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Emergence of a novel drug resistant H7N9 influenza virus: Evidence based clinical potential of a natural IFN-α for infection control and treatment

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Abstract

The novel avian H7N9 influenza virus has caused more than 130 human infections with 43 deaths in China. Because of the lack of existing immunity against H7 subtype influenza viruses in the human population and the absence of a licensed commercial vaccine, antiviral drugs are critical tools for the treatment of infection with this novel H7N9. Both M2-ion channel blockers and neuraminidase inhibitors are used as antiviral drugs for influenza infections of humans. The emerging H7N9 viruses are resistant to the M2-ion channel blockers because of a S31N mutation in the M2 protein; additionally, some H7N9 isolates have gained neuraminidase R292K substitution resulting in broad resistance to neuraminidase inhibitors. In this study we report that Alferon N can inhibit wild type and 292K H7N9 viruses replication *in vitro*. Since Alferon N is approved for clinical use, this would allow a rapid regulatory approval process for this drug under pandemic threat.

Key words: H7N9, Alferon N, neuraminidase inhibitors, Antiviral drug, Influenza, Tamiflu, Oseltamivir resistance.

Emergence of the novel H7N9

A new deadly influenza A H7N9 strain of avian influenza virus, first identified in China in March 2013 with human and avian cases, has been observed in various eastern and inland Chinese provinces[1, 2]. There have been a total of 132 confirmed cases with 43 deaths with people over 60 appearing to be more susceptible to the H7N9-associated severe respiratory disease[3-6]. Patients infected by H7N9 have exhibited viral pneumonia, acute kidney failure, acute respiratory distress syndrome, diffuse intravascular coagulation and septic shock. Although the H7N9 subtype can be highly virulent in humans, it exhibits low pathogenicity in avian hosts perhaps due in part to the absence of a multi-basic amino acid sequence at the hemagglutinin cleavage site[1, 7, 8]. Infected chickens, however, can shed virus very efficiently via the oropharynx, and we and others have observed limited shedding by the cloacal route despite no clinical signs of infection (unpublished data; Liu et al. and [9]). This unusual low pathogenicity of the avian H7N9 viruses makes an H7N9 infection in avian species hard to detect and to monitor. Experts remain fearful of the potential for this virus to mutate into a form easily transmissible between humans, although to date, there has been no clear confirmation of human-to-human transmission[4].

Genetic analysis shows that the novel H7N9 virus is a triple reassortant virus carrying genes from H7N3, H9N2 and H11N9 or H2N9 avian influenza A viruses. It is assumed that the novel H7N9 subtype virus was generated by a single or double independent reassortment event in ducks or chickens in China[10]. Most of the H7N9 viruses carry certain mammalian adaptation signatures (i.e. PB2-627K, HA-226L), which have been proved critical in mammalian adaption and transmission based on previous studies[11] and especially the 226L in the H7 HA protein based on our unpublished data (Liu et al. submitted). Virulence and transmissibility of this novel H7N9 influenza virus have been characterized in different mammalian animal models, including mice, ferrets, macaques and pigs[7, 9, 12-14]. In ferrets, the H7N9 virus showed transmission by direct contact among ferrets and limited transmissibility by respiratory droplets[7, 12-14]. Moreover, the novel H7N9 virus replicated efficiently in both upper and lower respiratory tracts of infected nonhuman primates (*cynomolgus macaques*). Emergent H7N9 viruses isolated from recent human cases have increased infectivity and lethality in mice when compared to avian

H9N2 viruses and genetically related H7N9 viruses isolated from wild birds before 2013[7, 12]. Swine are an important intermediate host for influenza viruses[15]; one of the concerns is that of H7N9 viruses may jump into pigs, they might replicate efficiently in this host, adapt to the mammalian receptor repertoire and reassort with endemic swine influenza viruses. Previous studies showed that pigs infected intranasally with H7N9 viruses did not transmit virus to contact pigs[14], while pigs infected intratracheally with H7N9 can transmit to sentinel animals with low efficiency (Liu et al., submitted). These facts warrant increased surveillance for H7N9 influenza viruses in pigs.

Antivirals and Tamiflu® resistance of the novel H7N9 virus

The novel H7N9 virus rapidly spread over a large geographic region in China after the first reported case in March 2013 with the live poultry markets thought to be the source of human infections, suggesting that H7N9 viruses might be silently prevalent in poultry in China[10]. Continuing detection in poultry and sporadic human infections raises concerns on the emergence of a H7N9 virus, which can efficiently transmit between humans. Vaccination is a very efficient way to prevent influenza infection; however, no commercial vaccine specific for H7N9 is currently available. Moreover, humans lack any immunological memory against H7N9, which is another concern of its potential to result in a pandemic if easy human to human transmission is acquired.

