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Short Communication

Inhibition of the growth of influenza viruses in vitro by 4-guanidino-2,4-dideoxy-*N*-acetylneuraminic acid

G.P. Thomas[a,*], M. Forsyth[a], C.R. Penn[b], J.W. McCauley[a]

[a] *AFRC Institute for Animal Health, Pirbright Laboratory, Ash Road, Woking, Surrey GU24 ONF, UK*

[b] *Department of Virology, Glaxo Group Research Limited, Greenford Road, Greenford, Middlesex UB6 OHE, UK.*

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Abstract

The sialidase inhibitor 4-guanidino-2,4-dideoxy-2,3-dehydro-*N*-acetylneuraminic acid was tested for growth inhibitory effects against a panel of avian influenza A viruses encompassing all nine neuraminidase subtypes. Growth in tissue culture of viruses from each subtype was inhibited by this compound at concentrations within a range previously found effective against human N1 and N2 viruses. This compound may prove a selective agent for the treatment (and prevention) of influenza virus infections.

Key words: 4-Guanidino-Neu5Ac2en; Influenza; Sialidase; Neuraminidase

Influenza A and B viruses possess two major surface glycoproteins, the haemagglutinin (HA) and the neuraminidase (NA), both of which interact with terminal sialic acid (SA) residues and adjacent sugar residues of glycoconjugates. The interaction of HA with SA-containing receptors is a prerequisite for infection (Wiley and Skehel, 1987), but the role of NA has yet to be clearly described. It is an essential gene (Liu and Air, 1993), is subject to immunological pressures (Webster et al., 1982) and some suggested roles include the release of virions from the surface of infected cells, the prevention of aggregation of virions, the facilitation of movement through mucus in the respiratory tract, as well as potentiation of pathogenicity through removal of carbohydrate from the HA (Palese and Compans, 1976; Col-

*Corresponding author. Fax: +44 (483) 232448.

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man and Ward, 1985; Burnet, 1948; Schulman and Palese, 1977; Nakajima and Sugiura 1979).

There are nine immunologically distinct NA subtypes in influenza A virus of which only two (N1 and N2) have appeared in humans: all nine occur in avian A strains (Webster et al., 1992). There is one serotype in influenza B viruses. Pandemic Influenza A virus strains are believed to arise through reassortment between currently circulating and previously unseen viruses, resulting in the presentation of new surface glycoproteins to which the population is immunologically naive and therefore susceptible ("antigenic shift": Woods et al. (1993); Webster et al. 1982). The reservoir for influenza virus genes is widely held to be the avian population, with transmission to the human population via ducks and pigs (Schafer et al., 1993; Sharp et al., 1993).

Analogues of sialic acid have previously been shown to be effective inhibitors of sialidase function (Meindl and Tuppy, 1969; Meindl et al., 1971; Palese and Compans, 1976), and recent improvements in drug design led to the development of 4-guanidino-2,4-dehydro-*N*-acetylneuraminic acid (4-guanidino-Neu5Ac2en) which was effective against human N1 and N2 viruses at concentration orders of magnitude lower than previous inhibitors (von Itzstein et al., 1993).

Given the potential introduction of another NA subtype into the human population through antigenic shift, we sought to determine the sensitivity to this compound of avian viruses with each of the NA subtypes. We performed plaque assays of viruses representative of each of the nine subtypes in the presence of 4-guanidino-Neu5Ac2en over the concentration range found effective against human N1 and N2 viruses, scoring reduction in both plaque number and plaque size, as well as inhibition of virus yield during growth in monolayer cultures. Two human N2 viruses were included for comparison with the results of Woods et al. (1993), in which this compound was assayed side by side several standard neuraminidase inhibitors. The results are presented in Table 1.

With the exception of the N1 and N7 strains we tested, plaque formation by viruses bearing each of the nine NA subtypes was inhibited at concentrations within the range found to be effective against a number of human N1 and N2 influenza A and influenza B viruses (von Itzstein et al., 1993; Woods et al., 1993). In our determinations, A/Aichi/1/68 had an IC_{50} under 1 nM, whereas Woods et al. (1993) reported a value of 14 nM for this strain. This difference may result from different passage histories of the virus, the cell culture conditions employed, or from the construction of semi-log plots, since Woods et al. (1993) recorded equal or greater differences for a given strain of different passage history. All nine avian subtypes displayed significant reduction in plaque size within this same range. In many cases this was accompanied by decreased plaque definition (not shown), as previously reported for other neuraminidase inhibitors (Palese and Compans, 1976). When inhibition of virus yield in single-double cycle growth experiments was measured, each virus exhibited an IC_{50} in this same range, with the exception of the N3 virus.

