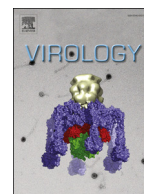




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Oseltamivir inhibits influenza virus replication and transmission following ocular-only aerosol inoculation of ferrets



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ABSTRACT

Ocular exposure to influenza virus represents an alternate route of virus entry capable of establishing a respiratory infection in mammals, but the effectiveness of currently available antiviral treatments to limit virus replication within ocular tissue or inhibit virus spread from ocular sites to the respiratory tract is poorly understood. Using an inoculation method that delivers an aerosol inoculum exclusively to the ocular surface, we demonstrate that oral oseltamivir administration following ocular-only aerosol inoculation with multiple avian and human influenza viruses protected ferrets from a fatal and systemic infection, reduced clinical signs and symptoms of illness, and decreased virus transmissibility to susceptible contacts when a respiratory infection was initiated. The presence of oseltamivir further inhibited influenza virus replication in primary human corneal epithelial cells. These findings provide critical experimental evidence supporting the use of neuraminidase inhibitors during outbreaks of influenza virus resulting in ocular disease or following ocular exposure.

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Introduction

Influenza virus is a highly contagious respiratory pathogen; high viral loads are expelled during coughing and sneezing of infected individuals, and inhalation of virus-containing aerosols by susceptible contacts represents a principal route of human infection (Milton et al., 2013; Tellier, 2009). However, like many respiratory pathogens, ocular exposure to influenza viruses constitutes a secondary route of virus entry, with the eye representing both a potential site of virus replication as well as a portal for establishing a respiratory infection (Belser et al., 2013b). While H7 subtype influenza viruses have typically been associated with ocular exposure and disease in humans, reports of both avian and human influenza viruses causing conjunctivitis have been documented (Belser et al., 2013b). Furthermore, ocular inoculation of mammalian models has demonstrated the ability of select influenza viruses to cause a fatal infection following ocular exposure, or to maintain a transmissible phenotype following ocular exposure, even in the absence of an ocular tropism (Belser et al., 2014, 2012a). Still, ocular exposure remains a poorly understood route of viral entry, limiting our ability to study

effective treatment options in humans exposed to influenza viruses by the ocular route or presenting with ocular symptoms.

Neuraminidase (NA) inhibitors represent the most widely used class of antiviral drug to treat individuals presenting with respiratory symptoms of influenza virus infection (Ison, 2015). Early administration of the NA inhibitor oseltamivir is associated with reduced clinical signs and symptoms of infection and reduced viral loads in respiratory tract tissues; oseltamivir prophylaxis is frequently prescribed during outbreaks of avian influenza virus where there is a risk of occupational exposure to the virus (Koopmans et al., 2004; Writing Committee of the Second World Health Organization Consultation on Clinical Aspects of Human Infection et al., 2008). Despite a paucity of experimental evidence examining the efficacy of antiviral treatments following infection by non-respiratory exposure routes, oseltamivir treatment and prophylaxis has nonetheless been prescribed during outbreaks in humans where conjunctivitis and/or ocular exposure has been reported (Koopmans et al., 2004; Tweed et al., 2004). Oral oseltamivir treatment has been shown to reduce viral pathogenesis and limit virus transmissibility in numerous mammalian models following inoculation with influenza viruses, though these studies have been limited to respiratory inoculation routes (Govorkova et al., 2007; Govorkova et al., 2011; Watanabe et al., 2013). As there are no antiviral drugs to treat viral conjunctivitis caused by respiratory viruses, it is necessary to assess the efficacy of currently available treatments to reduce viral replication in

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non-respiratory tissues such as the eye, in addition to examining drug efficacy when infection is initiated following virus exposure by non-respiratory routes.

A previous study from our laboratory demonstrated that oseltamivir inhibits H7 influenza virus replication in mice inoculated by the ocular route, with reductions in morbidity, mortality, and viral titers in both respiratory and ocular tissues in antiviral-treated mice (Belser et al., 2012b). However, there remains a need to better understand the capacity of currently available antiviral treatments to both inhibit virus spread from ocular sites to the respiratory tract and mitigate ocular disease caused by influenza virus infection. Here, we used a novel method of ocular-only aerosol (OA) inoculation of ferrets to investigate the ability of oseltamivir to prevent influenza virus infection, disease progression, and transmissibility following ocular exposure to influenza virus. We demonstrate that oral oseltamivir treatment effectively limits the establishment of a respiratory infection in ferrets exposed to aerosolized influenza virus by the ocular-only route, and reduces the transmission of viruses of multiple subtypes to naïve contacts when a respiratory infection results from ocular inoculation. Furthermore, we show that oseltamivir can limit influenza virus replication in human corneal epithelial cells.

