Antiviral Research 134 (2016) 144–152

Contents lists available at ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

Phenotypic and genotypic analysis of influenza viruses isolated from adult subjects during a phase II study of intravenous zanamivir in hospitalised subjects

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ARTICLE INFO

Article history:
Received 25 January 2016
Received in revised form
21 August 2016
Accepted 24 August 2016
Available online 26 August 2016

Keywords:
Influenza
Intravenous zanamivir
Antiviral susceptibility

ABSTRACT

Intravenous zanamivir (IVZ) is a neuraminidase (NA) inhibitor (NAI) under investigation for the treatment of subjects hospitalised with influenza. The study included 130 symptomatic, hospitalised adults with influenza. Subjects received IVZ for 5–10 days.

Viruses were cultured and analysed for susceptibility to zanamivir. Mean IC_{50}s (n = 50) (±SD) for influenza A/H1N1pdm09, A/H3N2 and influenza B were 0.20 ± 0.06, 0.26 ± 0.07 and 1.61 ± 0.35 nM, respectively, and are comparable to data observed for sensitive isolates.

A total of 185 NA and 180 haemagglutinin (HA) sequences were obtained from 123 subjects; the majority did not contain resistance substitutions. Four influenza A/H1N1pdm09 viruses from four subjects harboured NA resistance substitutions: three, Y155H, D199G and S247N, were present at Day 1 before IVZ exposure and the fourth, E119D/E, was detected at Post Treatment +5 Days but was not present at 5 other timepoints. Five subjects harboured virus with treatment-emergent NA substitutions not associated with resistance; N63D, V83A, W190C, M269K (A/H1N1pdm09) and R210K (A/H3N2). Viruses from fifteen subjects harboured HA resistance substitutions, (A/H1N1pdm09) one emerged during treatment: S162N (Day 5). Five viruses harboured treatment-emergent HA substitutions (A/H1N1pdm09) not associated with resistance: E81K, V108L, S164D, D168N and S185N. 10/92 subjects with A/H1N1pdm09 harboured a D222 HA substitution, which has been associated with increased virulence. The emergent substitutions are not associated with resistance but may have arisen due to selection pressure during IVZ treatment or by chance.

In this study, there was evidence for resistance selection in a post treatment sample but the resistant variant did not persist in later visit samples.

1. Introduction

Influenza infection causes substantial morbidity and mortality during epidemics and pandemics. Antivirals play an important role in the treatment of severely ill patients infected with influenza. Currently there are two classes of approved antivirals; adamantanes and neuraminidase (NA) inhibitors (NAI). There is wide spread resistance to adamantanes, therefore treatment with this class of drugs is not recommended (Dong et al., 2015). Currently four NAIs are approved for the prevention and treatment of influenza infection; oseltamivir, zanamivir, laninamivir (licensed in Japan only) and peramivir (licensed in China, Japan and South Korea).

Resistance is a key factor that can affect the efficacy of NAIs (Li et al., 2015). Resistance to zanamivir is rare and has never been detected in viruses isolated from immunocompetent individuals treated with zanamivir. However, there have been five reported cases of viruses with reduced susceptibility to zanamivir isolated from immunocompromised patients; a resistant influenza B virus with amino acid substitutions in the haemagglutinin (HA) (T198I)

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http://dx.doi.org/10.1016/j.antiviral.2016.08.023
0166-3542/

and the NA (R152K) (Gubareva et al., 1998), four cases of influenza A/H1N1pdm09 viruses with NA substitutions, three with a I223R and one with an E119G that conferred reduced susceptibility to zanamivir (Nguyen et al., 2010; Rousset et al., 2010; van der Vries et al., 2010; Tamura et al., 2015).

All of the approved NAIs are licensed for treatment of acute uncomplicated influenza infection based on prospective controlled studies. However, there remains an unmet medical need for treatment of severely ill patients. Intravenous zanamivir (IVZ) is a NAI suitable for treatment of hospitalised patients with influenza and is currently being assessed for safety and efficacy in adults and children in phase II and III studies. Safety and antiviral efficacy of IVZ in the adult cohort of the phase II study has been reported previously (Marty et al., 2013; Peppercorn et al., 2013). Here, we report the phenotypic and genotypic analyses of viruses isolated from adult subjects in that phase II study for their susceptibility to zanamivir.

2. Materials and methods

2.1. Study details

The study design has been previously described in detail (Marty et al., 2013). Briefly, this international open label, multicentre, single arm, phase II study (Clinical Trials registration NCT01014988; GSK NAI113678) was initiated at the onset of the 2009 influenza A H1N1 pandemic and included symptomatic, hospitalised subjects with laboratory-confirmed influenza. One hundred and thirty patients received intravenous zanamivir (median, 5 days; range, 1–11) a median of 4.5 days (range, 1–7) after onset of influenza; 83% required intensive care, 80% received oseltamivir before study entry (median exposure, 2 days). Subjects did not receive oseltamivir after study start. Subjects received IVZ (600 mg twice daily adjusted for renal impairment) for 5 days with the option to extend treatment for up to an additional 5 days at the investigator's discretion. The adult cohort was completed in September 2011.