4-Guanidino-Neu5Ac2en was derived from previous NA inhibitors (Neu5Ac2en, DANA, FANA: Meindl and Tuppy, 1969; Meindl et al., 1971) based on a knowledge of rigidly conserved residues at and about the active sites of the influenza A N2

Table 1
Growth inhibition of avian influenza viruses by 4-guanidino-Neu5Ac2en

		Plaque number[a]			Plaque size[b]			Virus yield[c]		
Subtype	Virus strain	IC50[d]	IC90	N[e]	IC50	IC90	N	IC50	IC90	N
(H7)N1	SD17[f]	≥ 1 μM	≥ 1 μM	3	220 ± 60 nM	≥ 1 μM	3	20 nM	≥ 1 μM	2
(H3)N2	X31 (human)	0.2 ± 0.12 nM	≥ 1 μM	3	–[g]	–[g]		3.5 nM	9 nM	2
(H3)N2	A/Aichi/1168 (human)	≤ 1 nM	7.5 ± 1 nM	3	–[g]	–[g]		60 nM	600 nM	2
(H7)N2	A/Ty/Eng/192-329/79	11 ± 2 nM	90 ± 10 nM	3	25 ± 12 nM	550 ± 10 nM	3	80 nM	≥ 1 μM	2
(H7)N3	A/FPV/Eng/1/63	5.5 ± 3.5 nM	95 ± 10 nM	3	0.7 ± 0.6 nM	≥ 1 μM	3	≥ 1 μM	≥ 1 μM	2
(H8)N4	A/Ty/Ont/6118/68	5 ± 2 nM	55 ± 20 nM	3	7 ± 3.5 nM	55 ± 10 nM	3	2.6 nM	9.5 nM	2
(H12)N5	A/Dk/Alb/60/76	3.5 ± 1.3 nM	42 ± 15 nM	3	11.3 ± 5 nM	60 ± 9 nM	3	10 nM	750 nM	2
(H11)N6	A/Dk/Eng/56	2.3 ± 0.1 nM	85 ± 2 nM	3	5 ± 2.6 nM	55 ± 5 nM	3	–[h]	–[h]	2
(H7)N7	SD1[f]	≥ 1 μM	≥ 1 μM	3	160 ± 20 nM	≥ 1 μM	3	150 nM	≥ 1 μM	2
(H5)N8	A/Dk/Ire/83	≤ 1 nM	1.3 ± 1 nM	3	1.3 ± 0.5 nM	65 ± 1 nM	3	3.3 nM	8.3 nM	2
(H11)N9	A/Dk/Mem/546/74	5 ± 2 nM	50 ± 7.5 nM	3	5.2 ± 1 nM	45 ± 5 nM	3	6.5 nM	80 nM	2

[a]Egg-grown viruses were diluted to give 30–60 plaques (uninhibited) on 3.5 cm MDCK monolayers in the presence of 0, 1 nM, 10 nM, 100 nM or 1 μM 4-guanidino-Neu5Ac2en. The drug was included in both inoculum and overlay. After 48–72 h incubation in DMEM, 0.35% bovine serum albumin, 2 μg/ml trypsin and 1% agar, the dishes were stained with 0.2% toludine blue in formal saline. Triplicate assays were performed at least three times. Plaques were defined as areas of the monolayer through which the cell sheet was destroyed to the level of the substrate plastic.

[b]Using a calibrated loupe at ×8 original magnification (permitting discrimination to 0.2 mm), plaque diameters were measured for the plaque number reduction assays reported.

[c]MDCK cultures were infected at approx. 1 pfu/cell. Medium containing 4-guanidino-Neu5Ac2en at suitable concentration was added and cells incubated for 3 h. The medium was removed, cell sheets rinsed three times with PBS, and fresh medium with inhibitor added. Supernatants were recovered after 20 h, and titrated on MDCK monolayers in the absence of drug.