Results

Efficacy of oseltamivir administration following OA inoculation with H5N1 virus

Oseltamivir treatment has been shown to reduce morbidity, mortality, and systemic spread of HPAI H5N1 viruses in ferrets inoculated by the traditional intranasal route (Boltz et al., 2008; Govorkova et al., 2007), but not following influenza virus infection by alternate exposure routes. As previous work found that the HPAI H5N1 virus A/Thailand/16/2004 (Thai/16) was lethal to ferrets following ocular inoculation (Belser et al., 2014, 2012a), ferrets inoculated by the OA route with Thai/16 virus were orally administered 25 mg/kg of body weight/day of oseltamivir phosphate, or distilled water as a control, from 2 h p.i. through day 5 p.i. Both treated and control ferrets were monitored daily for clinical signs of illness, and nasal wash (NW), conjunctiva wash (CW), and rectal swabs (RS) were collected on alternate days to assess virus replication.

Ferrets administered oseltamivir survived the H5N1 viral challenge, with transient weight loss and fever the only clinical signs and symptoms detected in this group (Table 1). While infectious virus was not detected in NW, CW, or RS samples during the acute phase of infection (Table 1, Fig. 1A), all treated ferrets seroconverted by day 21 p.i. (data not shown). In contrast, untreated ferrets inoculated by the OA route with the H5N1 virus succumbed to infection on days 5–7 p.i., presenting with weight loss, sustained fevers, nasal discharge, diarrhea, and neurological signs (Table 1). Virus was only detected sporadically and at low titer in NW (< 200 PFU/mL), CW (< 50 PFU/mL), and RS (< 600 PFU/mL) samples prior to euthanasia. However, postmortem necropsy revealed extensive systemic spread of virus in all untreated ferrets, including the respiratory tract, ocular tissue, and brain (Fig. 1B), with viral loads generally comparable to ferrets administered high doses of Thai/16 virus by the traditional intranasal route (Maines et al., 2005). In summary, we found that oseltamivir administration following OA inoculation with a HPAI H5N1 virus protected ferrets from a fatal, systemic infection.

Reduced virus transmissibility in ferrets receiving oseltamivir following OA inoculation

To examine a diverse range of viruses to which humans could potentially be exposed, we next challenged ferrets by the OA route

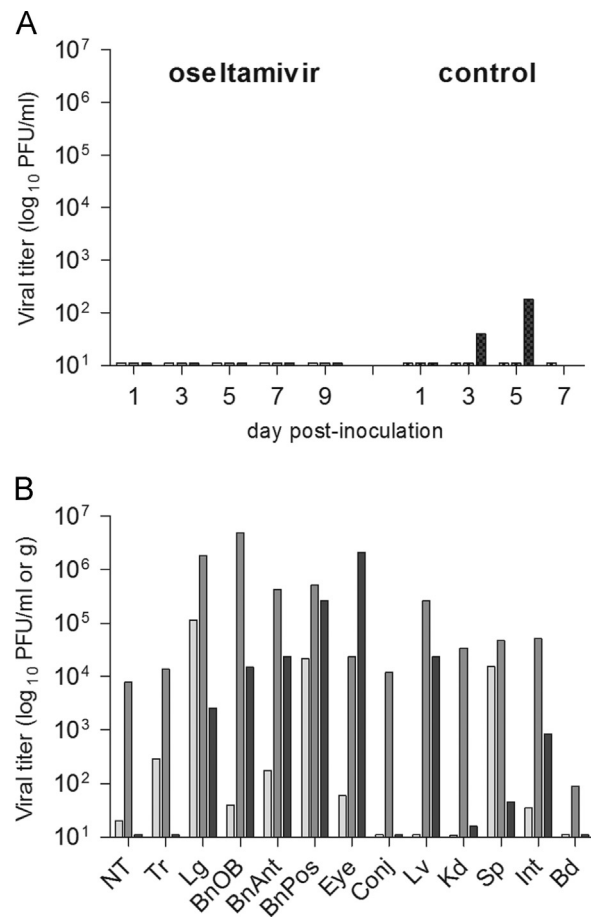


Fig. 1. Oseltamivir efficacy following OA inoculation of ferrets with HPAI H5N1 virus. Ferrets were inoculated by the OA route with Thai/16 virus at the doses reported in Table 1 and administered oseltamivir phosphate or distilled water as a control ($n=3$ per group). (A), viral titers in nasal wash specimens were determined on the indicated days p.i. (B), tissues collected from control-treated ferrets euthanized due to neurological symptoms days 5–7 p.i. were titrated for presence of infectious virus by standard plaque assay. NT, nasal turbinates; Tr, trachea; Lg, lung; BnOB, olfactory bulb; BnAnt, anterior brain; BnPos, posterior brain; Conj, conjunctiva; Lv, liver; Kd, kidney; Sp, spleen; Int, pooled intestine; Bd, blood. Bars represent individual ferrets. Limit of detection was 10 PFU/ml or g.

with two influenza A viruses recently associated with human infection: the HPAI H7N3 virus A/Mexico/InDRE7218/2012 (Mex/7218) isolated from a poultry worker with conjunctivitis (Lopez-Martinez et al., 2013), and a representative 2009 H1N1 pandemic virus A/Mexico/4482/2009 (Mex/4482) isolated from a patient with severe respiratory disease (Maines et al., 2009). Both viruses have been shown previously to cause a moderate infection in ferrets following inoculation by the OA (Mex/7218) or aerosol inhalation (AR) route (Mex/4482), and are capable of transmission in a ferret direct contact model (Belser et al., 2013a; Gustin et al., 2013). The efficacy of oseltamivir treatment to reduce virus virulence and transmissibility was examined for both viruses.