The study was conducted in accordance with all applicable regulatory requirements, including the principles of the Declaration of Helsinki. Before commencement of the study, all relevant study documentation was reviewed and approved by an ethics committee/institutional review board. Informed consent was obtained prior to any study procedures.

2.2. Viruses and cell cultures

Viruses were isolated from flocked throat swabs (Copan Flock Technologies) taken on Day 1 (prior to treatment), during treatment (Days 3 and 5) and post treatment (PT) timepoints (PT+2, +5, +9, +16 and + 23 Days) depending on hospitalisation status and symptomatology. If treatment was extended beyond Day 5, throat swabs were also collected on Days 7 and 10. Endotracheal (ET) samples were optional and collected between Days 3 and 5 of treatment. Viruses were propagated in the absence of zanamivir in Madin-Darby canine kidney (MDCK) cells by standard techniques (World Health Organisation, 2011).

2.3. NA activity inhibition assay

Susceptibility to zanamivir was carried out on MDCK cell culture supernatants by Quest Diagnostics using a NA Star influenza neuraminidase inhibitor resistance detection kit as described by the manufacturer (Applied Biosystems). Fold changes in the 50% inhibitory concentration (IC_{50}) were determined by comparing sample IC_{50}s with IC_{50}s of reference strains. Phenoype data was interpreted using the criteria recommended by the WHO; normal inhibition (<10-fold increase in IC_{50} over reference); reduced inhibition (10–100-fold increase in IC_{50} over reference); highly reduced inhibition (≥100-fold increase in IC_{50} over reference) (World Health Organisation, 2012).

2.4. Virus gene population sequence analysis

The NA and HA genes were sequenced directly from the clinical specimen and in cultured viruses that had resistance associated substitutions by Quest diagnostics and Viroclinics Biosciences. Briefly, viral RNA was extracted from throat swabs and the NA and HA genes amplified by either one round of reverse transcription-PCR (Quest diagnostics) or a nested PCR (Viroclinics Biosciences) using gene-specific primers. The resultant PCR products were sequenced using gene-specific primers. Nucleotide sequences were aligned and translated. Amino acid substitutions were shown in relation to a reference sequence (A/California/7/2009; A/Perth/16/2009; B/Wisconsin/1/2010) and compared to previously published resistance substitutions identified in the NA and HA (Nguyen et al., 2012; Tisdale, 2009). As part of the analysis process, QC filtering and trimming of the data was implemented to remove sequence anomalies. The respective subtype numbering is used throughout.

2.5. Nucleotide sequence accession numbers

The GenBank accession numbers of the NA and HA nucleotide sequences from all viruses analysed in this study are KX651620 to KX651980.

2.6. Minority species analysis

Minority species analysis was carried out in order to detect low levels of NA resistance substitutions that may be selected during treatment. Resistance is not generally selected prior to 4 days of NAI treatment (Aoki et al., 2007; Valinotto et al., 2010), therefore the NA gene in the last visit sample from each subject with a detectable viral load at or beyond Day 4 and virologic non-responders (any subject whose viral load has dropped from Baseline to Day 5 < 1.5 log copies/ml by quantitative RT-PCR) were analysed by Next Generation Sequencing (NGS). If resistance mutations were detected then the Day 1 sample and an additional During or Post treatment sample from the same subject were also analysed.

PCR samples were quantified with Quant-iT PicoGreen dsDNA Assay Kit or Qubit dsDNA HS Assay Kit (Invitrogen) and the quality checked on a 2100 Bioanalyzer High Sensitivity DNA Chip (Agilent). Samples consisting of a pool of four overlapping NA amplicons were used for library construction using the Beckman Coulter SPRIworks System I for Illumina Genome Analyzer. Libraries were sequenced using the Illumina MiSeq Reagent Kits v2 500 cycles for 250 bps paired end reads. The resultant sequences were then aligned to the reference NA-coding sequence (A/California/7/2009; A/Perth/16/2009; B/Wisconsin/1/2010) using the Bowtie 2 application (Version 2.0.0-beta7) (Langmead and Salzberg, 2012) with the “very fast local” alignment option. The resultant sequences were then aligned to the reference H1N1 NA-coding sequence A/California/07/2009 using the Bowtie 2 application (Version 2.0.0-beta7) (Langmead and Salzberg, 2012) with the “very fast local” alignment option. In order to identify clinically relevant resistance substitutions, only substitutions above a threshold of 5% of minority species were included in the analysis.

