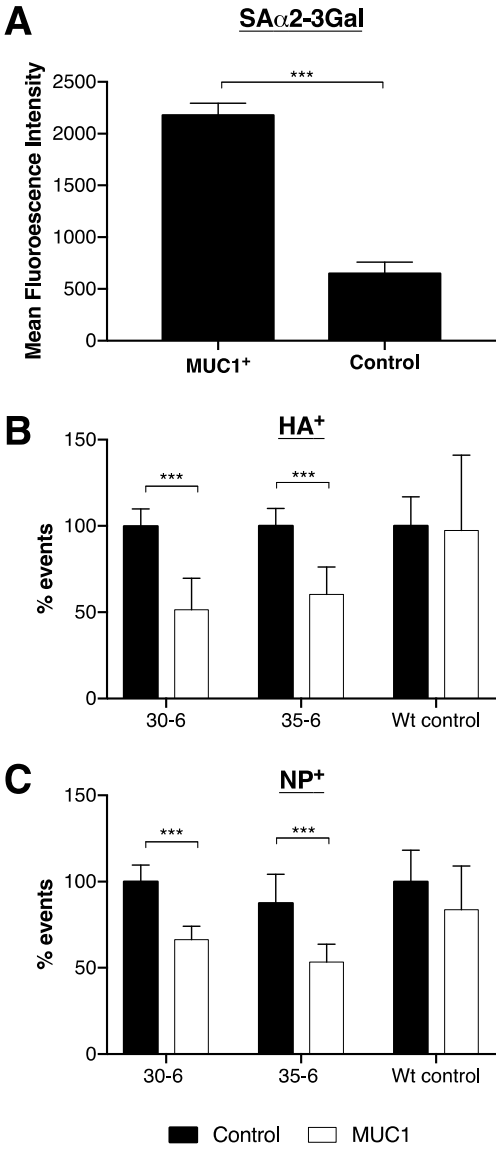

Fig. 6. Relative infection of CHO–K1 cells overexpressing MUC1. A) SAα2-3Gal expressed on uninfected CHO–K1 control and MUC1 + cells was detected with MAL-II and Streptavidin-APC and the mean fluorescence intensity was detected via flow cytometry. CHO–K1 control and MUC1 + cells were infected with an MOI of 1 of each of the viruses for 16 h. B) HA expressed at the surface was detected by staining with MAb clone 178 and goat anti-mouse IgG/IgM conjugated to Alexa Fluor 488 and flow cytometry. C) NP expressed intracellularly was detected by firstly permeablizing cells, then staining with anti NP-FITC and flow cytometry. Results were normalized to the average percentage of infected control samples for each viral infection group. (*** $p < 0.001$ unpaired Student's T test). 30-6, 35-6 are I106M low NA and HA mutants.

numbering is based on the pdm09 NA and HA sequences.

The P15 wild type NA has E382, whereas the P12 control virus, and the original sequence in the database have G382. All wild type or resistant plaques selected from the P15 had E382. Hence this does not appear to affect any of the phenotypes and is also found in several isolates in the Influenza Research Database (IRD).

3.8.1. NA sequences

The low NA mutants had a novel I106M substitution in the NA protein. The 30-6 and 35-6 mutants may have arisen independently since a silent mutation of T1155C was only seen in the 35-6 original mutant and its revertants. NA sequencing revealed there were three different types of revertants. There were revertants, with I106, or V106 conferring wild type drug sensitivity. A third type, maintained M106, but had an additional R363K change. These viruses formed medium plaques, and had NA activity, with a small decrease in sensitivity in plaque assay and enzyme inhibition assays. Thus the R363K mostly compensated for the severe deficiency incurred by the M106.

3.8.2. HA sequences

Surprisingly, HA sequencing did not reveal any differences between the P15 wild type and HA-deficient mutants. Nor were there HA sequence differences among those viruses that had altered agglutination abilities. The 30-6 48 h 2-1-1 wild type revertant virus had a N118S substitution in the HA2, and the 35-6 P4 2-2-2-3M had a K177N change (which corresponds to K163 in other N1 alignments), which introduced a potential new glycosylation site in HA1. This site is also potentially glycosylated in other N1 viruses (IRD). However, no other mutations correlated with the HA + or poor HA phenotypes.