Similar to inoculation with Thai/16 virus, ferrets treated with oseltamivir and inoculated with the HPAI H7N3 virus Mex/7218 did not shed infectious virus in NW samples, whereas virus was detected in NW samples from both untreated ferrets (Fig. 2A). Moreover, oseltamivir treatment of Mex/7218-inoculated ferrets was associated with reduced maximum weight loss (0–3%) compared with untreated ferrets (7.3–12.3%) (Table 1). Seroconversion to homologous virus was detected in two of three oseltamivir-treated ferrets and all untreated ferrets (data not shown). Transmission of virus to naïve cagemates was not detected in oseltamivir-treated ferrets (all contact ferrets remained

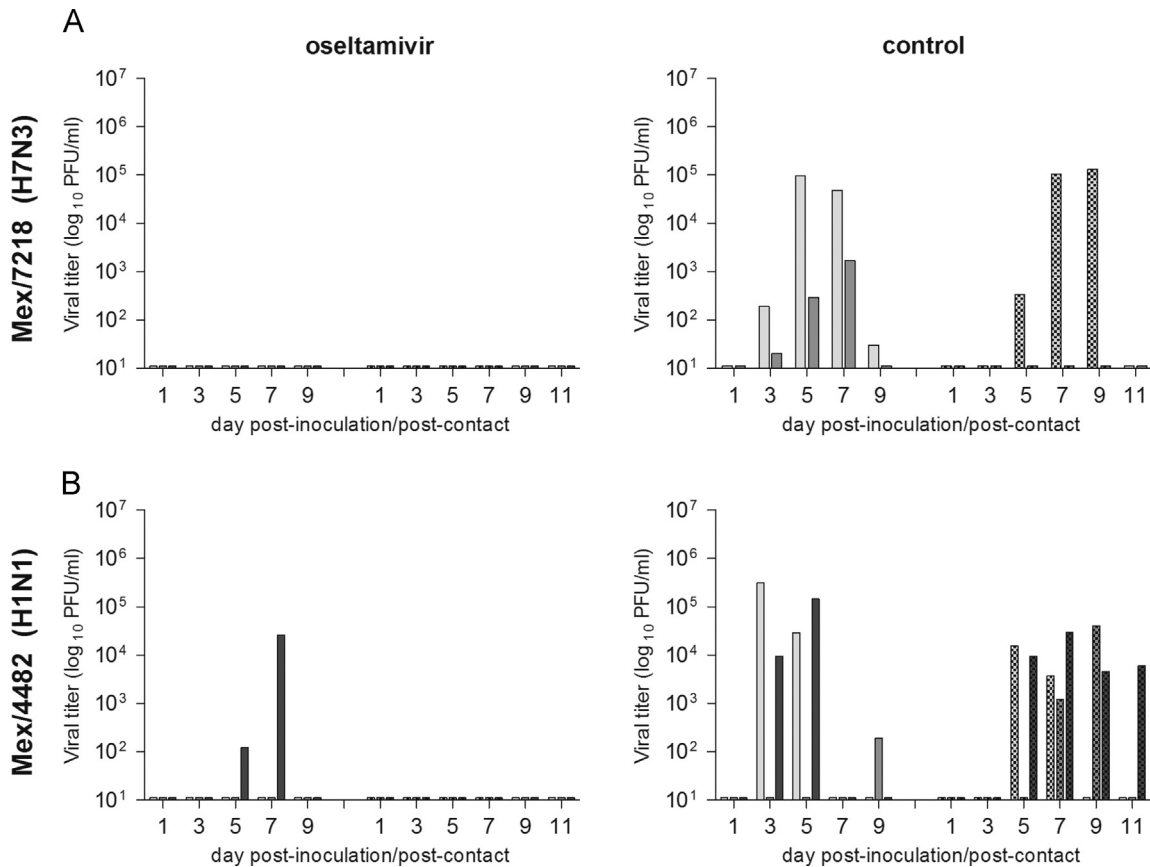


Fig. 2. Reduced influenza virus transmissibility in ferrets receiving oseltamivir following OA inoculation. Ferrets were inoculated by the OA route with Mex/7218 virus (A) or Mex/4482 virus (B) at the doses reported in Table 1 and administered oseltamivir phosphate (left column) or distilled water as a control (right column). A naïve ferret was placed in the same cage as each inoculated ferret at 24 h p.i. in order to assess virus transmission in the presence of direct contact. Nasal washes were collected from inoculated and contact ferrets on alternate days p.i. (solid bars) or p.c. (hatched bars). The limit of virus detection was 10 PFU/mL.

seronegative at day 21 p.c.), while one of two untreated ferrets transmitted virus in the presence of direct contact (seroconversion was not detected in the ferret for which infectious virus was not recovered).

