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Short Communication

4-Guanidino-Neu5Ac2en fails to protect chickens from infection with highly pathogenic avian influenza virus

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Abstract

The effectiveness of the novel sialidase inhibitor 4-guanidino-Neu5Ac2en, which is highly effective in mouse and ferret models of influenza virus infection (von Itzstein et al. (1993) Nature 363, 418–423), has been assessed as a prophylactic agent in the prevention of infection of chickens with highly pathogenic avian influenza viruses. At best a small delay in the onset of pyrexia and death was observed with one strain of fowl plague virus, but not with two other strains. These results demonstrate that a locally acting drug may be ineffective if virus can escape from the site of inoculation and replicate elsewhere.

Keywords: Sialidase inhibitor; 4-Guanidino-Neu5Ac2en; Influenza virus; Chicken

Influenza A viruses infect a wide range of animals: the most commonly affected are man, pigs, horses and species of aquatic birds. Influenza A viruses are classified on the basis of the antigenicity of the virus glycoproteins, the haemagglutinin (HA) and neuraminidase (NA) (WHO, 1971, 1980). Viruses which represent all the different

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subtypes of HA and NA have been isolated from avian species. Pathology in birds following infection with avian influenza viruses ranges from mild or inapparent to a lethal infection. This lethal infection is termed fowl plague and is caused by infection of poultry with highly pathogenic avian influenza (HPAI) viruses. Infection results in pantropic dissemination of virus within the bird. HPAI viruses are restricted to certain virus strains with the H5 and H7 subtypes of haemagglutinin. The HA of these strains has the characteristic of undergoing post-translation cleavage mediated by intracellular proteases to form fully functional HA consisting of HA_1, and HA_2 polypeptide chains (Stieneke-Grober et al., 1992, Walker et al., 1994). Fowl plague is not common in the western world; only 13 reports of outbreaks, epizootics or isolations have been made during the period from 1959 to 1992 (Alexander, 1993). Nevertheless, fowl plague remains a serious threat to the poultry industry: this is evinced from figures of a major epizootic in Pennsylvania and Virginia during 1983–1984 when > 17 million birds were slaughtered (USDA, 1984).

Control of HPAI virus infection through the use of therapeutics has been examined on a small scale using the compound 1-adamantanamine hydrochloride (amantadine) (Lang et al., 1970; Webster et al., 1985), but it has not been employed on a large scale due to the possibility of the rise of drug-resistant virus. In infections of humans with human influenza A viruses, resistant viruses have been readily selected (Belshe et al., 1989; Hayden et al., 1989) and in poultry, resistant viruses emerged under experimental conditions (Wainright et al., 1991) and under conditions which simulated natural transmission of avian influenza virus (Webster et al., 1985).

Recently, antiviral compounds have been developed which are based on sialic acid analogues that show high activity against and selectivity for human influenza A viruses and influenza B viruses both in tissue culture and in mouse and ferret disease models (von Itzstein et al., 1993; Woods et al., 1993). The most effective of these, 4-guanidino-2,4-dideoxy-2,3,didehydro-*N*-acetylneuraminic acid (4-guanidino-Neu5Ac2en), has been shown to inhibit the replication of avian influenza viruses containing neuraminidases of each of the 9 neuraminidase subtypes in tissue culture (Thomas et al., 1994). In this communication we report on the efficacy of 4-guanidino-Neu5Ac2en in the prevention of fowl plague.

Experiments using 6-week-old chicks were carried out using a regimen similar to that used with the mouse and ferret disease models (von Itzstein et al., 1993). Chicks were divided into groups of 8 or 10 birds. Drug was administered in sterile water at a dose of 1–1.5 mg/kg body weight twice daily for 5 days by intratracheal drops (75 μl dose) to half of the groups. On the day following the initiation of drug administration and less than 6 h following a dose of antiviral drug, all groups of animals were infected, again by the intratracheal route, with 100 μl of egg-grown HPAI virus diluted to approximately 10^7 PFU/ml in physiological saline. The chickens were then monitored twice daily for signs of disease and their body temperature was measured once or twice daily using a digital thermometer. All experiments were carried out under UK Home Office approval and under Category III containment as approved by the UK Ministry of Agriculture Fisheries and Food. The heads of birds that died or were culled during the experiment were retained for virus isolation.