Antiviral treatment is an important strategy in helping to control and prevent influenza infections. As recommended by the CDC in Atlanta, USA, antiviral treatment against influenza infections should be applied as early as possible, ideally within 48 hours after onset of illness. Antiviral drugs are readily available to treat influenza infections including this novel H7N9 virus. For influenza infections, M2-ion channel blockers (e.g., amantadine and rimantadine) and neuraminidase inhibitors (e.g., oseltamivir, zanamivir, and peramivir) have been mainly used as antiviral drugs and those compounds are approved by the Food and Drug Administration (FDA) [16, 17]. However, the H7N9 is resistant to both amantadine and rimantadine because a S31N mutation exists in the M2 protein, which confers M2-ion channel blocker resistance[18]. Therefore, the neuraminidase inhibitors are the only FDA approved compounds, which can be

used for the treatment of the novel H7N9 infection and other M2-ion channel blocker resistant influenza viruses[19].

Clinical studies show that oseltamivir treatment reduces the viral load in the respiratory tract of patients with H7N9 infection[5]. However, some patients with oseltamivir treatment failure had a poor clinical outcome. Further studies on the H7N9 viruses isolated from those patients showed that an R292K substitution occurred in the NA protein; this NA change is associated with resistance to all neuraminidase inhibitors including oseltamivir (Tamiflu®)[20]. Surprisingly, in humans treated with oseltamivir, the R292K substitution occurred as early as 2 days post treatment with the 292K genotype becoming the predominant virus population at 7 days post treatment. Neuraminidase inhibition assays using the resistant H7N9 virus showed 100-fold and 30-fold reduced susceptibility to the two FDA approved inhibitors, oseltamivir and zanamivir, respectively[5]. Subsequently, neuraminidase inhibitor resistance of H7N9 viruses in humans resulted in antiviral treatment failure and a poor clinical outcome. Further studies confirmed that H7N9 isolates with a dominant 292K genotype are resistant to zanamivir, peramivir, and oseltamivir, although this resistance can be masked by a mixed R/K genotype on position 292 of the NA protein in a viral quasispecies population[21]. Rapid emergence of antiviral drug resistant mutants after treatment with oseltamivir or zanamivir is raising concerns about the prevalence of such resistant H7N9 viruses in the humans and other mammalian populations. This emphasizes the importance of the development of new antiviral influenza drugs or the use of already developed FDA approved drugs for other infectious diseases, which might also work against influenza. Other influenza antivirals are under development, including thiazides that are anti-influenza molecules targeting the viral hemagglutinin at the post-translational level[22]; Favipiravir (T-705), a novel viral RNA polymerase inhibitor[23]; Aprotinin, a protease inhibiting the cleavage of the HA protein[24]. None of these compounds, however, have been approved for clinical treatment for influenza infection.

Alferon N inhibits replication of oseltamivir resistant and sensitive H7N9 viruses *in vitro*

Type I interferons (IFN) including IFN alpha and beta produced by host cells in response to the presence of viral pathogens, play a critical role in pathogen clearance during infection[25]. Previous studies showed that the 2009 pandemic H1N1 and the highly pathogenic H5N1 viruses

are highly sensitive to the antiviral actions of type I IFNs [26, 27]. Type I IFN upregulates the expression of several IFN-stimulated antiviral gene products, such as protein kinase R (PKR), 2',5'-oligoadenylate synthetase (OAS) and the Mx protein[28]. The activated PKR phosphorylates the eukaryotic initiation factor eIF2α, thereby inhibiting viral mRNA translation and viral replication[29]. OAS inhibits viral protein synthesis[28]; and the Mx protein interacts with viral components to block generation of new virus particles[26, 30].

