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Original Article

Consequences of resistance: *in vitro* fitness, *in vivo* infectivity, and transmissibility of oseltamivir-resistant influenza A viruses

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The development of drug resistance is a major drawback to any antiviral therapy, and the specific anti-influenza drugs, the neuraminidase (NA) inhibitors (NAIs), are not excluded from this rule. The impact of drug resistance depends on the degree of reduction in fitness of the particular drug-resistant virus. If the resistance mutations lead to only a modest biological fitness cost and the virus remains highly transmissible, the effectiveness of antiviral use is likely to be reduced. This review focuses on the fitness of oseltamivir-resistant seasonal H1N1 and H3N2, 2009 pandemic H1N1 (H1N1pdm09), and highly pathogenic H5N1 influenza A viruses carrying clinically derived NAI resistance-associated NA mutations.

Keywords Animal model, fitness, influenza virus, neuraminidase inhibitor, oseltamivir, transmissibility.

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Introduction

The proper use of neuraminidase inhibitors (NAIs) and worldwide monitoring for the presence and spread of drug-resistant influenza viruses are of the utmost importance. The clinical effectiveness of NAI antiviral treatment depends on many factors, including (but certainly not restricted to) the frequency of emergence of NAI-resistant viruses and the overall biological fitness of viruses carrying drug resistance–associated mutations. The segmented nature of the influenza virus genome and the high rate of misincorporation per nucleotide site make the emergence of resistant variants under selective drug pressure inevitable. NAI-sensitive viruses may possess fitness superior, equal, or inferior to that of NAI-resistant viruses. Drug-sensitive and drug-resistant influenza variants can be clearly dominant populations or can be a mixture of sensitive and resistant clones that can overgrow in different directions; a key issue being how successfully NAI-resistant viruses can compete with wild-type viruses in the absence of selective drug pressure.

Virus fitness can be defined as the summation of parameters that quantify the degree of virus adaptation to a given environment.[1] The key words in this definition are 'summation of parameters', necessitating evaluation of multiple parameters to determine virus fitness. Measuring the influenza virus fitness as the virus replicates in natural host organisms such as humans is difficult. Therefore, establishing basic concepts requires a simplified model system in which to test relevant variables. To gain insight into the biological fitness of NAI-resistant influenza viruses, scientists have applied different methods, which can be subdivided into the following categories (including initial confirmation of the resistance phenotype by phenotypic and genotypic methods[2–5]): (i) kinetic parameters of the neuraminidase (NA) in *in vitro* assays (relative NA activity, K_m, V_{max}, K_i, phenotypic and genotypic stability);[6–8] (ii) quantifying virus fitness *in vitro* and *ex vivo* (plaque morphology, virus yield in mono-infected cell cultures, replication kinetics under single- and multiple-cycle conditions, growth competition assays);[9–11] and (iii) infectivity and transmissibility in animal models.[12–15] BALB/c mice, Hartley strain guinea pigs, and ferrets have been used to evaluate the pathogenicity and transmissibility of NAI-resistant influenza viruses. In addition, innovations in modeling influenza virus infections in laboratory settings may more accurately reflect virus replication in humans and facilitate our understanding of the fitness of drug-resistant influenza viruses. Such new methods include reverse-genetics techniques,[13] immortalized cell lines representative of the human airway,[11,16] virus competition assays in *ex vivo* systems[5] and in animal models,[17,18] and aerosol delivery of

influenza virus to animals.[19] When used together, the data from these assays have proven to correlate with experimental, clinical, and epidemiologic data and partially explain the emergence of NAI-resistant strains.

In patients undergoing treatment, NAI resistance mutations have been found to be NA type- and subtype-specific and drug-specific. Clinically derived influenza A NAI-resistant variants of the N1 subtype most frequently carry H274Y or N294S amino acid substitutions in NA (N2 numbering used throughout the text). Viruses of the N2 subtype have carried E119V or R292K substitutions, and NAI-resistant variants of influenza B viruses have harbored R152K or D198N substitutions in NA. The experimental evidence suggests that amino acid substitutions at position 116, 117, 136, 247, 248, 252, or 276 in NA also reduce oseltamivir susceptibility of influenza viruses.[20–23] The contribution of these substitutions in clinical cases has not been reported.