3. Results

3.1. Study results

Adult enrolment completed in September 2011, with 130 subjects from 8 countries. Ninety two (71%) were infected with A/H1N1pdm09, sixteen (12%) A/H3N2, fourteen (11%) untyped influenza A, three (2%) influenza B and five (4%) an unknown subtype (positive for influenza by the local site laboratory but negative during study).

3.2. Susceptibility analysis

All samples were subjected to qualitative virus culture and those positive for influenza virus were analysed by phenotypic assay. A total of 50/611 samples from 39/129 subjects were culture positive; 37/128 Day 1 throat samples, 11/289 during treatment throat samples, 0/168 PT samples and 2/26 during treatment ET samples (Table 1). Matched Day 1 and during treatment or post treatment samples were obtained from 9/121 subjects. Mean IC_{50}s (±SD) for influenza all A/H1N1pdm09, A/H3N2 and influenza B were 0.20 ± 0.06, 0.26 ± 0.07 and 1.61 ± 0.35 nM, respectively. One A/H1N1pdm09 virus had a slightly raised IC_{50} (0.44 nM) (Table 2).

3.3. NA sequence analysis

A total of 185/513 NA sequences, 174 from throat and 11 from ET samples (153 influenza A/H1N1pdm09 (13 partial sequences), 30 A/H3N2 and 2 influenza B), from 123 subjects were obtained (Table 3). The number of NA sequences included 77/126 Day 1 samples, 86/250 during treatment and 22/137 post treatment.

The majority of the viruses (97.8%) analysed did not contain known resistance substitutions or substitutions in the NA active site (Nguyen et al., 2012). Four influenza A/H1N1pdm09 viruses from four subjects harboured NA resistance substitutions; Y155H (Day 1), D199G (Day 1), S247N (Day 1) and E119D/E (PT + 5 Days) (Table 4, N1 Numbering) (Monto et al., 2006; Ghedin et al., 2011; Hurt et al., 2011; L'Huillier et al., 2015). The Y155H and S247N substitutions were also present in the cultured virus that was used in the phenotype assays and only the S247N virus showed a fold shift in susceptibility to zanamivir (2.6).

Five subjects harboured treatment emergent NA amino acid substitutions in their final time-point sample that have not previously been associated with resistance (Table 5): N63D (Subject 19, Day 5), V83A (Subject 23, Day 10), W190C (Subject 9, PT+16), M269K (Subject 30, Day 3) (A/H1N1pdm09, N1 numbering), and R210K (Subject 3, PT+9, A/H3N2, N2 numbering).

Two further subjects developed substitutions in the NA not present at other time-points before or after their appearance and were not associated with resistance: S364N (Subject 4, PT+5, H1N1pdm09, N1 numbering) and S181Q (Subject 3, PT+2, H3N2, N2 numbering).

3.4. HA sequence analysis

A total of 180 HA sequences, 169 from throat and 11 from ET samples (148 A/H1N1pdm09 (27 partial sequences), 30 A/H3N2 and 2 influenza B), were obtained from 496 swabs/ET samples from 123 subjects. The majority of the viruses analysed did not contain resistance substitutions. Fifteen subjects harboured virus with resistance associated HA amino acid substitutions at one or more visit, the majority of which were present at Day 1 (Table 6). One virus (A/H1N1pdm09) harboured an emergent resistance substitution at a single timepoint: S162N (Subject 10, Day 5; H1 numbering) (Blick et al., 1998). The substitution was not present at other timepoints. An additional subject, 31, had a S162N amino acid substitution at Day 3 (A/H1N1pdm09) and in the ET sample, but a Day 1 sequence was not available.

Subject 34 harboured a number of different resistance amino acid substitutions at Day 3, Day 5 and PT+2 but not Day 1, PT+5 and PT+9. These substitutions were probably a sequence anomaly.

Five HA amino acid substitutions, not previously associated with resistance detected in influenza A/H1N1pdm09 viruses from four subjects emerged during treatment: E81K (Subject 9, PT+16), V108L (Subject 36, PT+2), S164D (Subject 6, ET), D168N (Subject 9, ET) and S185N (Subject 8, PT+5) (Table 7, H1 numbering). In addition, Subjects 5 and 18 had K160T (PT+2) and F111S (Day 3) substitutions that were not present at either earlier or later timepoints.

Five out of 92 A/H1N1pdm09 subjects (5%) harboured an HA amino acid substitution D222G in one or more virus samples (Table 8, H1 numbering), which has been associated with increased virulence of A/H1N1pdm09 viruses (Chan et al., 2011).

3.5. Minority species analysis

NGS analysis was carried out on 20 Day 1 samples and 61 During treatment or PT samples from 41 subjects and the results are shown

Table 1
Numbers of samples cultured and analysed by phenotypic analysis.