Oseltamivir treatment of ferrets inoculated by the OA route with Mex/4482 virus resulted in fewer ferrets shedding infectious virus in NW samples (1/3), reduced maximum weight loss (1.6–3.9%), and the absence of virus detected in naïve ferrets co-housed with inoculated animals (Table 1, Fig. 2B), compared with untreated ferrets, all of which shed virus in NW samples, exhibited greater maximum weight loss (5.8–13.7%), and transmitted virus to naïve ferrets (Table 1, Fig. 2B). Among naïve ferrets co-housed with oseltamivir-treated ferrets, only one ferret exhibited seroconversion to Mex/4482 virus (the cagemate to the inoculated ferret with positive NW detection), with no seroconversion detected in other contact ferrets (Fig. 2B and data not shown). These results show that oseltamivir treatment can lead to diminished pathogenicity and transmissibility of numerous virus subtypes following OA exposure.

Efficacy of oseltamivir following joint ocular-respiratory aerosol exposure of ferrets

In the absence of either respiratory or ocular protection, a joint ocular-respiratory exposure represents a probable exposure event for individuals in community settings. To more closely mimic a ‘natural’ human exposure, we exposed ferrets to aerosolized LPAI H7N9 A/Anhui/1/2013 (Anhui/1) influenza virus by the OA route with or without concurrent low-level (1.2–11 PFU) AR exposure (Table 1, Fig. 3A and B). Control ferrets received an ocular-only

exposure to virus (Fig. 3C). The virus Anhui/1, isolated from a fatal case at the beginning of the H7N9 outbreak in 2013, represents a current public health threat for which both respiratory and ocular exposure of humans is possible (Gao et al., 2013).

Unlike H5N1, H7N3, or H1N1 OA challenges, virus was detected in NW specimens of all oseltamivir-treated ferrets infected with H7N9 virus by either OA only or joint OA-AR exposure (Fig. 3A and B). Clinical signs and symptoms were generally comparable between oseltamivir-treated and untreated ferrets, and all inoculated ferrets mounted a respiratory infection (Table 1). Untreated ferrets inoculated with Anhui/1 virus efficiently transmitted virus to 100% of cagemates when placed in direct contact, with virus detected in NW specimens from all animals by day 3 p.c. (Fig. 3C). In contrast, transmission among ferrets exposed to Anhui/1 virus by the OA route or OA-AR route and treated with oseltamivir was less efficient, with transmission was detected in 2/2 and 2/4 ferret pairs, respectively (Fig. 3A and B). Compared with control ferrets, transmission events were delayed (with onset of virus detection in contact ferrets day 9 p.c.) or resulted in reduced replication in contacts (with peak titers in NW specimens from contact ferrets < 200 PFU/mL). In summary, we found that oseltamivir treatment limited the establishment of a transmissible respiratory infection in ferrets exposed to aerosolized LPAI H7N9 influenza virus by both ocular and joint ocular-respiratory routes.

Inhibitory activity of oseltamivir carboxylate in human respiratory and ocular cells

While our results in the ferret model agree with previous work demonstrating reduced viral loads in ocular tissue of