Oseltamivir-resistant seasonal H1N1 and H3N2 influenza A viruses

Until the end of 2007, the available clinical data indicated a low level of resistance to the NAI oseltamivir (<1% in adults and 4–8% in children >1 year of age).[2,24,25] However, a few studies reported an increased frequency of oseltamivir-resistant variants (18% and 27%) in drug-treated children.[26,27] Experimental data also suggested that the infectivity and replicative ability of oseltamivir-resistant seasonal influenza H1N1 viruses with H274Y (H275Y in N1 numbering) and H3N2 viruses with R292K NA mutations were less than that of the wild-type virus.[28,29] These findings led to the initial hypothesis that NAI-resistant viruses would be less infectious, less transmissible in humans, and, thus, unlikely to be of clinical consequence.

Importantly, further accumulation of experimental data suggested that influenza viruses carrying NAI resistance–associated NA mutations may not be attenuated. For example, the fitness of NAI-resistant viruses can depend on the NA subtype and location of the NA mutation(s) studied (Table 1). A reduction in the transmissibility of drug-resistant virus compared to that of wild-type virus was shown for an A/New Caledonia/20/99-like (H1N1) virus with the H274Y NA mutation in a direct contact ferret model,[30] for an A/Sydney/5/97-like (H3N2) influenza virus with the R292K NA mutation,[12] and for a recombinant A/Wuhan/359/95-like (H3N2) influenza virus with the R292K NA mutation.[13] However, an A/Wuhan/359/95-like (H3N2) virus with the E119V NA mutation was transmitted as efficiently as the wild-type virus.[13,30] In a guinea pig model, recombinant H3N2 influenza viruses carrying the E119V NA mutation or the double mutation, E119V and I222V, were not transmitted as efficiently by respiratory droplets as drug-sensitive variants (Table 1).[31]

The rapid dissemination of the influenza A/Brisbane/59/2007-like (H1N1) viruses carrying the H274Y NA mutation in the absence of antiviral drug pressure was reported worldwide during the 2007–2009 seasons.[32,33] It was suggested that the H274Y NA mutation may not compromise viral fitness and transmissibility in this H1N1 virus genetic background because of changes in NA enzymatic properties[8,34] and cell surface expression of NA.[35] The increased affinity of N1 NA of A/Brisbane/59/2007-like viruses for the substrate in NA activity assays was not compensated for by an increased affinity of the H1 hemagglutinin (HA) for sialic acid receptors.[8] The eventual dominance of viruses with the H274Y NA mutation may, however, have been due to a better balance between their HA and NA activities.[8,34] Interestingly, the reduction of NA activity conferred by the H274Y NA mutation has also been associated with a reduced cell surface expression of NA, possibly due to defects in the folding of NA or its transport to the cell membrane.[35] However, two other mutations in the NA gene (V234M and R222Q) can provide a compensatory effect by increasing NA surface expression, and these two substitutions did occur in the evolution of the H1N1 seasonal strain between 1999 and 2007.[35] This study was the first to indicate effects of the H274Y NA mutation on protein expression and to propose the concept of 'permissive' mutations for oseltamivir-resistant influenza viruses from the perspective of gene evolution. A more recent study has confirmed that substituting the identified 'permissive' residue (Q) for the 'unpermissive' residue (R) in the double H274Y/Q222R mutant virus was associated with a significant reduction of both affinity and activity of the NA enzyme resulting in a virus with a reduced replicative capacity *in vitro* and decreased replication in lungs of ferrets.[36]

Oseltamivir-resistant 2009 pandemic H1N1 influenza viruses

Concern about the spread of oseltamivir-resistant H1N1pdm09 influenza viruses prompted different groups to address the issue of the viruses' growth fitness *in vitro* and virulence and transmissibility in animal models.[37–45] One group of study results suggests that oseltamivir-resistant H1N1pdm09 viruses are not attenuated in pathogenicity or transmissibility and thus could spread among humans without loss of fitness (Table 1).[37–40] In studies using clinically derived H1N1pdm09 viruses carrying the H274Y NA mutation, oseltamivir-resistant virus was as virulent as its wild-type counterpart in mice and ferrets and was transmitted to co-housed animals; respiratory droplet transmission was not assessed in this research.[37] Similarly,

