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Reduced sialidase activity of influenza A(H3N2) neuraminidase associated with positively charged amino acid substitutions

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Abstract

Neuraminidase (NA) inhibitors (NAI), oseltamivir and zanamivir, are the main antiviral medications for influenza and monitoring of susceptibility to these antivirals is routinely done by determining 50% inhibitory concentrations (IC_{50}) with MUNANA substrate. During 2010–2019, levels of A(H3N2) viruses presenting reduced NAI inhibition (RI) were low (~0.75%) but varied year-on-year. The highest proportions of viruses showing RI were observed during the 2013–2014, 2016–2017 and 2017–2018 Northern Hemisphere seasons. The majority of RI viruses were found to contain positively charged NA amino acid substitutions of N329K, K/S329R, S331R or S334R, being notably higher during the 2016–2017 season. Sialidase activity kinetics were determined for viruses of RI phenotype and contemporary wild-type (WT) viruses showing close genetic relatedness and displaying normal inhibition (NI). RI phenotypes resulted from reduced sialidase activity compared to relevant WT viruses. Those containing S329R or N329K or S331R showed markedly higher K_m for the substrate and K_i values for NAIs, while those with S334R showed smaller effects. Substitutions at N329 and S331 disrupt a glycosylation sequon (NDS), confirmed to be utilised by mass spectrometry. However, gain of positive charge at all three positions was the major factor influencing the kinetic effects, not loss of glycosylation. Because of the altered enzyme characteristics NAs carrying these substitutions cannot be assessed reliably for susceptibility to NAIs using standard MUNANA-based assays due to reductions in the affinity of the enzyme for its substrate and the concentration of the substrate usually used.

INTRODUCTION

Oseltamivir and zanamivir are the main antivirals used to treat and prevent influenza A and influenza B infections. They act by competitively inhibiting the virus neuraminidase (NA) and are commonly referred to as NA inhibitors (NAIs) [1–3]. Their action leads to progeny viruses aggregating and not being released from the infected-cell surface [4, 5]. However, viruses have arisen with amino acid substitutions in the NA that confer highly reduced inhibition (HRI; resistance) or reduced inhibition (RI) by these antivirals; NA H275Y substitution in A(H1N1) viruses and NA E119V/I or R292K substitutions in A(H3N2) viruses confer RI/HRI phenotypes (reviewed in [6]). To monitor emergence and circulation of viruses with RI/HRI phenotypes, newly isolated viruses are routinely screened for NAI susceptibility. Assays to determine 50% inhibitory concentration (IC_{50}) values with NAIs commonly use the fluorogenic substrate 2′-(4-Methylumbel liferyl)-α-D-N-acetylneuraminic acid (MUNANA) to assess the sialidase activity of NA [7]. For routine monitoring of antiviral resistance, IC_{50} values for viruses are compared to a reference IC_{50} value, e.g. a median IC_{50} of viruses of the same subtype or that of a virus known to be susceptible to the NAIs. Antiviral susceptibility of influenza A viruses is classified as normal inhibition (NI, inhibited by concentrations of drug within ten-fold of the median IC_{50}), RI (10–100 fold increase compared to the median IC_{50}) or HRI (≥100 fold increase

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Abbreviations: GISAID, global initiative on sharing all influenza data; HA, haemagglutinin; HRI, highly reduced inhibition; IC_{50}, 50% inhibitory concentration; LDAO, lauryldimethylamine N-oxide; MS, mass spectrometry; MUNANA, 2′-(4-Methylumbelliferyl)-α -D-N-acetylneuraminic acid; NA, neuraminidase; NAI, neuraminidase inhibitors; NGS, next-generation sequencing; NI, normal inhibition; Perth/16, A/Perth/16/2009; RI, reduced inhibition; WT, wild-type.

One supplementary table and four supplementary figures are available with the online version of this article.

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compared to the median IC_{50}), as defined by a World Health Organisation working group [8].

During the 2016–2017 influenza season an increased frequency of viruses that had insufficient sialidase titre for the assessment of susceptibility to NAIs was observed but these viruses appeared to replicate as well as others that could be assessed [9]. A preliminary examination indicated that low sialidase activity might be associated with amino acid substitutions at NA residues 329, 331 and 334. Some viruses carrying NA substitutions of N329K or S331R have been previously reported to be associated with RI by NAIs [10–12], during the 2013–2014, 2014–2015 and 2016–2017 influenza seasons, but at the lower end of the RI range [9]. Analysis of the sialidase activity of viruses with these substitutions, and appropriate controls, was carried out to assess association of the substitutions with low sialidase activity.

METHODS

Propagation of viruses

Influenza viruses were propagated in MDCK (pre-2010) or MDCK-SIAT1 cells [13] (post-2010) as described [14].