3.9. Correlation of NA genotype and HA phenotype

Unexpectedly, there appeared to be a strong correlation with the NA genotype and the agglutination phenotypes (Table 1). While those with the NA M106 and R363 combination did not agglutinate CRBCs, those with NA M106 and K363 all agglutinated CRBC and rebound after elution. In contrast those with I106 or V106, bound weakly and rebound poorly after elution. Given the correlation between loss of NA and HA activity, and since some NAs have agglutinating activity (Mohr et al., 2015), we tried inhibition with 3Feq4Gu DFSA and a monoclonal anti HA antibody. The anti HA antibody inhibited all agglutination, but there was no inhibition of agglutination by the drug. This confirmed agglutination was through the HA only.

4. Discussion

An important feature for further development of the DFSA mechanism-based inhibitors of influenza NA (Kim et al., 2013) would be a high barrier to the emergence of resistance. We know that the closer the inhibitor is in structure to the natural substrate, the less likely that resistance can emerge and the NA retain function (Varghese et al., 1998). We were previously unable to select zanamivir resistant NA mutants after passaging a swine A(H1N1)pdm09 virus in zanamivir (McKimm-Breschkin et al., 2012).

There are two key features that distinguish the DFSA inhibitors from the current NAIs. The current NAIs are based on DANA, whereas the DFSA inhibitors are sialic acid derivatives, hence are even closer in structure to the natural substrate. Secondly, the NAIs are reversible competitive inhibitors, whereas the DFSAs are mechanism-based inhibitors, relying on the catalytic activity of the NA to cleave the DFSA and form a covalent bond to the Y406 in the active site. We predicted that a substitution leading to resistance to DFSA inhibitors would likely result in impairment of NA activity and it would be hard to isolate resistant mutants.

We passaged a human influenza A(H1N1)pdm09 virus, A/Perth/265/09, and an influenza B, B/Perth/211/01 virus in 3Feq4Gu DFSA for 15 passages to determine the likelihood of resistance emerging. We could not isolate any resistant influenza B viruses. Interestingly influenza B NAs have been shown to bind oseltamivir poorly, as full rotation of the E276 (N2 numbering) to accommodate the hydrophobic side chain does not occur (Oakley et al., 2010). However, as 3Feq4Gu DFSA is based on the natural sialic acid ligand, no structural changes are necessary for binding, and this class of inhibitor binds even better to the influenza B NAs than to influenza A NAs (Kim et al., 2013). This may contribute to the inability to select for resistant variants.

For the pdm A/Perth virus, a minor population of small plaques was visible by P15. Two picked plaques pp 30-6 and 35-6 had barely detectable NA or HA activity, despite infectivity > 10^7 pfu/ml. Surprisingly, their replication in MDCK cells was not adversely affected.