Table 1. Virus replication and transmissibility in animal models of oseltamivir-resistant seasonal H1N1 and H3N2, and H1N1pdm09 influenza A viruses

Influenza virus	NA mutation*	Replication *in vitro*	Transmissibility in animal model				References
			Ferrets		Guinea pigs		
			Contact	Aerosol	Contact	Aerosol	
Seasonal H1N1							
A/New Caledonia/20/99	H274Y	WT > R	WT > R	–	–	–	30
A/Brisbane/59/2007-like		WT = R	WT = R	–	–	–	15
Seasonal H3N2							
A/Wuhan/359/95-like	E119V	WT = R	WT = R	–	–	–	30
rgA/Wuhan/359/95		WT = R	WT = R	–	–	–	13
rgA/Panama/2007/99		WT = R	–	–	WT = R	WT > R	31
A/California/7/2004-like	E119V + I222V	WT = R	–	–	WT = R	WT > R	31
rgA/Panama/2007/99		WT = R	–	–	WT = R	WT > R	31
rg A/Wuhan/359/95	R292K	WT > R	WT > R	WT > R	–	–	13
A/Sydney/5/97		WT > R	WT > R	WT > R	–	–	12
Pandemic 2009 H1N1							
rgA/California/04/2009	H274Y	WT = R	WT = R	WT = R	WT = R	WT = R	40
rgA/Hansa Hamburg/01/2009		WT = R	WT = R	WT = R	WT > R	WT > R	40
A/Quebec/147365/2009		WT ≥ R	WT = R	WT ≥ R	–	–	37,42
A/Bethesda/NIH107-D0/2009		–	WT = R	–	–	–	39
A/Bethesda/NIH106-D0/2009		–	WT = R	–	–	–	39
A/Osaka/180/2009		WT ≥ R	–	WT ≥ R	–	–	38
A/Vietnam/HN32060/2009		WT ≥ R	–	WT ≥ R	–	–	38
A/Denmark/528/2009		WT ≥ R	WT = R	WT > R	–	–	41

rg, recombinant influenza virus; WT, wild-type virus; R, NAI–resistant virus; –, not determined; NA, neuraminidase.
*Amino acid numbering is based on that of N2 NA.
WT > R, fitness of the wild-type virus is greater than that of its oseltamivir-resistant counterpart; WT ≥ R, fitness of the wild-type virus is greater than that of its oseltamivir-resistant counterpart only at initial stages of infection or transmission; WT = R, fitness of the wild-type virus is similar to that of its oseltamivir-resistant counterpart; WT < R, fitness of the wild-type virus is less than that of its oseltamivir-resistant counterpart.

Kiso *et al.*,[38] concluded that the H274Y mutant virus undergoes aerosol transmission between ferrets, but there was a 2-day delay in transmission of one of the mutant strains.

Memoli *et al.*[39] also reported that replicative fitness, transmissibility, and virulence of NAI-resistant H1N1pdm09 mutants were comparable to those of the wild-type virus in the ferret model. These multidrug-resistant viruses were isolated from immunocompromised patients after just 9–14 days of NAI therapy, were resistant to the aminoadamantanes and to oseltamivir and peramivir, and maintained their ability to cause disease in ferrets. The resistant viruses replicated in both the upper and the lower respiratory tracts of ferrets, with no differences in the overall mean lung titers, duration of illness, or quality and quantity of lung pathology.[39]

Seibert *et al.*[40] used recombinant H1N1pdm09 viruses differing by a single H274Y amino acid substitution to show that the growth kinetics of the mutant viruses were similar to those of the wild-type viruses and that they were efficiently transmitted to guinea pigs and ferrets in both contact and droplet transmission studies (Table 1). Furthermore, when wild-type and H274Y mutant viruses were put into direct competition in the upper respiratory tract of guinea pigs, both viruses were detectable in three of four exposed guinea pigs, suggesting that the resistant virus is readily transmissible and equivalently fit.[40] However, a detailed analysis of their data reveals that one of the viruses tested had a 2-day delay in transmission to half of the contact-exposed guinea pigs. The other strain of H1N1pdm09 virus had a droplet transmission rate of 88% rather than 100%. The transmissibility and fitness of the H274Y mutant viruses were further studied in the ferret transmission model, in which ferrets inoculated with a 1:1 mixture of oseltamivir-sensitive and oseltamivir-resistant viruses transmitted both wild-type and mutant viruses by contact and aerosol transmission. These studies were performed using a small sample size, but the fact that the H274Y mutant viruses were transmitted by contact, by aerosol, and in competition with the wild-type virus in ferrets supports the conclusion made after analyzing the more rigorous guinea pig transmission data.[40]