Determination of kinetic parameters: K_m, K_i and IC_{50}

Sialidase activity was determined by incubating virus in tissue culture supernatant at 37 °C with a range of MUNANA concentrations, up to 2600 µM, in 32.5 mM MES buffer pH 6.5 containing 4 mM $CaCl_2$ and measuring changes in fluorescence at 460 nm (excitation 365 nm) using a JASCO FP-6300 spectrofluorometer. K_m and V_{max} were determined by the Michaelis-Menten equation (non-linear regression) with K_m values ≥0.5-fold the maximum substrate concentration being reported as >1200 µM. For direct comparison of V_{max} values between NAs from different viruses, virus concentration was determined using ELISA for nucleoprotein (NP) as described [14] and enzyme rate (arbitrary fluorescence $units_{460\ nm}$ per min) was normalised to virus concentration. K_i values were determined by comparing the enzymatic rates in the absence and presence of a known concentration of drug: oseltamivir carboxylate (Roche, UK) or zanamivir (GlaxoSmithKline). K_i values were calculated using the equation $K_i=K_m[I]/(((V_0/V_i)-1)*(K_m+[S]))$ where [I]=inhibitor concentration, [S]=substrate concentration, and V_0 and V_i are rates measured in the absence and presence of inhibitor, respectively. IC_{50} values were determined as described previously [15]. All graphs were plotted using GraphPad Prism software.

Genetic characterisation and phylogenetic analysis of viruses

Next-Generation Sequencing (NGS) was performed on an Illumina MiSeq platform following RNA extraction, RT-PCR using an MBT-universal three primer approach [16] and Illumina Nextera XT (Cat nos. FC-131–1096, FC-131–1002) library preparation and indexing. Sequences were analysed using DNASTAR Lasergene version 15.3: reference guided assemblies were performed using SeqMan NGen, and consensus sequences from the assemblies were generated using SeqMan Pro. Sequences of Haemagglutinin (HA) and NA genes of study viruses were submitted to the EpiFlu™ database of the Global Initiative on Sharing All Influenza Data (GISAID). Phylogenetic analyses and comparisons with reference sequences were performed using RAxML [17], with the Annotator program [18] used to indicate nodes defined by specific amino acid substitutions, and trees were drawn using the GGtree package in R [19].

Protein preparation and mass spectrometry

A/Brisbane/10/2007 was propagated in MDCK cells, A/Ukraine/7460/2016 and A/Ukraine/7726/2017 were propagated in MDCK-SIAT1 cells. Tissue culture supernatant was harvested at 72 h post-infection, clarified by centrifugation and virus was partially purified by ultracentrifugation using a 30% sucrose/PBS cushion at 100000 ***g*** for 60 min. Virus glycoproteins were extracted from the virus membrane by ultracentrifugation with 1% Lauryldimethylamine N-oxide (LDAO)/PBS and NA was purified from the supernatant by affinity chromatography using an oseltamivir–biotin conjugate [20] immobilized on a streptavidin Sepharose column and elution buffer containing 500 µM oseltamivir carboxylate. Proteins from either the eluate from the oseltamivir column or partially purified whole virus preparations were separated by SDS-PAGE and visualised by Coomassie staining. NA bands were excised and reduced and alkylated as described [21]. N-glycans were removed by enzymatic digestion with PNGase F at a concentration of 150 Units ml^{-1} (Roche Life Sciences) in 20 mM sodium hydrogen carbonate pH 7.0 at 37 °C overnight. The solution was removed and the protein digested in-gel with either trypsin and elastase (A/Brisbane/10/2007) or trypsin and chymotrypsin (Ukraine viruses) at 2 µg ml^{-1} (Promega) at 37 °C overnight or for 3 h. HPLC and tandem MS (MS/MS) were performed as described [21].

RESULTS

Identification and gene sequencing of viruses showing RI phenotype in IC_{50} assays

Global NAI susceptibility profiles for seasonal influenza viruses are characterised on an annual basis [10–12, 22, 23]. From 2010 to 2019, we screened a total of 7039 seasonal A(H3N2) viruses for NA activity using the standard MUNANA assay; all but 195 viruses (~3%) yielded measurable activity. In some seasons increased proportions of A(H3N2) viruses with sialidase activity below the measurable limit of the standard IC_{50} assay were detected: approximately 3, 7.5 and 6.5% in 2016–2017, 2017–2018 and 2018–2019 influenza seasons, respectively, compared to <3% previously. Of the viruses with insufficient NA activity to permit IC_{50} assay, approximately 44, 38 and 2% in the 2016–2017, 2017–2018 and 2018–2019 influenza seasons, respectively, were associated with positively charged amino acid substitutions (N329K, K/S329R, S331R or S334R – referred to as variant viruses hereafter) in NA.