Subtype	No. Subjects analysed/Total subjects	Number subjects with matched cultures [b]	Number of samples phenotyped/number of samples cultured											
			During treatment throat swabs					During treatment ET samples	Post treatment throat swabs					Total no. of on treatment or PT zanamivir samples analysed/Total no. samples	Total no. samples analysed/Total no. samples
			Day 1	Day 3	Day 5	Day 7	Day 10	Day 3–5	PT +2	PT +5	PT +9	PT +16	PT +23		
A/H1N1p[a]	32/92	8/85	30/91[c]	7/85	0/76[d]	0/23	2/19	2/23	0/34	0/28	0/28	0/24	0/25	11/226	41/456
A/H3N2	5/16	1/15	5/15	1/16	1/12	0/2	0/3	0/1	0/3	0/1	0/1	0	0	2/34	7/54
B	2/3	0/3	2/3	0/2	0/3	0	0	0	0/1	0	0	0	0	0/5	2/9
Unknown	0/18	0/18	0/19	0/18	0/17	0/7	0/6	0/2	0/6	0/5	0/4	0/3	0/5	0/50	0/92
Total	39/129	9/121	37/128	8/121	1/108	0/32	2/28	2/26	0/44	0/34	0/33	0/27	0/30	13/315	50/611

[a] A/H1N1p, influenza A/H1N1pdm09.
[b] Consists of a Day 1 and a post/during treatment sample.
[c] Includes 2 samples not in listings and 1 sample that was indeterminate.
[d] Includes an early withdrawal sample on Day 4.

Table 2
Zanamivir IC_{50} results for the different influenza virus subtypes isolated by cell culture.

Subtype	n	IC_{50} ± SD [nM] (range)			Fold change [a]		
		Day 1 (n = 29)	Post day 1 (n = 10)	All samples (n = 40)	Day 1	Post day 1	All samples
A/H1N1pdm09	31	0.21 ± 0.07 (0.14–0.44)	0.18 ± 0.04 (0.15–0.27)	0.20 ± 0.06 (0.14–0.44)	1.12 ± 0.4	0.96 ± 0.2	1.07 ± 0.4
A/H3N2	7	0.27 ± 0.08 (0.17–0.0.33)	0.23 ± 0.01 (0.22–0.24)	0.26 ± 0.07 (0.17–0.0.34)	0.19 ± 0.06	0.19 ± 0.01	0.19 ± 0.05
B	2	1.61 ± 0.35 (1.36–1.86)	NA	1.61 ± 0.35 (1.36–1.86)	0.29 ± 0.07	NA	0.29 ± 0.07

NB. One A/H1N1pdm09 virus had a slightly raised IC_{50} (0.44 nM, Fold-change 2.6).
NA, not applicable.
Two determinations for each sample were performed.
IC_{50} of reference virus, A/H1N1pdm09 0.19 nM, A/H3N2 1.35 nM, B 5.5 nM.
[a] Fold change compared to IC_{50} of reference virus.

Table 3
Numbers of samples analysed by NA genotypic analysis.

Subtype	No. Subjects analysed/Total subjects	Number subjects with matched swabs [a]	During treatment throat swabs (No samples sequenced/total analysed)					Post treatment throat swabs (No samples sequenced/total analysed)					During treatment ET samples	Total samples sequenced/Total samples analysed
			Day 1	Day 3	Day 5	Day 7	Day 10	PT +2	PT +5	PT +9	PT +16	PT +23	Day 3–5	
A/H1N1pdm09	92/92	82	65/90	31/77	19/72[b]	3/22	6/18	8/32	7/27	2/27	1/23	1/22	10/20	153/430
A/H3N2	10/10	9	10/15	8/16	7/12	0/2	1/3	2/3	0/1	1/1	0	0	1/1	30/54
Influenza A	14/14	0	0/13	0/1	0/1	0/1	0/1	0	0	0	0	0	0	0/17
Influenza B	2/3	0	2/3	0/1	0/2	0	0	0/1	0	0	0	0	0	2/7
Not typed	5/5	0	0/5	0	0	0	0	0	0	0	0	0	0	0/5
Total	**123/124**[c]	**91**	**77/126**	**39/95**	**26/87**	**3/25**	**7/22**	**10/36**	**7/28**	**3/28**	**1/23**	**1/22**	**11/21**	**185/513**

[a] Includes a Day 1 sample and a during/post treatment sample.
[b] Includes one Withdrawal sample taken on Day 4.
[c] Six H3N2 subjects were not sequenced.

Table 4
Summary of detected A/H1N1pdm09 NA resistance amino acid substitutions.