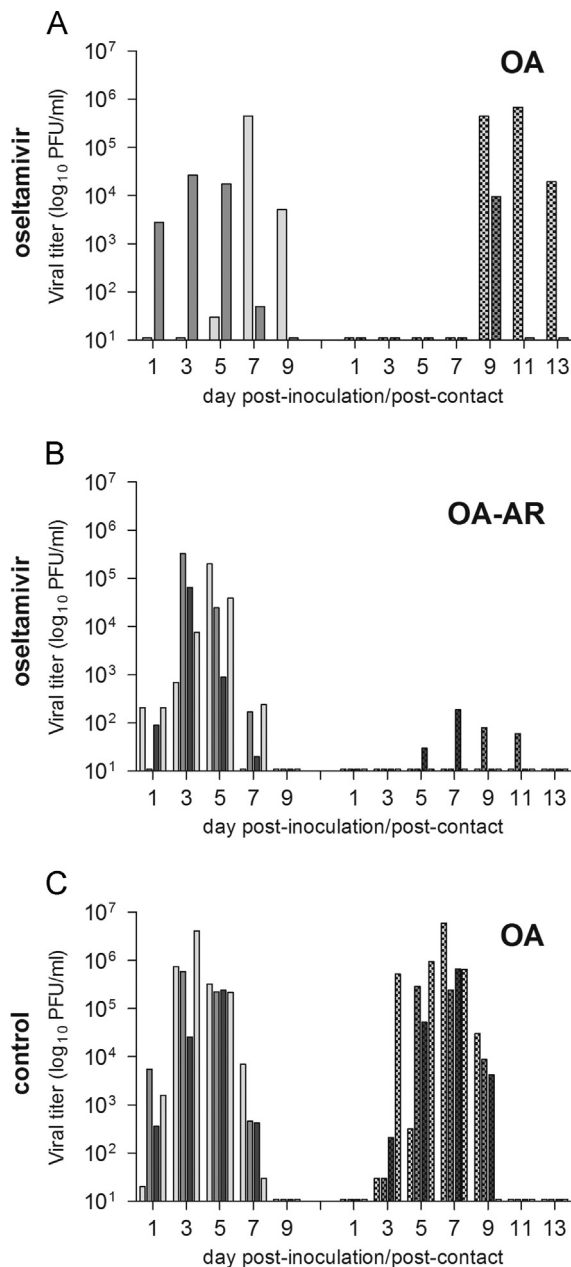


Fig. 3. Reduced H7N9 virus transmissibility in oseltamivir-treated ferrets receiving ocular-only or joint ocular-respiratory exposure. Oseltamivir-treated ferrets were inoculated by the OA route without (A) or with (B) respiratory (AR) exposure to Anhui/1 virus at the doses reported in Table 1, or inoculated by the OA route without respiratory exposure and administered distilled water as a control (C). A naïve ferret was placed in the same cage as each inoculated ferret at 24 h p.i. in order to assess virus transmission in the presence of direct contact. Nasal washes were collected from inoculated and contact ferrets on alternate days p.i. (solid bars) or p.c. (hatched bars). The limit of virus detection was 10 PFU/mL.

oseltamivir-treated mice (Belser et al., 2012b), the efficacy of oseltamivir specifically within human ocular tissue has not been studied. We examined the susceptibility of influenza viruses to oseltamivir carboxylate in primary human corneal epithelial cells (HCEpiC) and compared with the human bronchial epithelial cell line Calu-3 and MDCK cells. All cell types have been shown previously to support productive replication of human and avian influenza viruses (Belser et al., 2011b). Cells were infected with influenza viruses at a MOI of 0.001, and cultured in the presence of serially diluted non-cytotoxic

quantities of oseltamivir carboxylate to determine reductions of virus yield.

All influenza viruses tested demonstrated sensitivity to oseltamivir carboxylate in all cell types examined. Treatment with 100 μ m of oseltamivir carboxylate limited viral titers of H7N3, H7N9, and H1N1 viruses to $< 10^2$ PFU/ml at 24 h and $< 10^{3.3}$ PFU/ml at 48 h p.i (Fig. 4). At this concentration of drug, viral titers of the HPAI H5N1 virus Thai/16 were reduced by 4.3–5.8 logs compared with control-treated cultures at 24 h p.i. While the reductions in viral yield observed following oseltamivir carboxylate treatment varied between cell types in a strain-specific manner, with greater fold reductions in titer observed for viruses that replicated robustly in the absence of oseltamivir, dose-dependent inhibition was observed for all viruses tested regardless of replication efficiency. Comparable results were observed at a MOI of 0.01 (data not shown). These results indicate that, similar to human respiratory tract cells, treatment of human ocular cells with oseltamivir carboxylate effectively inhibits viral yields early after infection.

Discussion

Neuraminidase inhibitors are frequently employed to limit influenza virus infection and spread following respiratory exposure. Understanding how inoculation route and inoculation dose modulate the resulting severity of disease in humans has obvious implications for clinical treatment and mitigation of transmission to susceptible contacts. As the eye represents a secondary mucosal surface bearing permissive viral receptors, located proximal to the nasal passages and as such likely co-exposed during respiratory inhalation of virus, we chose ocular inoculation as a model non-respiratory inoculation route to study this property (Belser et al., 2013b). The utility of the ferret model to study viral conjunctivitis has been previously reported (Kirkeby et al., 2013); while influenza virus-infected ferrets do not typically display macroscopic ocular disease, the close physiologic relatedness between ferrets and humans warrants the use of this species to study ocular exposure and the efficacy of antiviral treatment following infection (Belser et al., 2011a).

Ocular inoculation of influenza viruses in mammalian models typically leads to delays and/or reductions in symptom onset and virus detection compared with traditional intranasal inoculation (Aamir et al., 2009; Belser et al., 2014, 2012a, 2009; Sun et al., 2009). Despite differential kinetics between inoculation routes, oseltamivir treatment administered orally for the standard 5 days p.i. was sufficient to abrogate detectable virus replication in the upper respiratory tract of treated ferrets with two HPAI viruses (Figs. 1 and 2), and further limit the establishment of a respiratory infection in ferrets exposed to aerosolized LPAI or 2009 pandemic influenza virus by the OA route. Each virus is processed differently during aerosolization; while challenge doses varied between viruses used in this study, inoculating doses of all ferrets for each virus were generally equivalent (within one log) (Table 1). Of note, the 2009 H1N1 virus Mex/4482 is highly infectious, with low inoculating doses (< 1 PFU of total OA exposure) sufficient to mount a productive respiratory infection in untreated ferrets (Table 1). This low inoculating dose was necessary to demonstrate oseltamivir efficacy as inoculation with high doses of this virus has been shown previously to limit abrogation of virus in NW specimens from drug-treated ferrets (Govorkova et al., 2011; Marriott et al., 2014). In accord, while viral titers in Anhui/1 OA and OA-AR virus-challenged ferrets were reduced and delayed compared with control ferrets, the sustained detection of virus in NW specimens from these groups may in part be due to the relatively higher inoculating dose use in this challenge (> 100 PFU) (Table 1).