Three strains of HPAI virus were used to infect birds: SD1 (H7N7), SD17 (H7N1)

and A/Duck/Ireland/83 (H5N8). SD1 and SD17 are reassortant avian influenza viruses made between A/FPV/Rostock/34 (H7N1) and A/FPV/Dobson/28 (H7N7) (Smith, 1985; McCauley and Penn, 1990). Each of these avian virus strains is sensitive to the inhibitor in vitro, and they represent the extremes of sensitivity in avian viruses that have been seen. A/Duck/Ireland/83 was the most sensitive avian influenza virus examined (exhibiting an IC_{50} of < 1 nM in plaque formation), but both SD1 and SD17 were less sensitive in the reduction of plaque numbers, but plaque size was sensitive to low levels of drug (SD1 and SD17 had an IC_{50} of 160 and 220 nM, respectively) (Thomas et al., 1994).

The results obtained from 3 experiments in which groups of 6-week-old chicks were infected are shown in Figs. 1 and 2. Fig. 1 shows the body temperature of individual birds measured up to the time of death or culling, and Fig. 2 shows the survival of birds following infection. With both control (virus alone) and drug-treated groups (virus plus drug), pyrexia followed virus infection: pyrexia was observed from the second or third day following infection, with body temperature rising to 43°C and falling rapidly before death or culling (Fig. 1B, D, H). Administration of 4-guanidino-Neu5Ac2en did not lower the degree of pyrexia or reduce the death rate following infection with any of these strains of HPAI virus (Fig. 1A, C, G). Pyrexia was maximal between 48 and 72 h following infection. In these experiments not all birds died with the dose they received: 2 birds (out of 8) survived infection with Duck/Ireland without drug and 1 out of 8 survived with drug. One bird out of 10 survived infection with SD17. It is our experience that the outcome of infection following intratracheal infection is more variable than infections initiated by intravenous inoculation. Thus it may be that the birds that survive infection had not been productively infected at the start of infection. Graphical representation of the survival of birds in each group is shown in Fig. 2. The groups infected with A/Duck/Ireland/83 (Fig. 2A) showed that 7 of the group of 8 birds and 6 of the 8 birds died in the treated and untreated groups, respectively. Death was recorded from the first report on day 3 in the absence of drug and from the later report on the same day in the presence of drug. In the groups of birds infected with SD1 (Fig. 2B) all 8 birds in the group treated with drug survived until 4 days postinfection but were dead by 5 days; in the untreated group death was recorded from day 3 and a single bird survived until day 5. Thus there seems to be no or very little effect on the progression of fever and death in the case of groups of birds infected with virus strains SD1 or A/Duck/Ireland/83.

By contrast, in the experiments in which chicks were infected with virus SD17, a clear delay in the onset of pyrexia and death was observed in one experiment. In the experiment shown in Fig. 2C, only 4 of the group of 8 birds survived until the end of the second day postinfection in the untreated group; but in the treated group, no deaths were recorded until the second report on day 3 (3 of 8) and 5 birds survived until day 4 postinfection. We used non-parametric analysis, the Wilcoxon rank sum test, to determine the statistical significance of this result. Birds were ranked according to the time at which they died or were culled in extremis and the rank was correlated with drug treatment. The probability that the rank correlated with drug treatment purely by chance was less than 0.01. In another earlier experiment involving SD17 infection of groups of birds in which reporting was daily, the efficacy of treatment with 4-guanidino-Neu5Ac2en