In contrast, by studying a different pair of H1N1pdm09 wild-type and oseltamivir-resistant viruses, Duan *et al.*[41] found that the resistant virus was not efficiently transmitted between ferrets by the respiratory droplet route. Furthermore, in co-infected animals, the wild-type virus outgrew the resistant mutant and was uniquely transmitted to contact animals (Table 1). The NA of the resistant virus had reduced substrate-binding affinity and catalytic activity *in vitro*, and the resistant virus exhibited slower initial growth in Madin-Darby canine kidney (MDCK) and MDCK-SIAT1 cells. This growth delay could have been caused by the delayed release of progeny virions from the host cell surface because of the reduced NA efficiency of the resistant virus. Such a delay would not affect the final virus yield in cell culture, but it could allow the host's first-line innate immune defense (e.g., macrophages or neutrophils) sufficient time to clear the virus from the respiratory tract of ferrets. The slightly reduced NA function and delayed growth of the H274Y mutant virus may have been more crucial in recipient ferrets that acquired the virus from the environment via natural routes, than in donor ferrets inoculated with a high dose of virus, because delayed viral shedding or inefficient transmission was observed in the recipient ferrets but not in the inoculated donor ferret.[41] The data reported by Hamelin *et al.*[42] confirmed that transmission of the H274Y mutant H1N1pdm09 virus by the airborne route (including aerosol and large droplets) is somewhat compromised, which may limit its widespread dissemination.

The novel NAI resistance-associated NA mutations, I222R and S247N, identified in clinically derived H1N1pdm09 viruses raised concerns about their effects on virus fitness. The I222R mutant was less pathogenic in ferrets than was the wild-type H1N1pdm09 virus but had similar replication ability *in vitro* and transmissibility in ferrets.[43] The results of another study showed that the wild-type, the H274Y mutant, and the I222R plus H274Y double mutant H1N1pdm09 viruses generated by reverse genetics had similar infectivity and transmissibility in ferrets.[44] The results of a study using reverse-genetics H1N1pdm09 viruses showed that viruses carrying the S247N NA mutation had reduced respiratory droplet transmissibility in guinea pigs, and the H274Y plus S247N double mutant had more efficient transmission compared to that of the wild-type virus.[45] However, when the reverse-genetics virus was rescued from another NA genetic H1N1pdm09 virus background, the S247N mutant and the H274Y plus S247N double mutant were less transmissible in guinea pigs than their wild-type counterpart.[45]

Thus, the data on the fitness deficit of the NAI-resistant mutants are so scarce that different groups have come to different conclusions about its relevance. These differences in interpretation might be partly due to the various investigators' use of H1N1pdm09 viruses with different genetic backgrounds and of different experimental protocols, for example, cage design, direction and strength of airflow, number of animals used. Moreover, ferrets are outbred animals, so individual differences in the susceptibility to influenza virus infection might add to variation in the data. Additionally, the reduced transmissibility of the oseltamivir-resistant H1N1pdm09 viruses could be explained by a number of factors. First, host physical exposure to virus is directly affected by the quantity of virus shed into the environment.[46] Other host variables such as the extent of inflammation could affect the amount of upper respiratory secretions and, thus, the release of infectious respiratory droplets. Second, efficient transmission to a naive host requires not only exposure to virus but also successful virus infection, effective replication, and simultaneous evasion of the first line of host innate immunity.[47] Anecdotal evidence from the clinic shows that, in most instances, contemporary drug-resistant variants of H1N1pdm09 were replaced by drug-susceptible variants when the selective pressure of oseltamivir was removed, suggesting that wild-type viruses possess superior fitness in humans. Reports of reduced aerosol transmissibility of oseltamivir-resistant viruses are consistent with the available epidemiologic data, which so far do not show a predominance of resistance.[48] However, the isolation of community-transmitted, oseltamivir-resistant H1N1pdm09 viruses[49,50] suggests that such viruses retain a certain level of transmissibility and may be more fit than at the start of the pandemic, reinforcing the need for continuous antiviral susceptibility surveillance. A conclusion from these studies is that H1N1pdm09 viruses can accommodate the single H274Y change without significantly impaired fitness and transmissibility and may not require other permissive NA changes, the phenomenon reported for A/Brisbane/59/2007-like (H1N1) viruses carrying the H274Y NA mutation.[35]