Subject no.	Visit	Viral load [b] (Log copies/mL)	Zanamivir IC_{50} (nM)[d]	Fold change in IC_{50}[a]	Prior oseltamivir treatment (Days)	Underlying illness	Clinical outcome	Amino acid substitution (From throat swab) (N1 numbering)
14	Day 1	5.18	NC		1	None	Non-fatal	D199G, N248D
	Day 10	4.95	NC					V106I, N248D
20	Day 1	3.7	0.15	1.1	0	Morbid obesity	Non-fatal	C49R, V106I, **Y155H**, N248D, D416N[c]
32	Day 1	4.28	0.44	2.6	0	None	Non-fatal	Y100F, S101L, V106I, F121I, C124R, F132L, **S247N**, N248D, I396M[d]
34	Day 1	5.44	0.19	1.1	2	Asthma, HIV	Fatal	V81A, V106I, V241I, N248D, N369K
	Day 3	5.25	NC					V81A, V106I, V241I, N248D, N369K
	Day 5	6.59	NC					W61W/R, V81A, V106I, V241I,S248D,N369K
	PT+2	4.26	NC					W61W/R, V81A, V106I, V241I, N248D, N369K
	PT+5	<2.7	NC					V81A, V106I, **E119D/E**, V241I, N248D,N369K
	PT+9	<2.7	NC					V81A, V106I, V241I, N248D, N369K

NC, not cultured.
Known resistance associated amino acid substitutions in **bold.**
[a] Fold change compared to IC_{50} of reference virus, IC_{50} of reference virus, A/H1N1pdm09 0.19 nM.
[b] Viral load in NP swab by qPCR, lower limit of detection = 2.7 $\log_{10}$ copies/mL, one determination performed.
[c] Also sequence of cultured virus.
[d] Two determinations performed.

in Supplementary Table 1. Amino acid summaries of all samples from subjects infected with a virus harbouring resistance substitutions identified by NGS above a threshold level of 5% are shown in Table 9. Eight resistance mutations were detected in seven viruses from six subjects: E119K (Subject 9, PT+5), E119D (two subjects, Subject 9, PT+16 and Subject 34, PT+5), L134S (Subject 14, Day 7), D199N (Subject 38, Day 3), S247I (Subject 34, PT+5), E277K (Subject 37, Day 5) and D294G (Subject 39, Day 1). None of the mutations were detected in other timepoints from the same subject. Only one of the mutations detected by NGS was also detected by population sequencing (E119D in Subject 34).

4. Discussion

The results shown here are from an open label single arm phase II study and the efficacy results are difficult to interpret without a comparator arm. However, a Phase III study has recently completed that compares IVZ with oral oseltamivir that may provide additional insight. Previous studies with orally inhaled zanamivir have shown that emergence of resistance is rare. The present study demonstrated that in viruses detected from subjects treated with IVZ there was no clear evidence for the emergence of resistance during treatment.

4.1. Susceptibility analysis

Many of the samples were of low viral load and could not therefore be cultured, resulting in a low number of IC_{50} values. Results from susceptibility analysis showed that the mean IC_{50}s for the influenza A/H1N1pdm09, A/H3N2 and influenza B viruses were

Table 5
Summary of AH1N1pdm09 and A/H3N2 treatment emergent NA amino acid substitutions not previously associated with resistance.

Subject no.	Visit	Subtype	Viral load [a] (Log copies/mL)	IC_{50} (nM)	Prior oseltamivir treatment (Days)	Underlying illness	Clinical outcome	Amino acid substitutions (From throat swab) (Subtype numbering)
4	Day 1	A/H1N1pdm09	5.04	NC	0	None	Fatal	V106I, V241I, N248D, N369K
	Day3	A/H1N1pdm09	4.22	NC				V106I, V241I, N248D, N369K
	Day 10	A/H1N1pdm09	3.29	NC				V106I, V241I, N248D, N369K
	PT+5	A/H1N1pdm09	<2.7	NC				V106I, V241I, N248D, S364N, N369K
	PT+9	A/H1N1pdm09	<2.7	NC				V106I, V241I, N248D, N369K
9	Day 1	A/H1N1pdm09	6.67	0.23	1	None	Non-fatal	V106I, N248D, V264I, N397K
	Day3	A/H1N1pdm09	5.03	NC				V106I, N248D, V264I, N397K
	Day 5	A/H1N1pdm09	4.2	NC				V106I, N248D, V264I, N397K
	PT+16[c]	A/H1N1pdm09	4.61	NC				V106I, **W190C,** N248D, V264I, N397K
	ET[c]	A/H1N1pdm09	8.08	0.27				V106I, N248D, V264I
19	Day 1	A/H1N1pdm09	6.11	NC	2	Diabetes Mellitus	Non-fatal	V106I, N248D, N386S, I436V
	Day 5	A/H1N1pdm09	3.96	NC				**N63D**, V106I, N248D, N386S, I436V
23	Day 1	A/H1N1pdm09	6.05	NC	2	None	Non-fatal	Q43K, V106I, N248D, T332K
	Day 5	A/H1N1pdm09	5.38	NC				Q43K, V106I, N248D, T332K
	Day 10	A/H1N1pdm09	3.94	NC				Q43K, **V83A**, V106I, N248D, T332K
30	Day 1	A/H1N1pdm09	7.72	NC	2	Cancer	Non-fatal	V106I, N248D
	Day 3[b]	A/H1N1pdm09	5.78	NC				N248D, **M269K**
3	Day 1	A/H3N2	6.14	NC	2	Diabetes Mellitus, Cirrhosis	Fatal	**V143M**, S367N, K369T, R400K, I464L
	Day 3	A/H3N2	5.54	NC				S367N, K369T, R400K, I464L
	Day 5	A/H3N2	5.51	NC				S367N, K369T, R400K, I464L
	PT+2	A/H3N2	2.86	NC				**S181Q,** S367N, K369T, R400K
	PT+9	A/H3N2	<2.7	NC				**R210K**, S367N, K369T, R400K, I464L