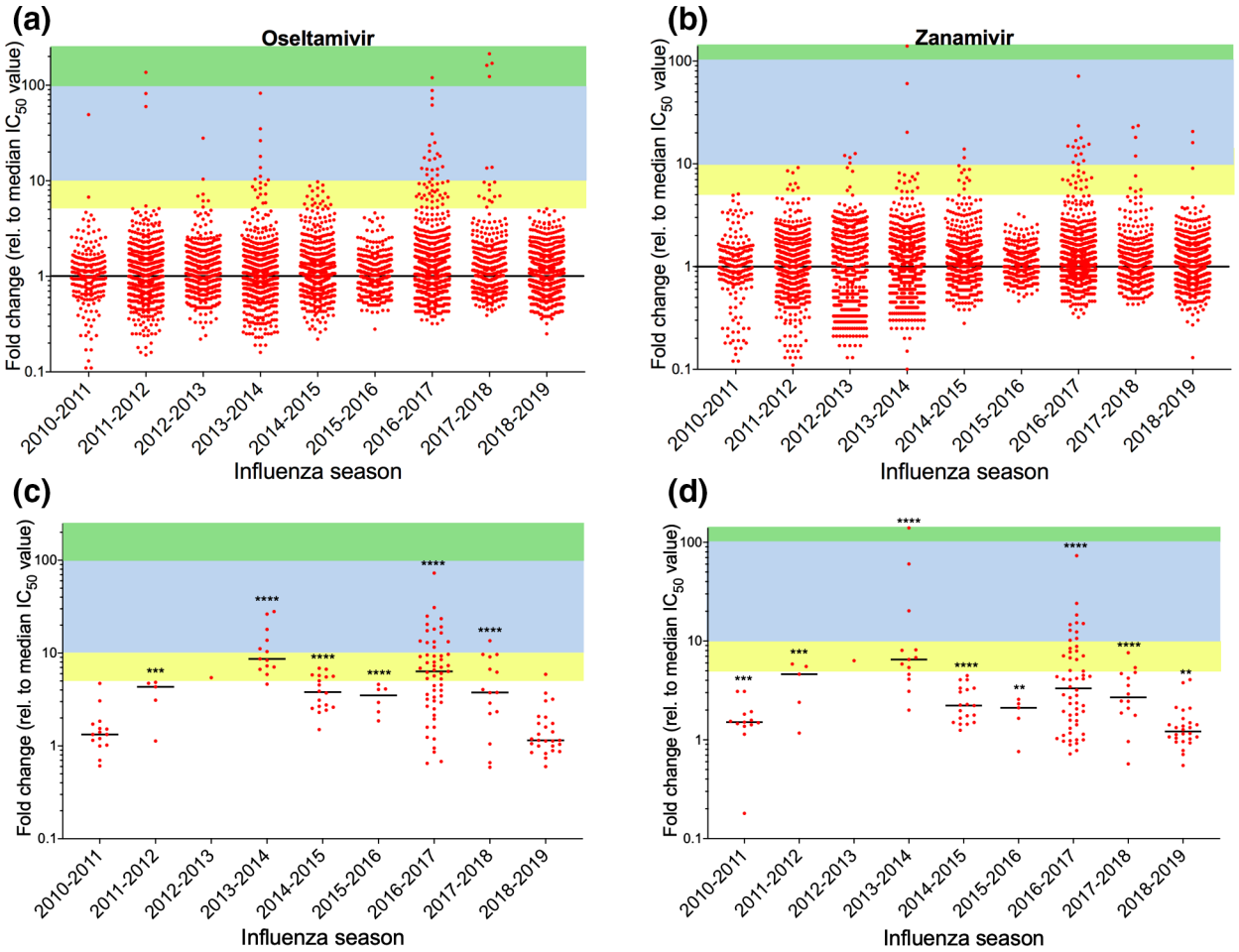

Fig. 1. NAI inhibition of seasonal A(H3N2) viruses during 2010–2019 using the MUNANA assay for routine susceptibility testing. IC_{50} values of viruses from each influenza season (sorted by collection date, October-September) using (a) oseltamivir and (b) zanamivir are plotted as fold increase over the median IC_{50} value of the respective year. Each data point represents antiviral inhibition of a virus isolate. The >5-fold, 10–100-fold (RI) and >100 fold (HRI) zones are shown in yellow, blue and green, respectively. (c) and (d) show a subset of viruses from each influenza season tested against oseltamivir and zanamivir, respectively, containing N329K, K/S329R, S331R or S334R substitutions in NA. Black lines indicate the median of each category. Mann-Whitney U test was used to determine statistical significance between the median of each season's subset (N329K, K/S329R, S331R and S334R containing viruses) versus the median of its respective influenza year. ***P*<0.01, ****P*<0.001; *****P*<0.0001.

In light of the low sialidase activity of some of these variant viruses and reports of RI in a proportion of them [10–12], we re-assessed inhibition of A(H3N2) variant viruses by oseltamivir and zanamivir. Fold changes in IC_{50} values of human A(H3N2) viruses compared to the median IC_{50} for each influenza season from 2010 to 2019 for oseltamivir and zanamivir are shown in Fig. 1(a, b), respectively. Variant viruses are shown as a subset in Fig. 1(c, d). Compared with the median IC_{50} values for each respective influenza season variant viruses can be seen to have significantly higher median IC_{50}s for at least six of the nine influenza seasons, although only a minority showed RI or HRI. These increased medians for variant viruses were most pronounced in each influenza season from 2013 to 2018, with the highest proportion (relating to NA S331R substitution) being detected in the 2016–2017 influenza season. Therefore, variant viruses show altered susceptibility to the NAIs with many having low sialidase activity. Of 45 viruses with RI phenotypes (viruses showing RI with either oseltamivir or zanamivir or both), all collected during the 2013–2014, 2016–2017 or 2017–2018 influenza seasons, approximately 70% were variant viruses bearing NAs with positively charged substitutions of N329K, K/S329R, S331R or S334R.