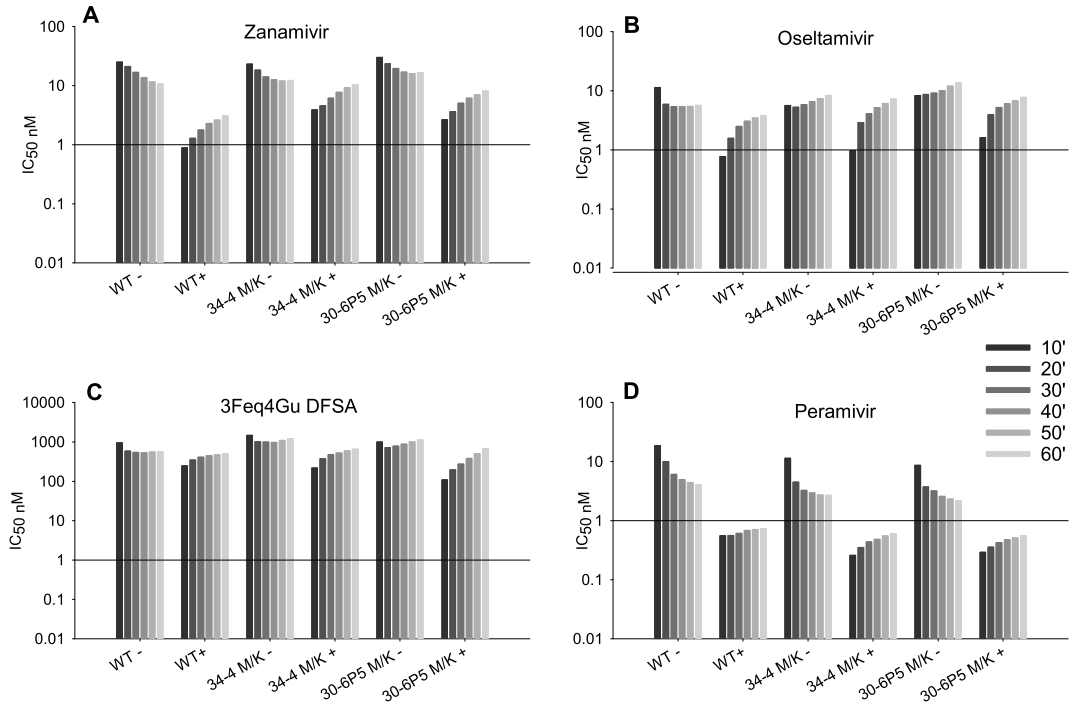

Fig. 7. IC_{50} Kinetics for revertants. While the I106M NA has minimal NA activity, revertants with an I106M and R363K have comparable NA activity to wild type. However, the double mutant has slightly different IC_{50} kinetics profiles for each of zanamivir, oseltamivir and 3Feq4Gu DFSA, but not peramivir, showing the R363K does not fully compensate for the I106M. A) Zanamivir B) Oseltamivir, C) 3Feq4Gu DFSA, D) Peramivir. 30-6 P5 = 30-6 P5 pp 1-1-2; 34-4 = 34-4 pp 3-1-1-2M. Samples were assayed in duplicate.

Using a CHO–K1 cell line expressing high levels of MUC1, creating a mucus like barrier, the low NA activity viruses bound and penetrated the cells poorly. These results suggest they would be unlikely to penetrate the mucus barrier *in vivo* thus resulting in reduced fitness, as previously seen for other low NA viruses (Abed et al., 2016; Ann et al., 2016; Tu et al., 2017; Zanin et al., 2015).

Sequencing showed that the low NA viruses had an I106M substitution in the NA, but no apparent changes in the HA, despite differences in the HA phenotype compared to the wild type viruses. The NA change was unstable to further passaging in the absence of inhibitor, with some reverting to the original I106 or V106, also seen in earlier pandemic strains (Xu et al., 2012).

Interestingly, an R363K substitution conferred an almost wild type phenotype to the M106 mutant. The original sequence in Genbank for the A/Perth/265/09 virus had K363, but we had plaque purified this virus due to mixed plaque morphology in the original sample and our parent virus had R363, so this virus reverted to the alternative parent sequence.

Structural modelling shows that amino acid 106 is remote from the NA active site (Fig. 8A). Several other substitutions have been reported at remote sites which affect NAI sensitivity, and these tend to be at the monomer/monomer interface (Fujisaki et al., 2012; Gubareva et al., 2017; McKimm-Breschkin et al., 2013). However, 106 is not at the interface. Additionally, as residue 363 is remote from residue 106 but K363 can mostly compensate for the M106 phenotype (Fig. 8A), this suggests a different mechanism of altered NA function. While the initial structures of the NA assumed the protein did not change upon substrate binding, it has been shown more recently that it is dynamic, with significant movement of two loops, designated the 150- (147–152) and 430- (428–440) loops (Amaro et al., 2007, 2011; Zhu et al., 2012). Movement of these loops impacts on the size of the active site cavity (Amaro et al., 2007, 2009, 2011) with the 150-loop predominantly in an open conformation in the unliganded enzyme, closing upon binding of inhibitor.