NC, not cultured.
Amino acid substitution in **bold** are selected or deselected during treatment.
[a] Viral load in NP swab by qPCR, lower limit of detection = 2.7 log_{10} copies/mL.
[b] Region coding V106I not available for Day 3 virus.
[c] Partial sequence.

comparable to data observed previously for sensitive isolates, 0.3 nM, 1.82 nM and 2.28 nM respectively (Leang et al., 2013; McKimm-Breschkin et al., 2003).

4.2. NA sequence analysis

The samples with resistant mutations were generally of low viral load making it difficult to ascertain the effect the substitutions had on susceptibility. Only one virus, from Subject 32, showed a shift in susceptibility to zanamivir of 2.6 fold that was caused by the known NA resistance substitution S247N but was within the normal susceptibility range in the WHO criteria for evaluating phenotype (Hurt et al., 2011; World Health Organisation, 2012). The S247N substitution was present at Day 1, (no other timepoints were available for analysis) and was not therefore selected by IVZ and did not arise as a result of oseltamivir pressure as the subject did not receive oseltamivir prior to entry in the study. The substitution did not appear to have a detrimental effect on viral clearance as the subject had no detectable virus in nasopharyngeal (NP) swabs at three time-points that were analysed by quantitative PCR (qPCR) on Day 1, Day 2 and Day 4.

Two resistance substitutions, Y155H and D199G, were present in Day 1 viruses from Subjects 20 and 14 respectively, and were not therefore selected by IVZ. In addition, Subjects 20 and 14 received only 2 and 1 days, of prior oseltamivir treatment, respectively, so it is unlikely that the amino acid substitutions were selected by oseltamivir during this time. A previous study has shown that the Y155H substitution gives rise to highly reduced inhibition to zanamivir but is dependent on the background in which it is presented (Monto et al., 2006). This is in line with the finding in this study as the Y155H substitution did not confer resistance to zanamivir as the cultured virus did not show a shift in susceptibility (Table 4). The D199G amino acid substitution is present in the Day 1 virus of subject 14 but not in the Day 10 virus, the only other timepoint available. Subject 14 received one day of oseltamivir treatment prior to the start of the study and it is unlikely this would have contributed to the selection of D199G. It is not known if the D199G, identified in this study, conferred resistance to NAIs as it was unable to be cultured.

A fourth resistance amino acid substitution, E119D/E (A/H1N1pdm09, N1 numbering), was identified in a PT+5 virus from Subject 34 and was not present at 5 other timepoints, before and after PT+5, from the same subject. Further analysis by NGS showed that the E119D/E amino acid substitution was found to be present in 71% of the virus population in PT+5 but was undetectable at Day 1, Day 3 and PT+9. Different variants at position 119 have been detected following oseltamivir treatment, in circulating viruses and in *in vitro* studies (Okomo-Adhiambo et al., 2010; Sheu et al., 2010). Recombinant viruses with the E119D substitution have been shown to confer shifts in susceptibility to zanamivir in A/H3N2 and influenza B viruses of 32 and 560 respectively (Zurcher et al., 2006; Jackson et al., 2005). The virus possessing the E119D/E from this study could not be cultured, as was the case in a similar study (Tamura et al., 2015). A virus harbouring the E119D was isolated from an immunocompromised child, was cultured and showed fold shifts in susceptibility of 825 and 25 to zanamivir and oseltamivir respectively (L'Huillier et al., 2015).

It is noteworthy that none of the viruses sequenced in this study harboured the Q136K, I223R or H275Y amino acid substitutions (N1 numbering). The H275Y substitution was not found in any virus analysed, despite 80% of subjects having had prior oseltamivir treatment; however, this may not be surprising since median duration of prior treatment with oseltamivir was 2 days (range 1–12) (Marty et al., 2013), and oseltamivir resistance does not generally arise until at least Day 4 of treatment (Aoki et al., 2007). The conditions that are required for selection of the I223R substitution are not fully understood. In the three reported cases to date, all of the patients were immunocompromised and were treated initially with oseltamivir followed by treatment with zanamivir; two were treated with IVZ and the third with inhaled zanamivir

Table 6
Summary of detected HA resistance amino acid substitutions.