A total of 6347 A(H3N2) viruses with collection dates from October 2010-September 2019 were sequenced by us and Fig. 2 shows phylogeny of the NA genes of the 207 (3.3%) variant viruses. A/Perth/16/2009 (Perth/16) is a common ancestor virus, specific amino acid substitutions at positions 329, 331 and 334, respectively, are marked as coloured branches on the tree, and NAI susceptibility

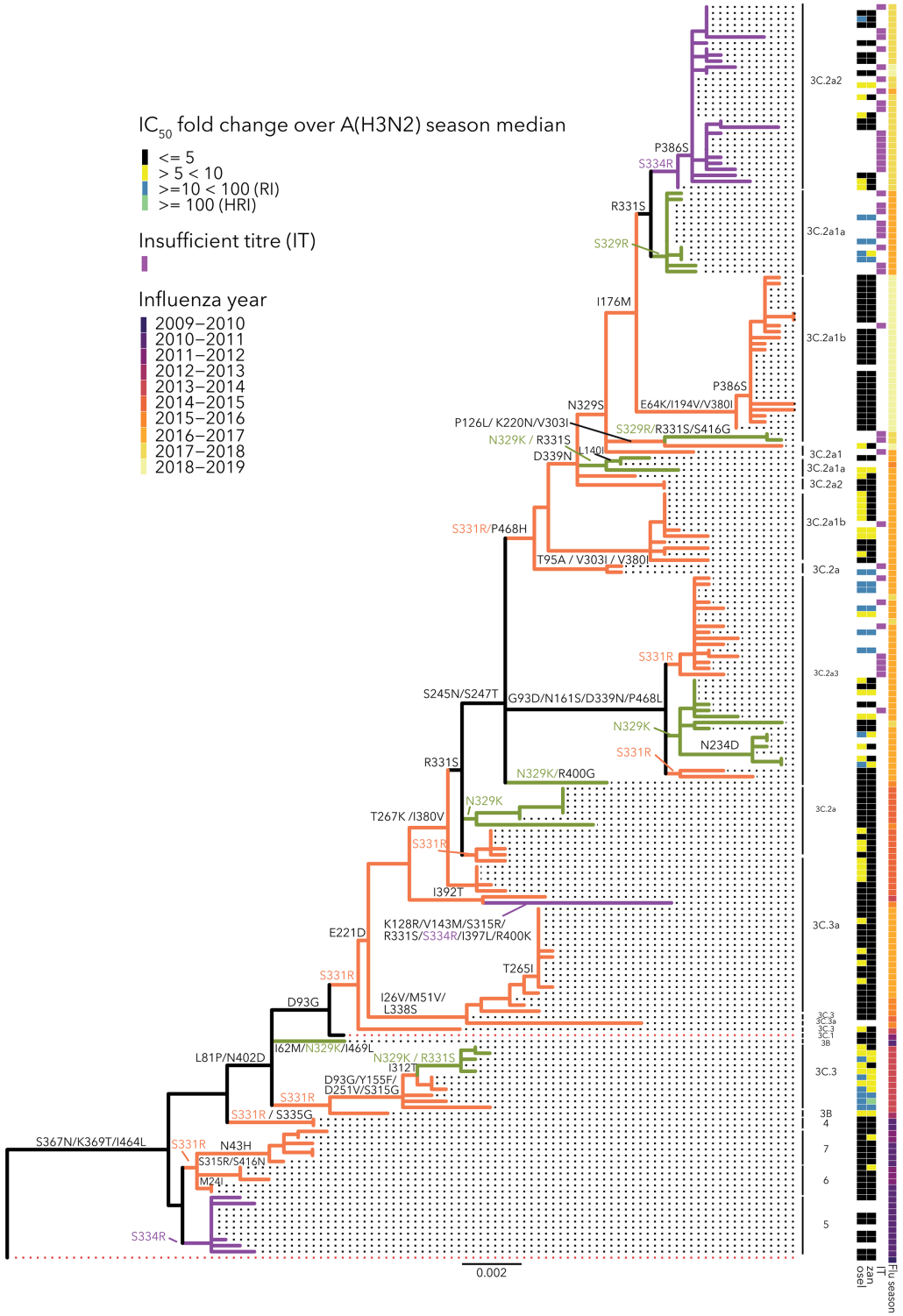

Fig. 2. Phylogenetic comparison of viruses encoding positively charged amino acid substitutions N329K, S329R, S331R and S334R in NA. NA gene phylogeny with annotations relating to H3 HA clades and subclades. Two previous vaccine viruses, A/Perth/16/2009 and A/Texas/50/2012, respectively are indicated by red dotted lines, included as references. Virus group defining amino acid substitutions are shown at nodes which are also coloured to indicate key substitutions in the text: N329K/S329R (green), S331R (orange) and S334R (purple). NAI (oseltamivir and zanamivir) and susceptibility profiles for each virus (as fold-change compared with the seasonal median value) are indicated by coloured bars. 'IT' = insufficient NA activity titre to perform standard IC_{50} assays with MUNANA. There are some viruses for which NA activity data were unavailable. Respective influenza seasons are also indicated by coloured bars.