Oseltamivir-resistant highly pathogenic H5N1 influenza viruses

Since the first human cases in 1997 in Hong Kong,[51,52] sporadic human infection with highly pathogenic avian influenza A(H5N1) virus has caused illness in more than 600 persons in 15 countries in Asia, the Middle East, Europe, and Africa, with an overall mortality rate of approximately 60%.[53] Because the seasonal influenza vaccine does not elicit effective immunity against H5N1 influenza viruses, we must rely on antiviral drugs to combat these deadly viruses; thus, the acquisition of NAI resistance by H5N1 influenza viruses is a serious public health concern. Oseltamivir-resistant H5N1 viruses with the H274Y NA mutation have been isolated from three patients during drug treatment or prophylaxis,[54,55] and those with the N294S NA mutation,

from two patients in Egypt.[56] In addition, the highly pathogenic A/Hanoi/30408/2005(H5N1) influenza virus that was isolated from a patient treated with oseltamivir had a mixed oseltamivir-sensitive and oseltamivir-resistant population.[55] Ten resistant clones that were randomly picked from plaques of the virus in MDCK cells possessed either a H274Y or N294S NA mutation.[55]

The available reports on the fitness of highly pathogenic oseltamivir-resistant H5N1 viruses are limited and focused on viruses of the two HA clades that have caused infection in humans: clade 1 and clade 2.2 (Table 2).[17,55,57–60] The virulence of H5N1 viruses carrying either a H274Y or N294S NA mutation was addressed using cell culture, mouse, and ferret models. In a mouse model, recombinant A/Vietnam/1203/2004-like (H5N1) influenza viruses possessing either the H274Y or N294S NA substitution exhibited lethality similar to that of the wild-type virus.[57] For the less-virulent A/Hanoi/30408/2005(H5N1) oseltamivir-resistant clone, the N294S NA substitution attenuated the virus in mice, although the degree of attenuation was lower than that caused by the H274Y NA substitution.[59] Le and collegues[55] reported that the oseltamivir-resistant A/Hanoi/30408/2005 (H5N1) influenza virus with the H274Y NA mutation was attenuated compared to the wild-type virus (Table 2), as reflected by less-efficient replication in the ferret upper respiratory tract. However, the A/Hanoi/30408/2005(H5N1) influenza virus does not cause severe infection in ferrets inoculated with 2×10^5 PFU per animal.[55] This mild infectivity is in contrast to the virulence of human A/Vietnam/1203/2004 (H5N1) virus in ferrets. Inoculation with A/Vietnam/1203/2004(H5N1) virus with a dose as low as 10 EID_{50} resulted in severe disease, and the deaths of two of three animals inoculated; higher doses (10^2 or 10^3 EID_{50}) caused high fever, substantial weight loss, anorexia, and extreme lethargy, and were lethal to all animals.[61] Compared to the wild-type virus, a recombinant A/Vietnam/1203/2004-like (H5N1) virus with the H274Y NA mutation replicated to similar titers in the upper respiratory tract of ferrets and caused similar disease signs; none of the animals survived when infected with either oseltamivir-resistant or -sensitive viruses.[17]