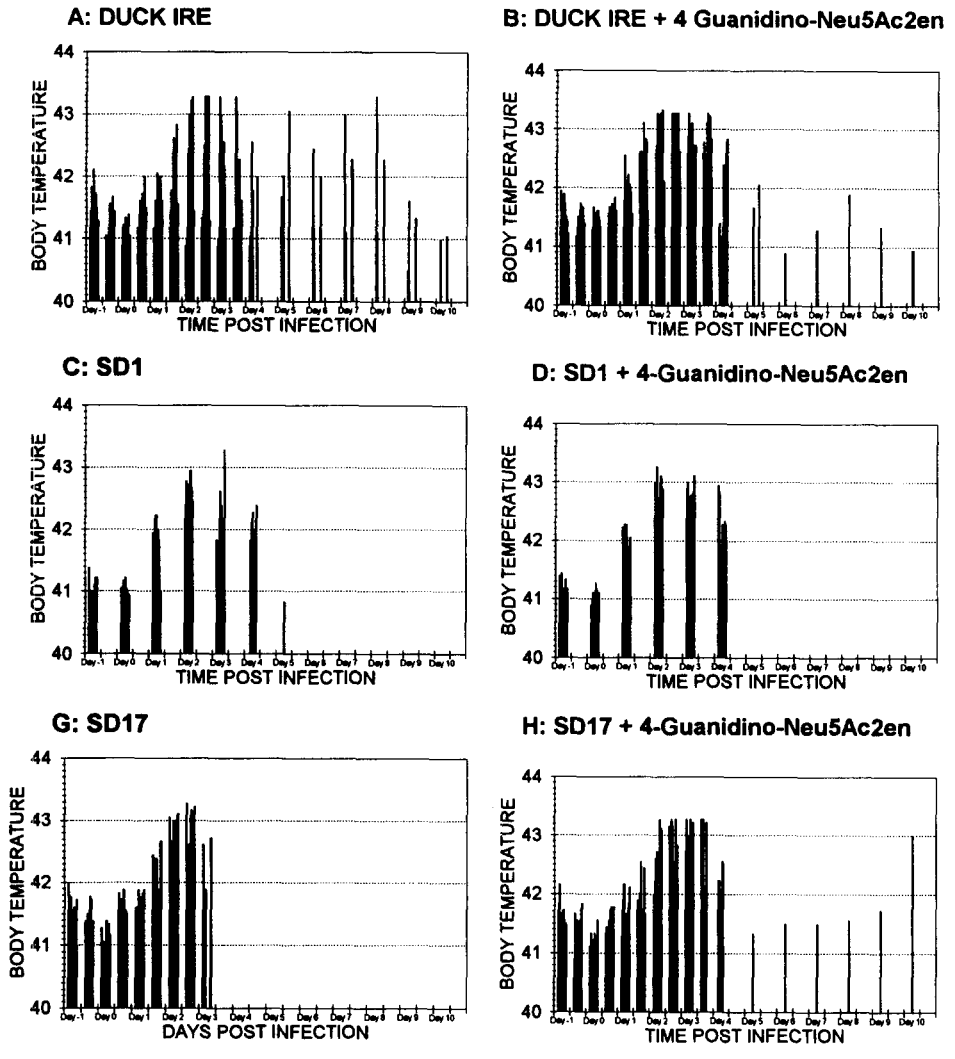


Fig. 1. The influence of administration of 4-guanidino-Neu5Ac2en on the body temperature of 6-week-old chicks following infection with avian influenza viruses. Drug was given at a dose of 1–1.5 mg/kg body weight twice daily for 5 days by intratracheal drops (75 μl dose in sterile water) to the groups of chicks in B, D and H; no drug treatment was given to the groups shown in A, C and G. On the day following the initiation of drug administration and less than 6 h following a dose of antiviral drug, all groups of animals were infected, again by the intratracheal route, with 100 μl of egg-grown HPAI virus diluted to approximately 10^7 PFU/ml in physiological saline. Infection was with A/Duck/Ireland/83 (H5N8) (G and H), with reassortant virus SD1 (H7N7) (C and D), and with reassortant virus SD17 (H7N1) (E and F). The chickens were monitored twice daily for signs of disease and their body temperature was measured once or twice daily using a digital thermometer. The temperature for each bird is shown by a bar in the figure until it died or was killed in extremis and thence was no longer recorded in the figure. Birds were infected on day zero.

was not demonstrable: 5 of 10 birds died on the fourth day after infection in the untreated group, but 4 of 10 birds died in the treated group.