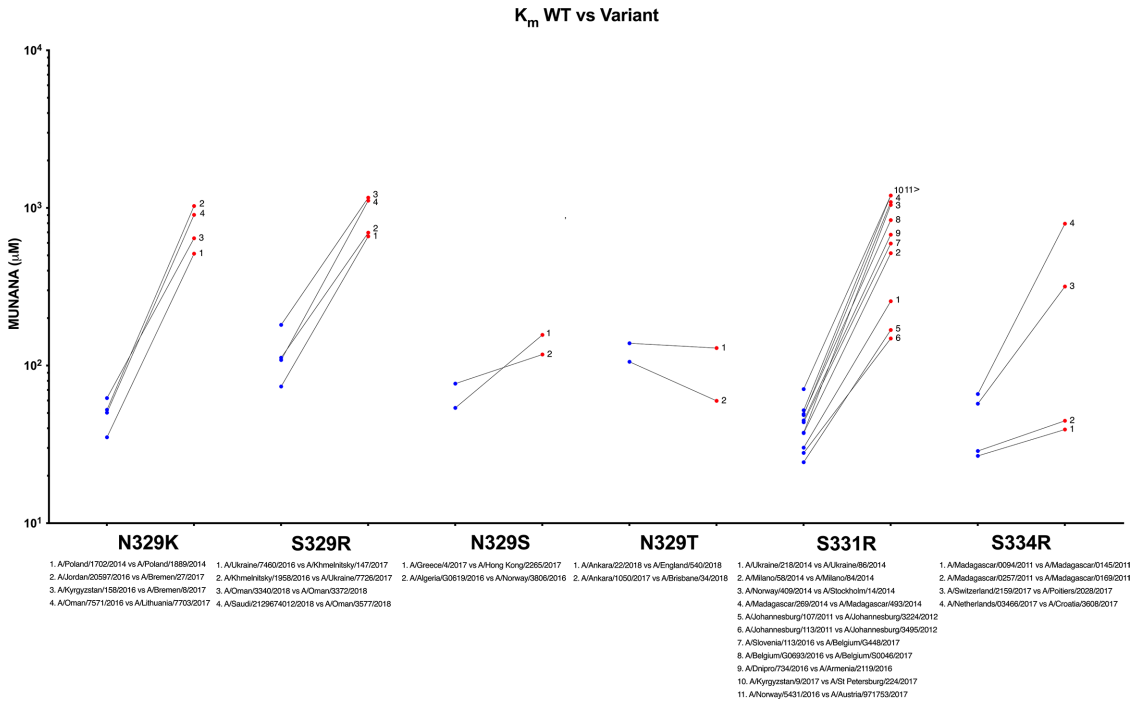

Fig. 3. K_m of viruses containing N329K, S329R, N329S, N329T, S331R and S334R substitutions using MUNANA as substrate. Enzymatic activity of NAs was determined by measuring the rate of change in fluorescence on cleavage of MUNANA. K_m was determined by the Michaelis-Menten equation. Mean K_m values ($n \geq 2$) of WT (blue filled circle) or their respective Variant viruses (red filled circle) are plotted as paired observations. Numbers denote virus pairs for comparison listed below the relevant substitution. '>' Indicates a value greater than, only a lower estimate could determined using the assay.

profiles of the viruses are indicated by a colour bar (fold-change in IC_{50} values relative to the median IC_{50} of the respective influenza season for each virus). The season in which viruses were collected and the corresponding clade of the HA gene for each virus is also shown. Full trees for HA and NA genes, including virus names, are shown in Fig. S1(a, b) (available in the online version of this article) respectively. Variant viruses carried HA genes in clade 3C.3 and all contemporary extant subclades of clade 3C.2a, with the variant viruses being detected mainly in geographically widespread cases, but with some evidence of clusters seen in Madagascar (2011), South Africa (2011–12) and Ukraine (2016–17) (Fig. S1a).

Enzyme kinetics of variant viruses

A genetically diverse subset of the variant viruses which either had insufficient NA activity for IC_{50} determination or displayed a RI phenotype, were selected and subjected to enzyme kinetics analyses using MUNANA as substrate. For each virus in this subset a relevant genetically similar WT virus-specific control, with a NI phenotype and bearing N/S329, S331 or S334 in NA, was selected. Since the substitutions N329K and S331R result in the loss of a potential NA glycosylation site viruses with either an N329S or N329T substitution, disrupting the glycosylation site without introduction of a positively charged amino acid, were also included, giving a total of 27 variant/WT virus pairs. Fig. S2 shows a NA phylogenetic tree for these variant/WT virus pairs and a set of reference viruses, with HA clades indicated. WT and variant viruses were incubated with increasing concentrations of MUNANA and reaction rates measured as change in fluorescence to allow calculation of K_m values. WT and variant virus K_m values are shown in Table S1 and plotted as paired observations (Fig. 3). Of the 23 variant/WT virus pairs involving introduction of a positively charged amino acid at residues 329, 331 or 334, 21 variant viruses showed marked increases in K_m for MUNANA, while virus pairs involving N329S/T substitution showed virtually no difference in K_m. In contrast, viruses with NA N329K or S329R or S331R substitutions showed 10–20-, 5–10- and 7–29-fold higher K_m compared to their respective WT viruses. Variant viruses with NA S334R substitutions showed two patterns: variant viruses in two pairs from Madagascar, collected in 2011, showed only 1.5-fold increases in K_m while those in two virus pairs collected in 2017 (A/Poitiers/2028/2017 and A/Croatia/3608/2017) showed 5.5-fold and 12-fold increases in K_m respectively compared to their WT controls.