Subject no.	Visit	Subtype	Viral load [a] (Log copies/mL)	Clinical outcome	Amino acid substitution (From throat swab) (Subtype numbering)
7 [b]	Day 1[c]	A/H1N1pdm09	3.1	Non-fatal	A134T, A141S, S183P, S203T, I295V,I321V, V479I
10	Day 1	A/H1N1pdm09	3.89	Non-fatal	P83S, D97N, S203T, R205K, I216V, V249L, I321V
	Day 3	A/H1N1pdm09	3.14		P83S, D97N, S203T, R205K, I216V, V249L, I321V
	Day 5	A/H1N1pdm09	4.62		P83S, D97N, **S162N**, S203T, R205K, I216V, V249L, I321V
11 [b]	Day 1	A/H1N1pdm09	5.44	Non-fatal	D35N, P83S, **S162N**, S185T, **A197T**, S203T, I321V
	Day 5	A/H1N1pdm09	NA		D35N, P83S, S185T, **A197T**, S203T,I321V
17 [b]	Day 1	A/H1N1pdm09	<2.7	Non-fatal	N31D, P83S, **S162N**, A186T, S203T,V234I, V272I, I321V
21 [b]	Day 1	A/H1N1pdm09	3.62	Non-fatal	P83S, **A134T**, S183P, S203T, I321V
22 [b]	Day 1	A/H1N1pdm09	6.74	Non-fatal	P83S, **A134T**, S183P, S203T, I321V
24 [b]	Day 1	A/H1N1pdm09	7.32	Non-fatal	P83S, S185T, **A197T**, S203T, I321V
	Day 3	A/H1N1pdm09	5.10		P83S, S185T, **A197T**, S203T, V250A/V, F255F/L, A256A/P, 261A/V, I321V
25 [b]	Day 1	A/H1N1pdm09	6.21	Non-fatal	E81K, P83S, S143G, S185T, **A197T**,S203T, I321V
	Day 3	A/H1N1pdm09	5.98		E81K, P83S, S143G, S185T, **A197T**,S203T, I321V
	Day 5	A/H1N1pdm09	2.77		E81K, P83S, S143G, S185T, **A197T**,S203T, I321V
26 [b]	Day 1	A/H1N1pdm09	6.84	Fatal	P83S, S143G, S185T, **A197T**, S203T,I321V
28	Day 1	A/H1N1pdm09	4.05	Non-fatal	P83S, S143G, P159Q, S162T, S185T,**A197T**, S203T, I321V
29 [b]	Day 1	A/H1N1pdm09	3.31	Non-fatal	P83S, **A134T**, S183P, S203T, K302R,I321V
31	Day 3	A/H1N1pdm09	6.15	Fatal	N31D, P83S, **S162N**, S164S/A, A186T,S203T, A256T, V272I, I321V
	ET	A/H1N1pdm09	5.2		N31D, P83S, **S162N**, A186T, S203T,A256T, V272I, I321V
33 [b]	Day 1	H3N2	6.85	Fatal	Q44H, T48A, Q57H, K62E, K92R,**A138S/A**, K144N, T212A, S214I,N312S
	Day 3	H3N2	5.8		Q44H, T48A, Q57H, K62E,K92R, **A138S/A**, K144N, T212A, S214I,N312S
	Day 5	H3N2	5.66		Q44H, T48A, Q57H, K62E, K92R, K144N, T212A, S214I, N312S
	Day 10	H3N2	5.36		Q44H, T48A, Q57H, K62E, K92R,K144N, T212A, S214I, N312S
34 [b]	Day 1	A/H1N1pdm09	5.44	Fatal	P83S, D97N, K160K/E, S162S/N, K163R/K,S164S/A,S203T, R205K,I216V,V249L,I321V
	Day 3[c]	A/H1N1pdm09	5.25		P83S, D97N, **E112Q/E**, S203T, R205K, I216V, V249L, I321V
	Day 5	A/H1N1pdm09	6.59		P83S, D97N, **V175L**, **H180H/L, Q189Q/R**, S203T, R205K, I216V,**V234I, P236S/P**, V249L, **V250L/V**, **I265I/L**
	PT+2[c]	A/H1N1pdm09	4.26		P83S, D97N,V249L, **N260T/N**, I321V
	PT+5	A/H1N1pdm09	<2.7		P83S,D97N,S162N,S164S/A, S203T, R205K, I216V, V249L,I321V
	PT+9	A/H1N1pdm09	<2.7		P83S, D97N, K160K/E, S162S/N, K163R/K, S164S/A,S203T,R205K,I216V,V249L,I321V
35 [b]	Day 1	A/H1N1pdm09	<2.7	Non-fatal	P83S, **A134T**, A141S, S183P, S203T,D222G, I295V, I321V

Known resistance amino acid substitution in **bold**, summarized in Tisdale, 2009.
[a] Viral load in NP swab by qPCR, lower limit of detection = 2.7 $\log_{10}$ copies/mL.
[b] received prior oseltamivir treatment.
[c] Partial sequence.