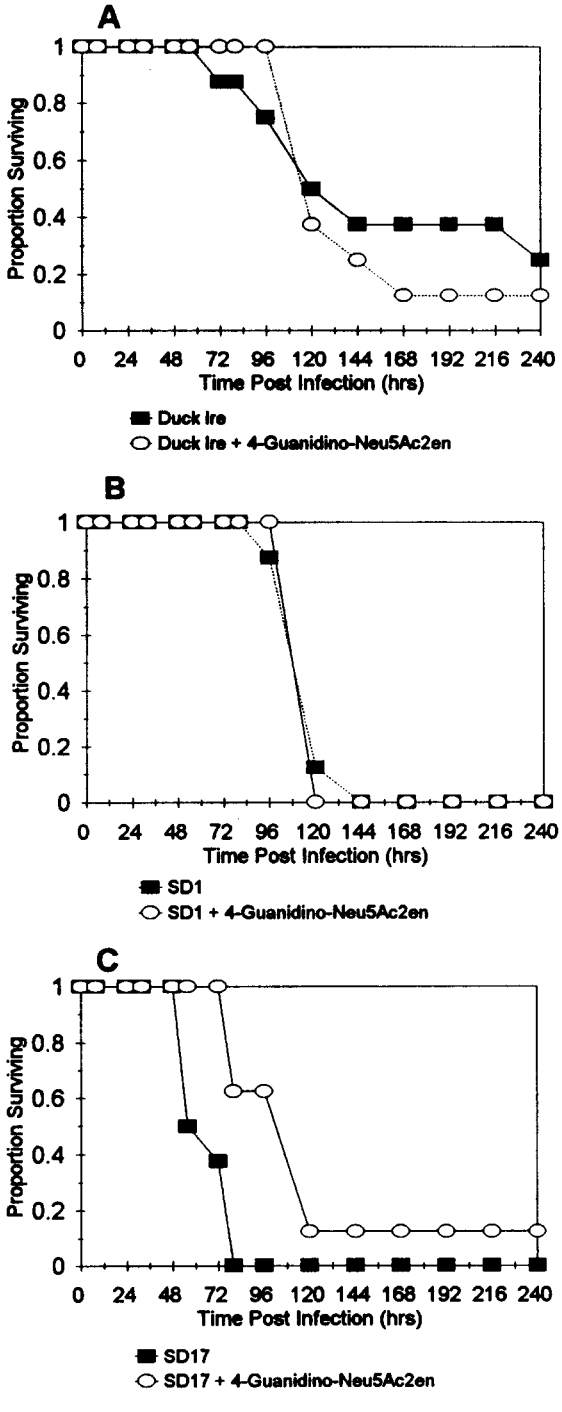
Further to the collection of data on pyrexia and death, virus recovered from infected birds was examined to determine whether increased resistance to the drug could account for the apparent inefficacy of the compound in vivo. For each experiment, virus was isolated from dead chicks by inoculation of brain homogenates into fertile hen eggs and, following plaque titration on MDCK cells, for each isolate approximately 50 pfu were plated in the presence of 4-guanidino-Neu5Ac2en at concentrations appropriate to the strain, as previously determined (Thomas et al., 1994). When plaque size or plaque number were assayed on MDCK cells in the presence of 4-guanidino-Neu5Ac2en at 160 nM (SD17) 220 nM (SD1) or 1 nM (Dk/Ireland/83), none of 16 SD17, 8 SD1 or 8 Dk/Ireland/83 isolates were significantly more or less resistant to the drug than were the viruses used to infect the chickens.

Experiments not shown here have also been carried out to demonstrate that the means of drug delivery was unlikely to affect the conclusions that could be drawn. A group of chicks which were given the drug-carrier alone (sterile water) were susceptible to infection, developed fever and died; another group which was given 4-guanidino-Neu5Ac2en in water did not show any disease signs. Furthermore, the antiviral drug 1-adamantanamine hydrochloride (amantadine) administered by the same route and frequency as the 4-guanidino-Neu5Ac2en and delivered at a dose of 10 mg/kg protected birds fully from any pyrexia, disease signs and death when challenged with the same lethal dose of Dk/Ireland/83.