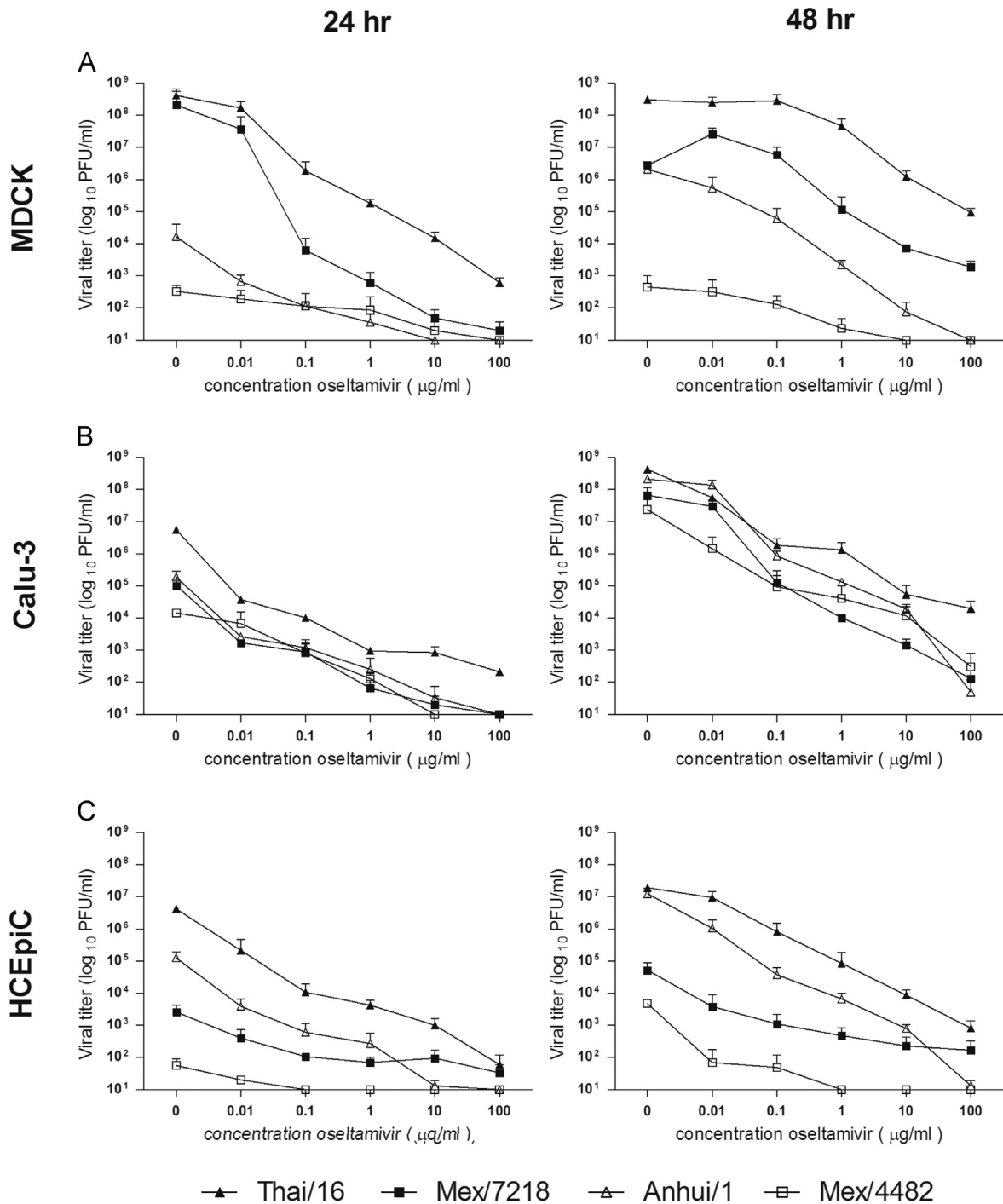


Fig. 4. Reduction of influenza virus yield by oseltamivir carboxylate in multiple cell types. MDCK (A), Calu-3 (B), or HCEpiC (C) cells were infected with H5N1, H7N9, H7N3, or H1N1 viruses at a MOI of 0.001 and cultured p.i. with medium containing the indicated quantity of oseltamivir carboxylate. 24 h (left column) or 48 h (right column) p.i., culture supernatant was sampled and titered for the presence of infectious virus by plaque assay. Values represent the mean + standard deviation for triplicate wells; limit of detection was 10 PFU/mL.