[d]IC_{50} and IC_{90} values were derived by linear extrapolation on semi-logarithmic plots of percentage inhibition (relative to uninhibited controls) vs. log inhibitor concentration.

[e]N represents the number of replicate assays performed for the three assays.

[f]SD17 and SD1 are reassortants between A/FPV/Rostock/34 (H7N1) and A/FPV/Dutch/27 (H7N7) (McCauley and Penn, 1990).

[g]For these viruses, plaque definition was too degraded to permit measurement.

[h]Yields of A/Dk/Eng/56 were extremely low under these conditions.

and N9 and influenza B neuraminidases (Varghese et al., 1983; Varghese and Colman, 1991; Tulip et al., 1991; Burmeister et al., 1992). The introduction of a guanidino group at carbon position 4 of the inhibitor affords additional interactions with residues forming a previously unoccupied pocket at the active site, resulting in increased selectivity since this pocket is unique to influenza virus NA amongst known sialidase structures (Crennell et al., 1993). We would predict, on the basis of their inhibition by 4-guanidino-Neu5Ac2en, that the neuraminidase subtypes yet to be crystallised (or sequenced in their entirety) will prove to have similar structures, including the pocket into which this sialidase inhibitor binds.

Since neuraminidase is not essential for infection (Liu and Air, 1993), and antibody-mediated inhibition does not prevent virus entry (Kilbourne et al., 1968), inhibition of virus growth by this compound must take place during subsequent rounds of infection. Most of the viruses tested here exhibited comparable sensitivity to 4-guanidino-Neu5Ac2en in each of the three assays: some strains did not. It is not immediately apparent why for a given virus such differences are observed in, albeit, indirect assays of neuraminidase activity. Reduction in plaque size is perhaps easiest to rationalize: progeny virions from an infectious centre have to be released from both one another and from the cell. If retained on remnant cell surfaces by virtue of HA-binding to SA-containing receptors, inhibition of NA function will restrict the number of virions free to infect neighbouring cells. Similarly, reduction of virus yield is reasonably explained if progeny particles are retained on receptors which otherwise would have been destroyed through the action of neuraminidase. Reduction in plaque number is less easily explained unless the virus enzyme is particularly sensitive to the inhibitor such that plaque growth is so severely restricted that a plaque is not formed because virus cannot spread from cell to cell. Woods et al. (1993) also found differences in sensitivity of viruses in plaque number and yield reduction assays, but direct assays of enzyme activity yielded more uniform IC_{50}s for the human N1 and N2 viruses they assessed. It is therefore possible that the relative importance of HA and NA in creation of plaques and release from cells may vary between viruses. In this context, we note that the most refractory strains in this study – SD17 (N1), A/FPV/Eng/1/63 (N3) and SD1 (N7) – each has an H7 HA with multiple basic residues at the cleavage site and which plaque in the absence of trypsin in MDCK cells. The inclusion of trypsin (for consistency) may have modified the requirements for plaque formation by these viruses.

Plaque formation presumably results from competition between HA and NA for SA-containing glycoconjugates. There are 6–10 times as many HA trimers than NA tetramers, and their extensions from the virion membrane are different, so that SA-containing glycoconjugates are more likely accessed by HA than NA. Similarly SA-receptors on the virion glycoproteins themselves will be more or less accessible to HA than NA. Virion-virion attachment must be minimised to maximise virus-cell interaction and therefore infection. The distribution of HA and NA spikes on virions has been investigated by immunoelectron microscopy and both have relatively even distributions when detected with polyclonal sera (Murti and Webster, 1986; Amano et al., 1992). As there are more HA spikes than NA spikes, the most likely interaction of a virion with a cellular receptor is binding via HA as opposed to

hydrolysis by NA. If the distribution of virus glycoproteins on the surface of infected cells behaves according to the fluid mosaic model (Singer and Nicholson, 1972), we would anticipate a similar relative distribution resulting in preferential retention of virions. That this is not the case (in normal infections) suggests that NA activity takes precedence at the budding stage, consistent with the growth inhibition observed in the presence of 4-guanidino-Neu5Ac2en.

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