Another loop spanning residues 363–370, has also been recently described in N1 NAs as impacting on substrate binding, (Du et al., 2018). We hypothesize that the additional extension of the positively charged R363 side chain, compared with the positively charged K363, may affect the precise position of the R368 (R371 N2 numbering) catalytic residue. Structurally, the longer side chain of R363 may facilitate H-bond contacts with e.g. the negatively charged D392 or F371 (backbone-carbonyl-oxygen) (Fig. 8C) thus restricting movement of the whole 363–370 loop (Du et al., 2018) with R368 at the tip. R363 may not significantly affect substrate binding due to potential flexibility and coupled motion of the 150 and 430 loops (Amaro et al., 2007, 2011).

Residue 106 is located behind the 430-loop facing the bulky W437 (Fig. 8B). An I436N substitution affects binding of all NAIs in the pdm H1N1 NA (Kwon et al., 2018) thus the 430-loop also plays an important role in the pdm H1N1 NA function. We hypothesize that the additional length of the M106 side chain, compared with V106 or I106, may be a steric factor in controlling the movement of the 430-loop. The longer side chain of M106 may facilitate van der Waals contacts to the neighbouring 430-loop, pushing it away to a more closed state with the 150-loop. This restriction may not significantly affect interactions of the substrate with K363 due to compensatory coupled motion of the 150 and 430 loops as well as small shifts of the 363–370 loop (Fig. 8C). However, the combination of the M106 with the R363 becomes too restrictive to the shifts of the 363–370 loop and positioning of the catalytic R368 residue and thus this combination renders the NA poorly functional.

While many NA mutations result in a decrease in NA activity, compensating HA mutations which reduce the affinity of the HA for the receptor, are often seen especially in cell culture (McKimm-Breschkin et al., 1998, 2012, 2013). However, for our low NA mutants, there was an unexpected correlation between low NA activity and absence of HA activity, but then reversion to regain both NA activity and HA activity, despite no changes in the HA. Although deep sequencing can detect low levels of mutations, since the low NA and HA phenotypes were the dominant population, there should have been detectable changes with Sanger sequencing. We propose that the lack of binding to CRBCs by the