Human cases of influenza resulting from a known ocular exposure are typically self-limiting, though documented instances of probable person-to-person transmission have been reported (Koopmans et al., 2004). Furthermore, viruses not typically associated with an ocular tropism, such as the 2009 H1N1 pandemic virus, have nonetheless been found capable to cause ocular symptoms in infected individuals (Mansour et al., 2012). Thus, our finding that oseltamivir treatment following ocular exposure reduces virus transmission to naïve contacts when a respiratory infection is established supports the use of antiviral treatments in

outbreak settings where ocular symptoms such as conjunctivitis are the primary manifestation of disease. Comparable results between ferrets receiving an ocular-only aerosol dose of Anhui/1 virus and those receiving a concurrent ocular and low-level (~10 PFU) respiratory exposure demonstrates the utility of antiviral treatment following a more rigorous virus exposure (Fig. 3). This is of particular note as select avian influenza viruses have caused concurrent ocular and respiratory disease (Avian influenza, 2007; Tam, 2002; Tweed et al., 2004; Writing Committee of the Second World Health Organization Consultation on Clinical Aspects of

Table 1
Infectivity and pathogenesis of influenza A viruses in ferrets inoculated by the ocular aerosol route and given antiviral treatment.

Virus	Subtype	Tamiflu ^a	Ocular dose ^b	Resp dose ^c	Clinical signs			Virus detection		
					Wt loss ^d	Fever ^e	Lethality ^f	NW ^g	CW ^g	RS ^g
Thai/16	H5N1	Yes	28–88	< 1	7.2 (4–10)	1.5	0/3	0/3	0/3	0/3
		No	34–60	< 1	9.4 (5–7)	2.3	3/3 (5–7)	1/3	1/3	2/3
Mex/7218	H7N3	Yes	15–216	< 1	2.0 (4–8) 2/3	1.4	0/3	0/3	0/3	0/3
		No	94–114 ^h	< 1	9.8 (11–12)	1.8	0/2	2/2	1/2	0/2
Mex/4482	H1N1	Yes	0.08–0.44	< 1	2.9 (6–8)	none (0/3)	0/3	1/3	0/3	0/3
		No	0.36–0.84	< 1	11.0 (9–12)	1.2	0/3	3/3	1/3	0/3
Anhui/1	H7N9	Yes	214–520 ^h	< 1	6.8 (10) 1/2	1.4 (1/2)	0/2	2/2	0/2	0/2
		Yes	116–840	1.2–11	5.8 (2–12) 3/4	1.7 (3/4)	0/4	4/4	0/4	0/4
		No	780–1220	< 1	4.3 (8–10)	2.1	0/4	4/4	1/4	2/4

^a Ferrets received 25 mg/kg of body weight/day oseltamivir phosphate given orally twice daily from 2 h p.i. through day 5 p.i. or control (distilled water).

^b Presented ocular dose (expressed in PFU) is calculated as the virus concentration in the aerosol passed over the ocular surface multiplied by the exposure time (20 min for all viruses with the exception of Thai/16 at 10 min).

^c Estimation of presented respiratory dose (expressed in PFU) is calculated as the virus concentration in the aerosol present in the secondary holding chamber multiplied by the exposure time. Ferrets with a respiratory dose < 1 PFU were considered to be inoculated by the ocular-only aerosol route.

^d Mean maximum weight loss, expressed as a percentage, among ferrets with detectable weight loss (with the day range p.i. of peak weight loss given in parentheses). The number of ferrets with detectable weight loss/total number of ferrets in group is reported when not all ferrets presented with this clinical sign.

^e Mean maximum rise in body temperature, in degrees centigrade, among ferrets with fevers (the number of ferrets with fevers/total number of ferrets in group is reported in parentheses when not all ferrets presented with this clinical sign). The baseline temperature range was 37–39 °C. Fever was defined as a rise of > 1 °C from baseline.

^f Number of ferrets that succumbed to infection or were euthanized due to severe illness/total number of ferrets tested (with the days of death p.i. given in parentheses).

^g Number of ferrets with positive virus detection in nasal wash (NW), conjunctival wash (CW), or rectal swabs (RS)/total number of ferrets. Limit of virus detection was 10 PFU/ml.

^h An additional ferret received an ocular dose of 300 PFU (Anhui/1) or 110 PFU (Mex/7218) but did not shed virus in NW specimens and did not seroconvert; each is excluded from this analysis.

Human Infection et al., 2008), and in the absence of personal protective equipment, simultaneous exposure of both respiratory and ocular tissues is likely. Our results further suggest that oseltamivir prophylaxis of naïve cagemates would offer comparable levels of protection as has been observed following traditional intranasal inoculation of ferrets (Oh et al., 2014). Previous work in the ferret model has demonstrated that oral administration of oseltamivir does not typically generate drug-resistant variants during the acute phase of infection (Govorkova et al., 2007, 2011); nonetheless, future studies examining ocular exposure to oseltamivir-resistant influenza viruses and the role of oseltamivir drug-resistance in the context of ocular infection is warranted.