Alferon N (IFN-α-n3) is a natural IFN alpha product derived from human leukocytes. In previous studies using SARS virus, Alferon N was the only IFN-based drug to be active against SARS virus at clinically achievable serum levels with the failure of other tested IFN-based clinical products [31]. Additionally Alferon N has a better safety profile than recombinant IFNs [32]. We tested antiviral activity of Alferon N against the novel H7N9 virus in human alveolar epithelial cells (A549) using two human H7N9 isolates: A/Anhui/1/2013 (Anhui/1/H7N9) and A/Shanghai/1/2013 (Shanghai/1/H7N9). Both viruses were isolated from human cases in China. Sequence information showed that the Anhui/1/H7N9 contains the NA-292R residue and is sensitive to NA inhibitors; in contrast, the Shanghai/1/H7N9 contains a NA-292K residue that is associated with resistance to NA inhibitors. The genetic signatures of both viruses are summarized in Table. 1. Oseltamivir (Tamiflu®), an NA inhibitor, was tested on A549 cell as a well-known and widely used anti-influenza drug.

Confluent A549 cells in 48-well plates were treated with different units of Alferon N (10,000 IU/ml, 1,000 IU/ml, 100 IU/ml obtained from Hemispherx Biopharma) for 4 hours before infection, and untreated cells served as controls; Thereafter, both Alferon N treated and untreated A549 cells were infected with the Anhui/1/H7N9 and Shanghai/1/H7N9 viruses at a multiplicity of infection (MOI) of 0.01 under BSL3/BSL3Ag conditions. Combination of a short natural IFN-α pre-incubation and a MOI of 0.01 simulate the conditions of an *in vivo* natural infection. A relatively small number of cells are initially infected resulting in the induction of Type 1 IFNs that in turn induce innate immune responses in uninfected cells. The supernatants were collected at 12 hours, 24 hours, 36 hours and 48 hours post infection and titrated for virus content on MDCK cells in 96-well plates as described previously[33]. For comparison, Oseltamivir (Tamiflu® obtained from Roche) was tested against both H7N9 viruses on A549 cells using a

concentration of 0.3 μg/ml, 0.2 μg/ml, 0.1 μg/ml and 0.01 μg/ml with treatment starting at the time of infection.

The results showed that oseltamivir (Tamiflu®) was able to inhibit the replication of NA inhibitor sensitive Anhui/1/H7N9 virus at a concentration ranging from 0.1 μg/ml to 0.3 μg/ml (Figure 1A); in contrast, oseltamivir (Tamiflu®) treatment with the same doses did not inhibit the NA inhibitor resistant Shanghai/1/H7N9 virus, confirming that the 292K residue in NA of Shanghai/1/H7N9 conferred resistance to oseltamivir. Antiviral activity of Alferon N was found against both the Shanghai/1/H7N9 and Anhui/1/H7N9; clear inhibition of virus replication was found at 36 and 48 hours post infection (Figure 1B). Alferon N with a concentration of 10,000 IU/ml significantly reduced Anhui/1/H7N9 virus load at 36 hours post infection, and all three doses (100 IU/ml, 1000 IU/ml and 10,000 IU/ml) significantly inhibit replication of oseltamivir sensitive Anhui/1/H7N9 and oseltamivir resistant Shanghai/1/H7N9 at 48 hours post infection (Figure 1B). Our data demonstrates that Alferon N is effective against both oseltamivir sensitive and resistant H7N9 viruses and provides sufficient evidence to justify the costly *in vivo* studies (BSL3/BSL3Ag) required for regulatory approval.

Expert commentary and five-year view

The emergence of NA inhibitor resistant strains of the novel H7N9 virus in treated patients requires alternative antiviral compounds for human therapy, which will be critical in case of a potential pandemic. Our results show that both oseltamivir (Tamiflu®) and Alferon N have significant inhibitory effect on the NA inhibitor sensitive H7N9 virus. In contrast, Alferon N, not Tamiflu®, had an inhibitory effect on the neuraminidase resistant Shanghai/1/H7N9 virus. These results provide evidence for a new therapeutic strategy to mitigate the public health hazards associated with H7N9 infection in humans and the potential spread of NA inhibitor resistant H7N9 and other influenza viruses of potential high virulence for humans.

The emergence of influenza pandemic is unpredictable. No universal influenza vaccine is available so far. The licensed anti-influenza drugs (M2-ion channel blockers and neuraminidase inhibitors) are limited in clinical treatment of infected patients because of rapid emergence of drug resistant mutations in the targeted genes. One of the concerns of potential influenza

pandemic is high mortality (e.g. H5N1, H7N9). The rapid disease progression characteristic of pandemic influenza viruses and the emergence of resistance to inhibitors of viral function will require other approaches to limit or terminate disease and spread of the virus. In addition to the rapid regulatory approval of Alferon N or other anti-influenza drugs for this indication, the development of novel anti-influenza drugs using different mechanisms or targeting at different genes (e.g. polymerase genes, nucleoprotein gene and hemagglutinin gene etc.), innate immune responses, and/or combination therapy is necessary for better pandemic defense.