Virus inhibition by oseltamivir and zanamivir was measured by comparing the sialidase rates in the absence and presence of a known concentration of drug and calculating K_i values as described in Methods. In this way, K_i assays can also be used to determine antiviral inhibition for isolates

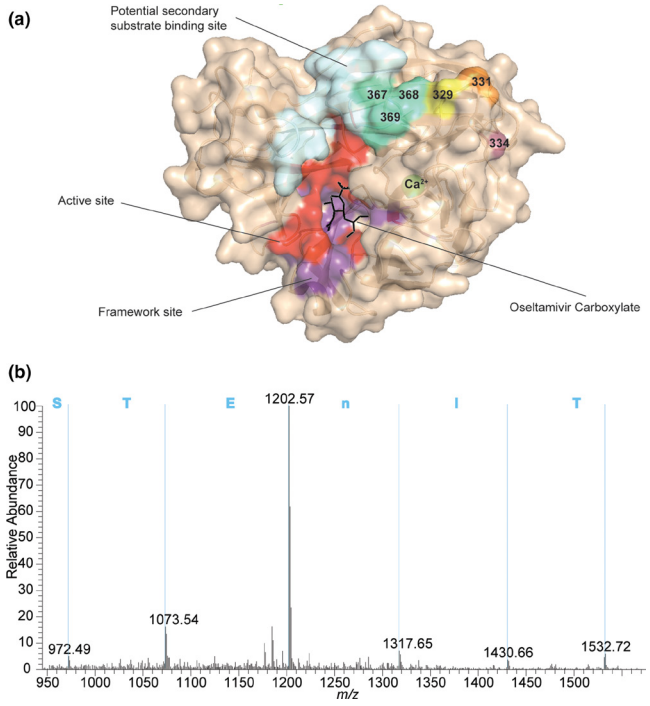

Fig. 4. Structure of N2 NA and representative mass spectra indicative of N-linked glycosylation at $N_{367}ET$. (a) Structure of the N2 NA of A/Tanzania/205/2010 in complex with oseltamivir carboxylate (black) [28], PDB ID: 4gzp. Coloured residues 329 (yellow), 331 (orange) and 334 (pink), potential secondary binding site residues (cyan) containing the 367–369 glycosylation sequon (dark cyan), and calcium ion (green). Active site residues (red) and framework residues (purple) are defined in Yen *et al.*, 2006 [34]. (b) MS/MS spectra for A/Ukraine/7726/2017 NA corresponding to the peptide M362GRTInETSRLGYETF identified at 1% FDR. For simplicity, consecutive y ion series are highlighted. 'n'=deamidated asparagine.

which show insufficient titre to be tested under routine NAI susceptibility monitoring IC_{50} assay conditions. For competitive inhibitors of an enzyme, K_m, K_i and IC_{50} values are all linked by the equation $K_i = IC_{50}/(1 + [S_0]/K_m)$ where $[S_0]$ represents the MUNANA concentration (μM). K_i values for the assays performed are represented in Fig. S3 and shown in Table S1. The K_i measurements for both NAIs show a similar pattern, as expected, to the K_m measurements. Again, variant virus/WT pairs with the substitutions N329S/T showed low or no difference in K_i for NAIs, while those with N329K or S329R showed 6–35- and 8–69-fold difference compared to their WT counterparts for oseltamivir and zanamvir, respectively. Similarly, variant viruses with S331R also showed increased K_i values: 7–29- and 3–71-fold higher K_i values compared to their WT counterparts for oseltamivir and zanamvir, respectively. For variant viruses containing S334R the two from Madagascar, collected in 2011, showed only small differences in K_i for NAIs, while those from 2017 show marked increases in K_i (up to 22-fold higher), compared to their respective WT viruses.

V_{max} values for a subset of variant viruses were also calculated. This was done by normalising enzyme rate (in arbitrary $fluorescence_{460nm}$ units $time^{-1}$) to virus concentration determined by ELISA for influenza NP. Michaelis-Menten plots are shown in Fig. S4. Unlike the significantly elevated K_m values obtained for NAs of variant viruses compared to their respective WT controls, the V_{max} value estimates varied somewhat: equal estimates of the V_{max} were observed for one pair (A/Ukraine/7460/2015 versus A/Khmenitsky/147/2017), and were only as much as three-fold higher for A/Slovenia/113/2016 versus A/Belgium/G448/2017, and A/Madagascar/0094/2011 versus A/Madagascar/0169/2011, despite much larger differences in K_m, up to 25-fold higher.