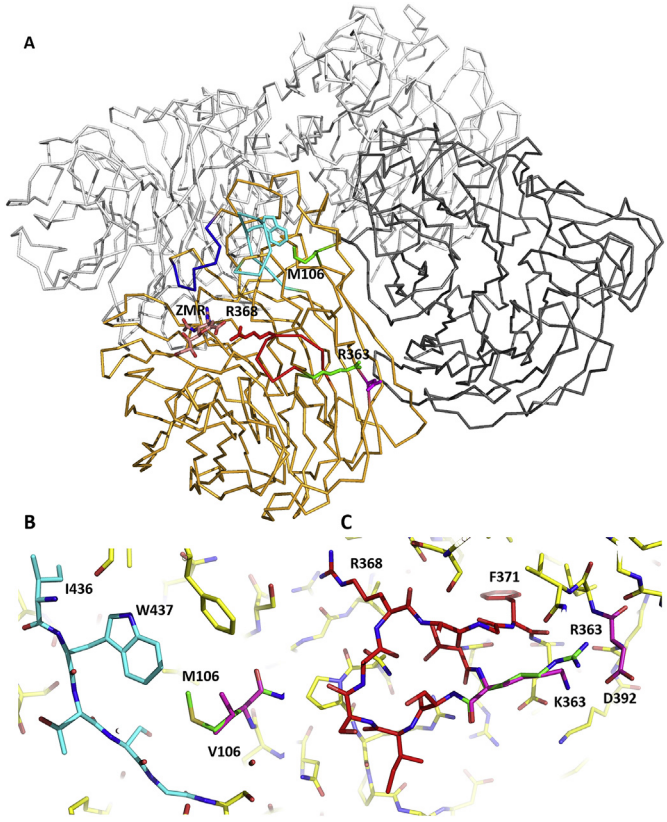

Fig. 8. A) Structure of N1 tetramer (based on 4B7N structure) in ribbon representation with symmetry related chains shown in shades of grey. Zanamivir (ZMR) is shown in pink in the active site. The 150-loop is shown in blue. (A,B) M106 is in green and facing W437 of the 430-loop in cyan. (A,C) The 363-370-loop is in red with R368 facing ZMR and R363 (green) close to D392 (magenta) and F371. The marked residues and the inhibitor are shown in stick representation. The mentioned residues are not on the interface of the tetramer. The figure was generated using PyMol (Schrodinger, 2010). (See web version for colours).

low NA mutants is due to lack of sufficient cleavage of the sialic acids on the NA and HA proteins on the virion, sterically limiting access to the RBC receptors. Subsequent changes in the NA that restore its function enable cleavage of the sialic acids on the virion surface glycoproteins, thus allowing binding to RBC. The M106 plus K363 NA appears to cleave differently from the wild type I106 or V106 NAs, resulting in different HA/HAE phenotypes. In contrast to other mutants lacking NA activity, exogenous NA was not required for virus replication in cell culture (Stray et al., 2000). This suggests that there may be sufficient NA activity, so that sterically the HA can still access receptors on the MDCK cells. A mutant lacking NA activity was previously shown to bind MDCK cells as well as the wild type virus, and it was proposed that there are also non-sialic acid receptors on cells (Stray et al., 2000). They also observed a discrepancy between MDCK cell binding and hemagglutination and suggested that receptors used by influenza virus for infection of permissive cells may be distinct from those mediating agglutination of erythrocytes. This could also provide an explanation for our observations.

Thus, as predicted, it was difficult to generate mutants with reduced sensitivity to 3Feq4Gu DFSA *in vitro*. Although the mutants generated were resistant to all the inhibitors in cell culture, they were also genetically unstable, and likely to be highly compromised in their fitness *in vivo*. We could also only find a single M106 sequence in the thousands of pdm09 like viruses in the IRD, so this is clearly a rare occurrence. Others have also had difficulty selecting for resistant variants to DFSA inhibitors (Tai et al., 2015) with NA mutations, although they reported that viruses able to grow in high concentrations of inhibitor had HA mutations. Thus DFSA inhibitors are both potent and have a high barrier to resistance, supporting their development as potential future therapeutics for influenza.

Declarations of interest

None apart from J.L. M-B and SGW being named inventors on a patent held by the University of British Columbia.

Acknowledgements

We thank Dr Richard Liggins and his colleagues at the Centre for Drug Research and Development (CDRD) for valuable discussions. We thank the Canadian Institutes for Health Research, Genome BC and CDRD for financial assistance.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.antiviral.2019.104542.