In a ferret model, the N294S NA substitution may be introduced less frequently than the H274Y substitution under NAI selective pressure.[60] However, H5N1 viruses with the N294S NA substitution caused considerable pathogenicity in inoculated ferrets, underscoring the importance of monitoring the emergence of the N294S NA mutation in circulating H5N1 viruses.[60] In recombinant clade 2.2 A/Turkey/15/2006-like (H5N1) influenza viruses possessing different NAI resistance–associated NA mutations, viruses with the H274Y mutation conferred a high level of oseltamivir resistance (mean IC_{50}, >900-fold that of wild-type) and possessed virulence similar to that of the wild-type virus in ferrets. Virus with N294S conferred a moderate level of oseltamivir resistance (mean IC_{50}, >60-fold that of wild-type) and was associated with significantly higher virus titers ($P < 0{\cdot}01$) and more inflammation in the lungs of ferrets than the wild-type virus.[58] In a competitive ferret model in which animals were co-inoculated with different ratios of oseltamivir-resistant and oseltamivir-sensitive recombinant H5N1 viruses, the H274Y NA mutation affected the fitness of the two H5N1 viruses differently: the fitness of resistant A/Vietnam/1203/2004-like (H5N1) virus was undiminished as compared to that of its drug-sensitive counterpart; yet, the fitness of the resistant A/Turkey/15/2006-like (H5N1) virus was impaired.[17] In addition, a I254V NA mutation was identified in A/Vietnam/1203/2004-like (H5N1), and a E276A mutation was identified in A/Turkey/15/2006-like (H5N1) genetic backgrounds, which could potentially

Table 2. Fitness of highly pathogenic H5N1 influenza A viruses carrying H274Y and N294S NA mutations

H5N1 influenza virus (HA clade)	NA mutation*	Virus replication *in vitro*	Pathogenicity *in vivo*		References
			Mice	Ferrets	
rgA/Vietnam/1203/2004 (clade 1)	H274Y	WT = R	WT = R	WT = R	17,57,60
	N294S	WT = R	WT = R	WT = R	
A/Hanoi/30408/2005 (clade 1)	H274Y	–	WT > R	WT > R	55,59
	N294S	–	WT > R	WT > R	
rgA/Turkey/15/2006 (clade 2.2)	H274Y	WT = R	–	WT > R	17,58
	N294S	WT ≥ R	–	WT < R	

rg, recombinant influenza virus; WT, wild-type virus; R, NAI–resistant virus; –, not determined; HA, hemagglutinin; NA, neuraminidase.
*Amino acid numbering is based on that of N2 NA.
WT > R, fitness of the wild-type virus is greater than that of its oseltamivir-resistant counterpart; WT ≥ R, fitness of the wild-type virus is greater than that of its oseltamivir-resistant counterpart only at initial stages of infection or transmission; WT = R, fitness of the wild-type virus is similar to that of its oseltamivir-resistant counterpart; WT < R, fitness of the wild-type virus is less than that of its oseltamivir-resistant counterpart.

exert a compensatory effect on the fitness of H5N1 viruses carrying the H274Y NA mutation.[17]

One important conclusion from these studies is that a particular NAI resistance-associated marker can cause different effects on fitness in different H5N1 virus genetic and virulence backgrounds. Deficiency in NA function caused by an NAI resistance mutation may not be deleterious for highly pathogenic H5N1 viruses because of the extremely efficient replication of these viruses.

Conclusion

The effects of NAI resistance NA mutations on the fitness and transmissibility of influenza viruses may vary depending on several factors: location of the mutation (catalytic or framework residue), NA type/subtype, virus genetic background, existence of permissive secondary NA mutations, degree of NA functional loss, and an appropriate functional NA–HA balance. In addition, differences in the host's immune response and genetic background can contribute to such variation. No single measure can easily describe the extent of fitness of influenza viruses carrying drug resistance mutations. A summation of data on weight loss, duration of illness, clinical score, pathologic changes, and contact and aerosol transmissibility are required to more accurately reflect the viral fitness deficit in animal models. Understanding how drug resistance mutations affect the frequency and mode of transmission is crucial to designing public health measures aimed at controlling epidemics and pandemics of influenza. Information from a variety of experimental model systems should be combined to evaluate the overall biological fitness of influenza viruses carrying NAI resistance markers. Such knowledge clearly needs to be revised specifically for each novel influenza virus that emerges either as a seasonal strain by drift or as a pandemic virus by antigenic shift. Many other factors, including heterogeneous mixing of populations and stochastic effects, may influence whether a particular mutant virus predominates in the population: therefore, the fitness deficit must be determined for multiple strains of drug-resistant virus to better predict or explain the epidemiologic observations. The risk of emergence of drug-resistant influenza viruses with undiminished fitness should be monitored closely and considered in pandemic planning.

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Conflicts of interest

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