Mass spectrometry analysis of N-linked glycosylation of NA of A(H3N2) viruses

As N329 forms part of a glycosylation sequon in N2 NA (highlighted in Fig. 4a) mass spectrometry (MS) was used to determine whether the site was utilised in representatives of the study viruses, using methods described briefly in the Methods section and previously [21]. As a positive control NA from A/Brisbane/10/2007, a high yield egg-propagated prototype virus (from a period when ~98% of viruses contained N329), was used. Two different proteases were used for NA digestion to ensure assignment of the same site in multiple peptides, thereby providing confirmation of deamidation detection at the same site. Background deamidation, determined by the ratio of asparagine deamidation to amidation when asparagine was not in a glycosylation motif, was very low (~1%). Good quality MS/MS spectra were obtained at a 0–3% false discovery rate (FDR). Gene sequencing identified eight potential N-linked glycosylation sites on A/Brisbane/10/2007 NA, of which seven showed deamidation (and thus glycosylation) rates of 100% on sequons $N_{61}IT$, $N_{70}TT$, $N_{86}WS$, $N_{146}DT$, $N_{200}AT$, $N_{234}GT$ and $N_{329}DS$, while $N_{402}RS$ showed a rate of 35%. Thus, all N-linked glycosylation sites were shown to be used and, importantly for this study, 100% glycosylation of the $N_{329}DS$ glycosylation sequon was confirmed.

All 27 virus pairs analysed in this study (Fig. S2) possess a NA $N_{367}ET$ glycosylation sequon and all viruses with collection dates after 2012 onwards have lost the glycosylation sequon at residues 402–404. NA $N_{367}ET$ is associated with a potential secondary binding site for sialic acid, as was $N_{402}RS$ [24–27] (highlighted in Fig. 4a: structure [28]). It has been reported that: (i) mutation affecting the secondary binding site is associated with reduced NA activity [29]; and (ii) charged amino acids, like those studied here in the proximity of the $N_{367}ET$ sequon, can influence glycosylation [30–33]. Hence, we investigated the effects of S329R substitution on glycosylation at the $N_{367}ET$ site. NA containing 329R (A/Ukraine/7726/2017) or 329S as a control (A/Ukraine/7460/2016) was purified and digested with chymotrypsin to generate recoverable peptides encompassing residue 367: good quality MS/MS spectra (1% FDR) were obtained for the control NA while for 329R NA most spectra were scored at 5% FDR, and only one at 1% FDR (Fig. 4b). Both NAs showed 100% deamidation of N367, indicating no difference in glycan occupancy at this

site between variant and WT viruses, showing that S329R substitution has no effect on glycosylation occupancy at site N_{367}. Notably, among the 27 virus pairs analysed (Fig. S2), three viruses detected in Madagascar and Johannesburg in 2011 gained a glycosylation sequon, N_{245}AT, as did all of those with collection dates after 2014. All eight glycosylation sites in the two viruses from Ukraine (N_{61}IT, N_{70}TT, N_{86}WS, N_{146}NT, N_{200}AT, N_{234}GT, N_{245}AT and N_{367}ET) showed deamidation (and thus glycosylation) rates of close to 100%.

DISCUSSION

This study of a set of variant viruses with either insufficient NA activity to allow IC_{50} determination for NAIs or a RI phenotype showed such viruses to have increased K_m estimates of the NAs for the MUNANA substrate and altered K_i estimates with oseltamivir and/or zanamivir, compared to WT controls. For competitive inhibitors K_m, K_i and IC_{50} are linked, consequently a high K_m can result in an elevated IC_{50} and viruses being assigned RI phenotypes. In contrast to the effects on NA, K_m for variant viruses studied here E119V substitution in the framework site of N2 NA, associated with antiviral resistance [34], resulted in no difference in K_m compared to the control (Table S1), while showing an average 256-fold and 152-fold increase in IC_{50} and K_i, respectively, with oseltamivir, and 2.7-fold and 2.5-fold increases in IC_{50} and K_i, respectively, with zanamivir. While the reasons behind human A(H3N2) variant viruses studied here having greatly elevated NA K_m estimates are not clear, it must be noted that the IC_{50} values determined with MUNANA are still less than those for influenza B viruses displaying a NI phenotype with oseltamivir carboxylate [35] and so we expect that antiviral treatment of cases infected with examples of these variant viruses will remain effective.

The concentrations of MUNANA in routine assays for NA activity are 60 µM [15] or 150 µM [36] which is far below the K_m estimates of many NAs with either N329K, K/S329R, S331R or S334R substitutions. Therefore, enzyme activity measured in these assays with such variant viruses would be much lower than the V_{max}, explaining the apparent lack of NA activity, preventing assessment of NAI susceptibility, despite variant viruses having infectious titres similar to WT NI viruses (data not shown). Moreover, where NA activity is used to verify propagation of viruses that do not agglutinate guinea pig red blood cells (RBCs) then variant viruses like those studied here may score as false negatives if the assay is performed with 60 µM or 150 µM MUNANA. If assays were performed with ~1.5 mM MUNANA substrate, then viruses carrying such NA substitutions would have sufficient sialidase activity to be assessed.