References

Abed, Y., Boivin, G., 2017. A review of clinical influenza A and B infections with reduced susceptibility to both oseltamivir and zanamivir. Open Forum Infect. Dis. 4 ofx105.
Abed, Y., Bouhy, X., L'Huillier, A.G., Rheaume, C., Pizzorno, A., Retamal, M., Fage, C., Dube, K., Joly, M.H., Beaulieu, E., Mallett, C., Kaiser, L., Boivin, G., 2016. The E119D neuraminidase mutation identified in a multidrug-resistant influenza A(H1N1)pdm09 isolate severely alters viral fitness in vitro and in animal models. Antivir. Res. 132, 6–12.
Amaro, R.E., Cheng, X., Ivanov, I., Xu, D., McCammon, J.A., 2009. Characterizing loop dynamics and ligand recognition in human- and avian-type influenza neuraminidases via generalized born molecular dynamics and end-point free energy calculations. J. Am. Chem. Soc. 131, 4702–4709.
Amaro, R.E., Minh, D.D., Cheng, L.S., Lindstrom Jr., W.M., Olson, A.J., Lin, J.H., Li, W.W., McCammon, J.A., 2007. Remarkable loop flexibility in avian influenza N1 and its implications for antiviral drug design. J. Am. Chem. Soc. 129, 7764–7765.
Amaro, R.E., Swift, R.V., Votapka, L., Li, W.W., Walker, R.C., Bush, R.M., 2011. Mechanism of 150-cavity formation in influenza neuraminidase. Nat. Commun. 2, 388.
Ann, J., Abed, Y., Beaulieu, E., Bouhy, X., Joly, M.H., Dube, K., Carbonneau, J., Hamelin, M.E., Mallett, C., Boivin, G., 2016. Impact of a large deletion in the neuraminidase protein identified in a laninamivir-selected influenza A/Brisbane/10/2007 (H3N2) variant on viral fitness in vitro and in ferrets. Influenza Other Respir. Viruses 10, 122–126.
Barrett, S., Mohr, P.G., Schmidt, P.M., McKimm-Breschkin, J.L., 2011. Real time enzyme inhibition assays provide insights into differences in binding of neuraminidase inhibitors to wild type and mutant influenza viruses. PLoS One 6, e23627.
Dharan, N.J., Gubareva, L.V., Meyer, J.J., Okomo-Adhiambo, M., McClinton, R.C., Marshall, S.A., St George, K., Epperson, S., Brammer, L., Klimov, A.I., Bresee, J.S., Fry, A.M., Oseltamivir-Resistance Working, G., 2009. Infections with oseltamivir-resistant influenza A(H1N1) virus in the United States. J. Am. Med. Assoc. 301, 1034–1041.
Doyle, T.M., Hashem, A.M., Li, C., Van Domselaar, G., Larocque, L., Wang, J., Smith, D., Cyr, T., Farnsworth, A., He, R., Hurt, A.C., Brown, E.G., Li, X., 2013. Universal anti-neuraminidase antibody inhibiting all influenza A subtypes. Antivir. Res. 100, 567–574.
Du, W., Dai, M., Li, Z., Boons, G.J., Peeters, B., van Kuppeveld, F.J.M., de Vries, E., de Haan, C.A.M., 2018. Substrate binding by the second sialic acid-binding site of influenza a virus N1 neuraminidase contributes to enzymatic activity. J. Virol. 92.
Fujisaki, S., Takashita, E., Yokoyama, M., Taniwaki, T., Xu, H., Kishida, N., Sato, H., Tashiro, M., Imai, M., Odagiri, T., 2012. A single E105K mutation far from the active site of influenza B virus neuraminidase contributes to reduced susceptibility to multiple neuraminidase-inhibitor drugs. Biochem. Biophys. Res. Commun. 429, 51–56.
Gubareva, L.V., Besselaar, T.G., Daniels, R.S., Fry, A., Gregory, V., Huang, W., Hurt, A.C., Jorquera, P.A., Lackenby, A., Leang, S.K., Lo, J., Pereyaslov, D., Rebelo-de-Andrade, H., Siqueira, M.M., Takashita, E., Odagiri, T., Wang, D., Zhang, W., Meijer, A., 2017. Global update on the susceptibility of human influenza viruses to neuraminidase inhibitors, 2015-2016. Antivir. Res. 146, 12–20.
Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp. Ser. 41, 95–98.
Hurt, A.C., Ernest, J., Deng, Y.M., Iannello, P., Besselaar, T.G., Birch, C., Buchy, P., Chittaganpitch, M., Chiu, S.C., Dwyer, D., Guigon, A., Harrower, B., Kei, I.P., Kok, T., Lin, C., McPhie, K., Mohd, A., Olveda, R., Panayotou, T., Rawlinson, W., Scott, L., Smith, D., D'Souza, H., Komadina, N., Shaw, R., Kelso, A., Barr, I.G., 2009. Emergence and spread of oseltamivir-resistant A(H1N1) influenza viruses in oceania, south east