Key Issues

- Antiviral treatment is an important strategy in controlling and preventing influenza viral infections. Current pharmaceuticals approved for the prophylaxis and/or treatment of influenza inhibit the function of the neuraminidase and M2 ion channel viral proteins.
- High mutational rates are characteristic of influenza viruses resulting in viruses with resistance to neuraminidase or M2 inhibitors. Such viruses present a high-risk human public health threat for emerging avian influenza viruses with high pathogenicity for humans such as H7N9.
- Some H7N9 isolates have gained resistance to neuraminidase inhibitors. Alferon N (natural interferon) has broad anti-viral activities and inhibits the replication of both wild type and neuraminidase inhibitor resistant H7N9 viruses.
- An imminent pandemic threat from H7N9 or other avian influenza viruses with resistance to neuraminidase and M2 inhibitor demands research on novel anti-influenza drugs targeting different viral genes and/or innate immune responses.

Acknowledgments & competing interests disclosure:

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References

Papers of special note have been highlighted as:

• Of interest

• • Of considerable interest

1. Gao R, Cao B, Hu Y, et al. Human infection with a novel avian-origin influenza A (H7N9) virus. N Engl J Med 368(20), 1888-1897 (2013).

• Detailed introduction of human infected by novel H7N9 in China

2. Mei Z, Lu S, Wu X, et al. Avian influenza A(H7N9) virus infections, Shanghai, China. Emerg Infect Dis 19(7), 1179-1181 (2013).
3. Salathe M, Freifeld CC, Mekaru SR, Tomasulo AF, and Brownstein JS. Influenza A (H7N9) and the importance of digital epidemiology. N Engl J Med 369(5), 401-404 (2013).
4. Li Q, Zhou L, Zhou M, et al. Preliminary Report: Epidemiology of the Avian Influenza A (H7N9) Outbreak in China. N Engl J Med, (2013).
5. Hu Y, Lu S, Song Z, et al. Association between adverse clinical outcome in human disease caused by novel influenza A H7N9 virus and sustained viral shedding and emergence of antiviral resistance. Lancet 381(9885), 2273-2279 (2013).

• • Detailed description of drug resistant H7N9 infection in patients.

6. To KK, Chan JF, Chen H, Li L, and Yuen KY. The emergence of influenza A H7N9 in human beings 16 years after influenza A H5N1: a tale of two cities. Lancet Infect Dis 13(9), 809-821 (2013).

• Review of H7N9 and H5N1 in multiple aspects.

7. Zhang Q, Shi J, Deng G, et al. H7N9 influenza viruses are transmissible in ferrets by respiratory droplet. Science 341(6144), 410-414 (2013).
8. Zhou J, Wang D, Gao R, et al. Biological features of novel avian influenza A (H7N9) virus. Nature 499(7459), 500-503 (2013).
9. Watanabe T, Kiso M, Fukuyama S, et al. Characterization of H7N9 influenza A viruses isolated from humans. Nature 501(7468), 551-555 (2013).
10. Lam TT, Wang J, Shen Y, et al. The genesis and source of the H7N9 influenza viruses causing human infections in China. Nature, (2013).
11. Hatta M, Gao P, Halfmann P, and Kawaoka Y. Molecular basis for high virulence of Hong Kong H5N1 influenza A viruses. Science 293(5536), 1840-1842 (2001).
12. Belser JA, Gustin KM, Pearce MB, et al. Pathogenesis and transmission of avian influenza A (H7N9) virus in ferrets and mice. Nature 501(7468), 556-559 (2013).
13. Richard M, Schrauwen EJ, de Graaf M, et al. Limited airborne transmission of H7N9 influenza A virus between ferrets. Nature 501(7468), 560-563 (2013).
14. Zhu H, Wang D, Kelvin DJ, et al. Infectivity, transmission, and pathology of human-isolated H7N9 influenza virus in ferrets and pigs. Science 341(6142), 183-186 (2013).
15. Ma W, Kahn RE, and Richt JA. The pig as a mixing vessel for influenza viruses: Human and veterinary implications. J Mol Genet Med 3(1), 158-166 (2008).
16. Schmidt AC. Antiviral therapy for influenza : a clinical and economic comparative review. Drugs 64(18), 2031-2046 (2004).
17. Beigel J and Bray M. Current and future antiviral therapy of severe seasonal and avian influenza. Antiviral Res 78(1), 91-102 (2008).
18. Grambas S, Bennett MS, and Hay AJ. Influence of amantadine resistance mutations on the pH regulatory function of the M2 protein of influenza A viruses. Virology 191(2), 541-549 (1992).