The human eye and surrounding conjunctiva possess numerous discrete cell types which are permissive to influenza virus infection (Belser et al., 2011b; Chan et al., 2010; Michaelis et al., 2009). While the mechanisms underlying viral conjunctivitis associated with H7 subtype viruses are poorly understood, high viral loads leading to increased inflammation in ocular tissue may represent a contributing factor (Chan et al., 2010). The majority of antiviral drugs available to treat viral conjunctivitis are targeted towards herpesvirus and adenovirus infection; ocular disease caused by RNA viruses such as influenza or RSV currently lack commercially available antiviral drug treatments (Skevaki et al., 2011). The high bioavailability of oseltamivir following oral administration (Davies, 2010; Wattanagoon et al., 2009) likely contributes to the reduced viral titers observed in ocular tissue following ocular inoculation of either mice or ferrets with influenza viruses (Belser et al., 2012b). Our finding that oseltamivir reduces influenza virus titers in human ocular cells in a dose-dependent manner is in agreement with these studies (Fig. 4).

Influenza virus pathogenicity and transmissibility in mammals represent multifactorial traits, with numerous host and viral factors contributing to disease severity. Recent advances in mammalian modeling of influenza viruses have illustrated that exposure route and dose can similarly influence the presentation of clinical signs and symptoms of disease (Gustin et al., 2012). Accordingly, prevention and control measures must be evaluated in settings which reflect the heterogeneity intrinsic in human exposure. While this study examined ocular-only virus exposure, using fixed concentrations of virus for each ferret challenge and a fixed exposure time, further modulation and characterization of inoculation parameters in mammalian models will allow for both a broader understanding of the inherent variability in influenza virus virulence as well as provide greater guidance in the development, assessment, and use of anti-influenza stratagems.

Methods

Viruses

Influenza A viruses used in this study are shown in Table 1. Virus stocks were propagated in the allantoic cavity of 10-day-old embryonated hens' eggs (Maines et al., 2005), and virus stock titers were determined by standard plaque assay using Madin-Darby canine kidney cells (MDCK) (ATCC, Manassas, VA) (Zeng et al., 2007). All experiments were conducted under biosafety level 3 containment, including enhancements as required by the U.S. Department of Agriculture and the National Select Agent Program (Chosewood and Wilson, 2009).

Inoculation and assessment of drug efficacy in ferrets

Male Fitch ferrets (Triple F Farms) were 6-to-8 months old and serologically negative by hemagglutination inhibition assay to currently circulating influenza viruses (H1N1 [2009 pandemic], H3N2 [A/Perth/16/2009], and B [Yamagata and Victoria lineages]). Ferrets were housed in a Duo-Flo Bioclean mobile environmental enclosure (Lab Products, Seaford, DE) for the duration of each experiment. Ocular-only aerosol (OA) inoculation of ferrets were performed by passing aerosolized virus through aerosol-delivery goggles fitted on sedated ferrets (Belser et al., 2014). The ocular dose presented to each ferret was calculated by multiplying the concentration of virus in the aerosol by the exposure time; all ferrets inoculated by the OA-only route received <1 PFU of respiratory exposure. Ferrets which received ≥ 1 PFU of respiratory exposure (measured by sampling the air inside the secondary animal holding chamber) were either excluded from the study or studied for joint ocular-respiratory exposure (Table 1). Ferrets received control (distilled, sterile water) or 25 mg/kg of body weight/day oseltamivir phosphate (Sequoia Research Products, United Kingdom) suspended in 15% fructose orally twice daily (12.5 mg/kg per dose) from 2 h post-inoculation (p.i.) through day 5 p.i.

Ferrets were monitored daily for morbidity and clinical signs of infection for 14 days p.i. (Maines et al., 2005). Any ferret which lost > 25% of its preinoculation body weight or exhibited neurological dysfunction was euthanized and submitted to postmortem examination. Virus shedding was measured in nasal wash, conjunctival wash, and rectal swab samples (Belser et al., 2012a). Naïve ferrets were placed in the same cage as inoculated ferrets to measure virus transmission in the presence of direct contact, with serum collected 17-to-23 days p.i./post-contact (p.c.) to measure seroconversion by hemagglutinin inhibition (Maines et al., 2006). Animal research was conducted in an Association for Assessment and Accreditation of Laboratory Animal Care International-accredited facility, under the guidance of the CDC's Institutional Animal Care and Use Committee.

Cell culture and viral infection

The bronchial epithelial cell line Calu-3 (ATCC) was cultured on 6-well membrane inserts (Zeng et al., 2007). Primary human corneal epithelial cells (HCEpiC, ATCC) were grown to confluence in 12-well plates in serum-free medium. MDCK cells were grown to confluence in 6-well plates. Virus was added apically at a multiplicity of infection (MOI) of 0.01 or 0.001 for one hour before washing. To assess reduction of influenza virus yields by oseltamivir carboxylate in each cell type, cell type-specific serum-free media containing indicated concentrations (0–100 μ M) of oseltamivir carboxylate (Sequoia) was added to the cultures (Ilyushina et al., 2006), with the addition of 300 μ g/liter *N*-*p*-tosyl-L-phenylalanine chloromethyl ketone (TPCK-trypsin) in HCEpiC and MDCK cultures. Aliquots of apical culture supernatants were harvested at indicated times p.i. and titered for the presence of infectious virus by standard plaque assay.

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