Thus our experiments show that 4-guanidino-Neu5Ac2en fails to act in vivo as a prophylactic agent against HPAI. This result contrasts with the prophylaxis obtained with 4-guanidino-Neu5Ac2en following the infection of mice or ferrets with human influenza viruses of an intermediate in vitro sensitivity to the drug compared with that of these avian strains (von Itzstein et al., 1993). The failure to show prophylaxis against HPAI could result from any one of several causes, but the possibility that selection of increased resistance of the virus to the drug occurred during in vivo infection has been excluded.

In these experiments it had been intended that drug would be delivered to the same location as the initial site of virus replication since it is known that 2-deoxy-2,3-didehydro-D-*N*-acetylneuraminic acid (Neu5Ac2en) is very rapidly excreted by mice (Nohle et al., 1982) and 4-guanidino-Neu5Ac2en may be eliminated likewise in the chicken. Experience obtained from using the compound in mice indicates that the effective concentration of the drug is rapidly lost when administered systemically (von Itzstein et al., 1993). Since fowl plague is caused by a systemic infection and virus replication is not restricted to respiratory epithelial cells, it was felt that it would be necessary to inhibit the earliest possible stages of virus replication by delivering drug and virus to the trachea.

It is possible that during the early stages of infection only partial inhibition of virus replication had occurred and that virus could have escaped from the respiratory tract to spread to locations where 4-guanidino-Neu5Ac2en was present at too low a concentration to be effective. The dose of drug chosen in these experiments was higher than that shown to be effective in both mice and ferrets (von Itzstein et al., 1993), although the


A
Proportion Surviving
1
0.8
0.6
0.4
0.2
0
0 24 48 72 96 120 144 168 192 216 240
Time Post Infection (hrs)
Duck Ire
Duck Ire + 4-Guanidino-Neu5Ac2en
B
Proportion Surviving
1
0.8
0.6
0.4
0.2
0
0 24 48 72 96 120 144 168 192 216 240
Time Post Infection (hrs)
SD1
SD1 + 4-Guanidino-Neu5Ac2en
C
Proportion Surviving
1
0.8
0.6
0.4
0.2
0
0 24 48 72 96 120 144 168 192 216 240
Time Post Infection (hrs)
SD17
SD17 + 4-Guanidino-Neu5Ac2en

possibility that this dose of drug was inappropriate for chicks has not been examined. Another possibility for the failure to demonstrate a prophylactic effect of 4-guanidino-Neu5Ac2en, and for which there exists some evidence, is that the development of fowl plague is not dependent upon neuraminidase activity. Breuning and Scholtissek (1986) described a reassortant avian influenza virus with a *ts* lesion in the virus neuraminidase that resulted in failure of the neuraminidase to be correctly processed at 41°C and the production of virus with no neuraminidase activity. The mutant virus was, nevertheless, still highly pathogenic for chicks. Although in some cases it is possible to delay the onset of pyrexia and death but not to eliminate it, our failure to demonstrate a protective effect of 4-guanidino-Neu5Ac2en, is consistent with any of the above possibilities.

The potential for HPAI control by antivirus agents remains, but the results presented here show that control through the action of 4-guanidino-Neu5Ac2en directed against the neuraminidase is ineffective as tested in the experiments carried out. Extrapolation from antiviral effects demonstrated in vitro to activity in vivo is dependent on appropriate and compatible choice of drug and disease models, since factors such as distribution of both drug and virus within the animal may be important. Antiviral drugs which act at local sites are probably unsuitable for virus infections in which virus is disseminated from the site of initial infection and is able therefore to replicate elsewhere in an animal (as is the case for HPAI virus, but not, for example, in influenza virus infection in man or the ferret). In the experiments that we have described here, it is likely that our inability to demonstrate an effective prophylactic response against fowl plague by a sialidase inhibitor which is effective in vitro is an example of this limitation.

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Fig. 2. The influence of 4-guanidino-Neu5Ac2en treatment on the survival of 6-week-old chicks following infection with avian influenza viruses. The surviving proportion of chicks treated as described in Fig. 1 is plotted against time in days. A: chicks infected with A/Duck/Ireland/83 (H5N8). B: infection with reassortant virus SD1 (H7N7). C: infection with reassortant virus SD17 (H7N1).

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