19. Hay AJ and Hayden FG. Oseltamivir resistance during treatment of H7N9 infection. Lancet 381(9885), 2230-2232 (2013).
20. Yen HL, Herlocher LM, Hoffmann E, et al. Neuraminidase inhibitor-resistant influenza viruses may differ substantially in fitness and transmissibility. Antimicrob Agents Chemother 49(10), 4075-4084 (2005).
21. Yen HL, McKimm-Breschkin JL, Choy KT, et al. Resistance to neuraminidase inhibitors conferred by an R292K mutation in a human influenza virus H7N9 isolate can be masked by a mixed R/K viral population. MBio 4(4), (2013).
22. Rossignol JF, La Frazia S, Chiappa L, Ciucci A, and Santoro MG. Thiazolides, a new class of anti-influenza molecules targeting viral hemagglutinin at the post-translational level. J Biol Chem 284(43), 29798-29808 (2009).
23. Furuta Y, Gowen BB, Takahashi K, Shiraki K, Smee DF, and Barnard DL. Favipiravir (T-705), a novel viral RNA polymerase inhibitor. Antiviral Res, (2013).
24. Zhirnov OP, Klenk HD, and Wright PF. Aprotinin and similar protease inhibitors as drugs against influenza. Antiviral Res 92(1), 27-36 (2011).
25. Antonelli G. Biological basis for a proper clinical application of alpha interferons. New Microbiol 31(3), 305-318 (2008).
• • Review of biological basis of alpha interferons
26. Haasbach E, Droebner K, Vogel AB, and Planz O. Low-dose interferon Type I treatment is effective against H5N1 and swine-origin H1N1 influenza A viruses in vitro and in vivo. J Interferon Cytokine Res 31(6), 515-525 (2011).
27. Osterlund P, Pirhonen J, Ikonen N, et al. Pandemic H1N1 2009 influenza A virus induces weak cytokine responses in human macrophages and dendritic cells and is highly sensitive to the antiviral actions of interferons. J Virol 84(3), 1414-1422 (2010).
28. Silverman RH. Viral encounters with 2',5'-oligoadenylate synthetase and RNase L during the interferon antiviral response. J Virol 81(23), 12720-12729 (2007).
29. Garcia MA, Gil J, Ventoso I, et al. Impact of protein kinase PKR in cell biology: from antiviral to antiproliferative action. Microbiol Mol Biol Rev 70(4), 1032-1060 (2006).
30. Haller O, Staeheli P, and Kochs G. Interferon-induced Mx proteins in antiviral host defense. Biochimie 89(6-7), 812-818 (2007).
31. Tan EL, Ooi EE, Lin CY, et al. Inhibition of SARS coronavirus infection in vitro with clinically approved antiviral drugs. Emerg Infect Dis 10(4), 581-586 (2004).
32. Strayer DR, Carter WA. Recombinant and natural human interferons: analysis of the incidence and clinical impact of neutralizing antibodies. J Interferon Cytokine Res.32(3), 95-102 (2012).
• • Review of clinical outcomes following treatment of a wide range of diseases with recombinant interferons and natural interferons.
33. Liu Q, Qiao C, Marjuki H, et al. Combination of PB2 271A and SR polymorphism at positions 590/591 is critical for viral replication and virulence of swine influenza virus in cultured cells and in vivo. J Virol 86(2), 1233-1237 (2012).

Figure legend

Figure 1. Sensitivity of human H7N9 influenza viruses to Tamiflu® and Alferon N *in vitro*. A549 cells were infected with Shanghai/1/H7N9 (containing K at NA residue 292) or Anhui/1/H7N9 (containing R at NA residue 292) viruses at MOI of 0.01. Supernatants were collected at 12, 24, 36 and 48 hours post infection and were titrated in MDCK cells, all studies were conducted in BSL3/BSL3Ag laboratory located at Biosecurity Research Institute (BSL3/BSL3Ag) located at Kansas State University. All data were analyzed by using analysis of variance (ANOVA) in GraphPad Prism version 5.0 (GraphPad software Inc, CA); a P-value < 0.05 was considered statistically significant. Bars represent the mean values ± SEM for 3 independent replicates. (*: $p<0.05$; **: $p<0.01$)