asia and South Africa. Antivir. Res. 83, 90–93.
Hurt, A.C., Iannello, P., Jachno, K., Komadina, N., Hampson, A.W., Barr, I.G., McKimm-Breschkin, J.L., 2006. Neuraminidase inhibitor-resistant and -sensitive influenza B viruses isolated from an untreated human patient. Antimicrob. Agents Chemother. 50, 1872–1874.
Kim, C.U., Lew, W., Williams, M.A., Liu, H., Zhang, L., Swaminathan, S., Bischofberger, N., S, C.M., Mendel, D.B., Tai, C.Y., Laver, W.G., Stevens, R.C., 1997. Influenza neuraminidase inhibitors possessing a novel hydrophobic interaction in the enzyme active site:design, synthesis, and structural analysis of carbocyclic sialic acid analogues with potent anti-influenza activity. J. Am. Chem. Soc. 119, 681–690.
Kim, J.H., Resende, R., Wennekes, T., Chen, H.M., Bance, N., Buchini, S., Watts, A.G., Pilling, P., Streltsov, V.A., Petric, M., Liggins, R., Barrett, S., McKimm-Breschkin, J.L., Niikura, M., Withers, S.G., 2013. Mechanism-based covalent neuraminidase inhibitors with broad-spectrum influenza antiviral activity. Science 340, 71–75.
Kwon, J.J., Choi, W.S., Jeong, J.H., Kim, E.H., Lee, O.J., Yoon, S.W., Hwang, J., Webby, R.J., Govorkova, E.A., Choi, Y.K., Baek, Y.H., Song, M.S., 2018. An I436N substitution confers resistance of influenza A(H1N1)pdm09 viruses to multiple neuraminidase inhibitors without affecting viral fitness. J. Gen. Virol. 99, 292–302.
Lackenby, A., Besselaar, T.G., Daniels, R.S., Fry, A., Gregory, V., Gubareva, L.V., Huang, W., Hurt, A.C., Leang, S.K., Lee, R.T.C., Lo, J., Lollis, L., Maurer-Stroh, S., Odagiri, T., Pereyaslov, D., Takashita, E., Wang, D., Zhang, W., Meijer, A., 2018. Global update on the susceptibility of human influenza viruses to neuraminidase inhibitors and status of novel antivirals, 2016-2017. Antivir. Res. 157, 38–46.
McKimm-Breschkin, J.L., 1990. The use of tetramethylbenzidine for solid phase immunoassays. J. Immunol. Methods 135, 277–280.
McKimm-Breschkin, J.L., 2013. Influenza neuraminidase inhibitors: antiviral action and mechanisms of resistance. Influenza Other Respir. Viruses 7 (Suppl. 1), 25–36.
McKimm-Breschkin, J.L., Rootes, C., Mohr, P.G., Barrett, S., Streltsov, V.A., 2012. In vitro passaging of a pandemic H1N1/09 virus selects for viruses with neuraminidase mutations conferring high-level resistance to oseltamivir and peramivir, but not to zanamivir. J. Antimicrob. Chemother. 67, 1874–1883.
McKimm-Breschkin, J.L., Sahasrabudhe, A., Blick, T.J., McDonald, M., Colman, P.M., Hart, G.J., Bethell, R.C., Varghese, J.N., 1998. Mutations in a conserved residue in the influenza virus neuraminidase active site decreases sensitivity to Neu5Ac2en-derived inhibitors. J. Virol. 72, 2456–2462.
McKimm-Breschkin, J.L., Williams, J., Barrett, S., Jachno, K., McDonald, M., Mohr, P.G., Saito, T., Tashiro, M., 2013. Reduced susceptibility to all neuraminidase inhibitors of influenza H1N1 viruses with haemagglutinin mutations and mutations in non-conserved residues of the neuraminidase. J. Antimicrob. Chemother. 68, 2210–2221.
Meijer, A., Lackenby, A., Hungnes, O., Lina, B., van-der-Werf, S., Schweiger, B., Opp, M., Paget, J., van-de-Kassteele, J., Hay, A., Zambon, M., European Influenza Surveillance, S., 2009. Oseltamivir-resistant influenza virus A (H1N1), Europe, 2007-08 season. Emerg. Infect. Dis. 15, 552–560.