Amino acid substitutions which alter NA phenotype may be a consequence of changes in virus HA as the balance of HA receptor-binding and NA activity has been shown to be important for virus replication/fitness [37–39]. It has previously been shown that recently circulating A(H3N2) viruses have low avidity for α2,6-sialyllactosamine [14]. Since 2014, A(H3N2) viruses of HA clades 3C.2a and 3C.3a have emerged [9], with the large majority of clade 3C.2a viruses showing markedly altered receptor-binding properties and being unable to agglutinate mammalian RBCs, associated with the acquisition of a glycosylation site in antigenic site B of the HA [40, 41]. The HAs of viruses studied here (HA clades are indicated in Fig. S1) have poor receptor-binding potential. Therefore, the NA of recent A(H3N2) viruses may not need to be as efficient as previously, with fewer sialic acid residues needing to be removed from cellular receptors and virus glycoproteins to allow efficient release of viruses from infected cell surfaces. Consequently, virus replication would become less dependent on the sialidase activity of the virus. Indeed, it has been reported that some A(H3N2) viruses with deficient NAs can be rescued and propagated in tissue culture cells [42].

In this context, NA K_m values of WT (control) viruses showed a gradual increase over the years, up to three-fold for viruses isolated in 2017–2018 compared with to those isolated in 2011; the K_m of these viruses in turn are approximately one- to two-fold higher than the K_m for viruses collected in 1968 (Table S1). The V_{max} estimates for WT viruses show some increase from 1968 for most viruses, with the variant viruses showing somewhat higher V_{max} estimates in most cases (Fig. S4).

It is unclear why positively charged amino acid substitutions at NA positions 329, 331 and 334 alter NA kinetic properties. NA substitutions N329K, K/S329R, N329S, N329T and S331R result in the loss of a glycosylation sequon (N_{329}DS). Substitutions N329S and N329T did not significantly alter NA enzymatic properties, while substitutions N329K, K/S329R and S331R had marked effects on these properties. Hence, loss of glycosylation at N329 *per se* is unlikely to be a major factor influencing NA kinetics associated with the positively charged substitutions. NA residues 329, 331 and 334 are not located near the catalytic site, identified by the presence of oseltamivir carboxylate in the structure (Fig. 4a), so are unlikely to directly affect enzymatic activity. However, they are close to the proposed secondary sialic acid binding site [24–27]. The glycosylation sequon N_{367}ET is associated with the proposed secondary binding site but no difference in glycosylation efficiency was observed between variant S329R and WT S329 NAs. The triple serine SxxSxS loop, residues 367 to 372, and W403 which are highly conserved in avian NAs have been shown to be important for interaction with sialic acid and haemadsorption binding by NA [27, 43, 44]. Only one (S370) of the triple serine loop residues is conserved in human influenza N1 and N2 NAs which show reduced haemadsorption and cleavage of multivalent substrates [25, 45]. Therefore, it seems unlikely that the secondary binding site was utilised by the viruses in our study.

Residues 329, 331 and 334 are not close to the surfaces that form interfaces between subunits of the NA so substitutions at these positions would not be expected to affect inter-subunit interactions. Conversely, they are near the calcium cation present in the tight binding site located close to the catalytic site [46] so could potentially affect Ca^{2+} binding. However, previous studies have reported that this calcium ion affects V_{max} but not K_m [47].

While NA N329K/R, S331R and S334R had marked effects on K_m, there was variation in the extent of these effects (compared

to WT NAs) on kinetic parameters in different NA backbone sequences as observed for viruses carrying NA S331R or S334R substitutions (Table S1). This is consistent with the observation that WT viruses from 1968 containing 331R showed low K_m values (Fig. S4). Therefore, the precise context of residues, such as those focused on in this study, might lead to different phenotypes. It has, for example, been shown in former seasonal H1N1 viruses, that additional substitutions in N1 NA were required for accommodation of the oseltamivir resistance-conferring H275Y substitution [48].

A question arises as to what induced the emergence of variant viruses with the NA amino acid substitutions studied here. Immune selection is a possibility. Supportive of this view are observations that NA substitutions D329N and N334S have been identified in monoclonal antibody (mAb)-escape variants of a reassortant H1N2 virus [49] and N329D (N2 numbering) has been identified in mAb-escape variants carrying N9 NA [44]. Furthermore, N1 NA K329E substitution antigenically distinguished A/New/Caledonia/20/1999 and A/Solomon Islands/3/2006 from the later A/Brisbane/59/2007 vaccine virus [50]. Therefore, although the NA substitutions N329K, K/S329R, S331R or S334R can result in reduced sialidase activity, they may confer immune evasion. However, the low prevalence of seasonal A(H3N2) viruses with NA substitutions N329K, K/S329R, S331R or S334R in recent years, suggests either low level immune selection or selection against such variant viruses in order to maintain the balance between HA and NA activities.

In summary, this study shows that positively charged amino acid substitutions in NA, which are not associated with active or framework sites of the enzyme, may have substantial effects on the kinetic properties of the enzyme. It is important to recognise that such viruses cannot be assessed reliably for susceptibility to NAIs using standard MUNANA-based assays due to the differences in the affinity of the enzyme for the substrate. Surveillance laboratories could consider using a higher MUNANA concentration in assays for monitoring isolation and NAI susceptibility testing of such viruses (we have used up to 2600 µM MUNANA). Nevertheless, we submit that, the concentration of drug in the human body under standard treatment regimens is likely to remain effective against such variant viruses.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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