Mohr, P.G., Deng, Y.M., McKimm-Breschkin, J.L., 2015. The neuraminidases of MDCK grown human influenza A(H3N2) viruses isolated since 1994 can demonstrate receptor binding. Virol. J. 12, 67.
Oakley, A.J., Barrett, S., Peat, T.S., Newman, J., Streltsov, V.A., Waddington, L., Saito, T., Tashiro, M., McKimm-Breschkin, J.L., 2010. Structural and functional basis of resistance to neuraminidase inhibitors of influenza B viruses. J. Med. Chem. 53, 6421–6431.
Schrodinger, L., 2010. The PyMOL Molecular Graphics System. Version 1.3r1.
Stray, S.J., Cummings, R.D., Air, G.M., 2000. Influenza virus infection of desialylated cells. Glycobiology 10, 649–658.
Tai, S.H., Agafitei, O., Gao, Z., Liggins, R., Petric, M., Withers, S.G., Niikura, M., 2015. Difluorosialic acids, potent novel influenza virus neuraminidase inhibitors, induce fewer drug resistance-associated neuraminidase mutations than does oseltamivir. Virus Res. 210, 126–132.
Tu, V., Abed, Y., Barbeau, X., Carbonneau, J., Fage, C., Lague, P., Boivin, G., 2017. The I427T neuraminidase (NA) substitution, located outside the NA active site of an influenza A(H1N1)pdm09 variant with reduced susceptibility to NA inhibitors, alters NA properties and impairs viral fitness. Antivir. Res. 137, 6–13.
Varghese, J.N., McKimm-Breschkin, J.L., Caldwell, J.B., Kortt, A.A., Colman, P.M., 1992. The structure of the complex between influenza virus neuraminidase and sialic acid, the viral receptor. Proteins 14, 327–332.
Varghese, J.N., Smith, P.W., Sollis, S.L., Blick, T.J., Sahasrabudhe, A., McKimm-Breschkin, J.L., Colman, P.M., 1998. Drug design against a shifting target: a structural basis for resistance to inhibitors in a variant of influenza virus neuraminidase. Structure 6, 735–746.
von Itzstein, M., Wu, W.Y., Kok, G.B., Pegg, M.S., Dyason, J.C., Jin, B., Van Phan, T., Smythe, M.L., White, H.F., Oliver, S.W., Colman, P.M., Varghese, J.N., Ryan, D.M., Woods, J.M., Bethell, R.C., Hotham, V.J., Cameron, J.M., Penn, C.R., 1993. Rational design of potent sialidase-based inhibitors of influenza virus replication. Nature 363, 418–423.
Xu, R., Zhu, X., McBride, R., Nycholat, C.M., Yu, W., Paulson, J.C., Wilson, I.A., 2012. Functional balance of the hemagglutinin and neuraminidase activities accompanies the emergence of the 2009 H1N1 influenza pandemic. J. Virol. 86, 9221–9232.
Zanin, M., Marathe, B., Wong, S.S., Yoon, S.W., Collin, E., Oshansky, C., Jones, J., Hause, B., Webby, R., 2015. Pandemic swine H1N1 influenza viruses with almost undetectable neuraminidase activity are not transmitted via aerosols in ferrets and are inhibited by human mucus but not swine mucus. J. Virol. 89, 5935–5948.
Zhu, X., Yang, H., Guo, Z., Yu, W., Carney, P.J., Li, Y., Chen, L.M., Paulson, J.C., Donis, R.O., Tong, S., Stevens, J., Wilson, I.A., 2012. Crystal structures of two subtype N10 neuraminidase-like proteins from bat influenza A viruses reveal a diverged putative active site. Proc. Natl. Acad. Sci. U. S. A. 109, 18903–18908.
Zurcher, T., Yates, P.J., Daly, J., Sahasrabudhe, A., Walters, M., Dash, L., Tisdale, M., McKimm-Breschkin, J.L., 2006. Mutations conferring zanamivir resistance in human influenza virus N2 neuraminidases compromise virus fitness and are not stably maintained in vitro. J. Antimicrob. Chemother. 58, 723–732.