Table 7
Summary of H1N1pdm09 treatment emergent HA amino acid substitutions not previously associated with resistance.

Subject no.	Visit	Viral load [a] (Log copies/mL)	Clinical outcome	Amino acid substitution (From throat swab) (N1 numbering)
5 [b]	Day 1	6.46	Non-fatal	S74N, P83S, S203T, K283E, I321V
	Day 3	3.55		S74N, P83S, S203T, K283E, I321V
	Day 5	4.61		S74N, P83S, S203T, K283E, I321V
	PT+2	NA		S74N, P83S, K160T, S203T, K283E, I321V
	PT+5	NA		S74N, P83S, S203T, K283E, I321V
	ET[c]	NA		S74N, P83S
6	Day 1	7.76	Non-fatal	P83S, S203T, I321V
	ET	7.37		P83S, **S164D**, S203T
8 [b]	Day 1	5.34	Non-fatal	L32I, P83S, I321V, I460V, V520A
	Day 3	3.40		L32I, P83S, I321V, I460V, V520A
	Day 5	<2.7		L32I, P83S, I321V, I460V, V520A
	PT+5[c]	<2.7		L32I, P83S, **S185N**, I460V, V520A
9 [b]	DAY1	6.67	Non-fatal	E68G/E, P83S, N125D, S203T, I321V
	DAY3[c]	5.03		P83S, N125D, S203T
	DAY5	4.20		P83S, N125D, S203T, I321V
	PT+16	4.61		**E81K**, P83S, N125D, S203T, I321V
	ET[c]	8.08		N125D, **D168N**, S203T, I321V
18 [b]	Day 1	5.72	Non-fatal	**K22E**, P83S, S203T, K211R, D222E, I321V
	Day 3[c]	5.38		P83S, **F111S**, S203T, K211R, D222E, I321V
	Day 5	5.17		P83S, S203T, K211R, D222E, I321V
36	Day 1	7.28	Non-fatal	P83S, D97N, S162S/N, K163R/K, S164S/A, S185T, S203T, I321V
	PT+2[c]	3.58		P83S, D97N, **V108L**, I321V

Amino acid substitutions selected or deselected during treatment in **bold**.
[a] Viral load in NP swab by qPCR, lower limit of detection = 2.7 $\log_{10}$ copies/mL.
[b] received prior oseltamivir treatment.
[c] Partial sequence.

Table 8
Summary of H1N1pdm09 HA amino acid substitutions associated with virulence.

Subject no.	Visit	Viral load [a] (Log copies/mL)	Clinical outcome	Amino acid substitution (N1 numbering)
12 [b]	DAY 1	4.41	Non-fatal	N64S, P83S, S185T, S203T, **D222D/G**, P282P/L, I321V
	DAY 3	3.82		P83S, S185T, S203T, I321V
	ET	4.94		P83S, S164S/A, S185T, S203T, **D222D/G**, I321V
13 [b]	DAY 1	3.91	Fatal	P83S, S185T, S203T, **D222D/G**, I321V
15 [b]	DAY 1	3.00	Non-fatal	P83S, D97N, S162S/N, K163R/K,S185T, S203T, **D222G**, I321V
16 [b]	DAY 1	3.44	Non-fatal	P83S, D97N, K119N/K, S203T, R205K, I216V, **D222G**, V249L, I321V
35 [b]	DAY 1	<2.7	Non-fatal	P83S, A134T, A141S, S183P, S203T,**D222G**, I295V, I321V

Amino acids associated with virulence in **bold**.
[a] Viral load in NP swab by qPCR, lower limit of detection = 2.7 $\log_{10}$ copies/mL.
[b] received prior oseltamivir treatment.

(Nguyen et al., 2010; Rousset et al., 2010; van der Vries et al., 2010). Although 80% of subjects in this study had prior oseltamivir treatment and a significant number were immunocompromised, no viruses harbouring I223R were selected. The Q136K amino acid substitution confers high level resistance to zanamivir but it has only been found in cultured virus and never in a clinical specimen (Hurt et al., 2009; Okomo-Adhiambo et al., 2009; Yates et al., 2013).

Five emergent NA amino acid substitutions N63D, V83A, W190C and M269K (A/H1N1pdm09), and R210K (PT+9, A/H3N2) were detected that have not previously been associated with resistance and were not located in or near the active site, but may have arisen due to selection pressure during IVZ treatment or by chance. Three of the subjects (3, 9 and 23) with emergent substitutions died during the study and had detectable virus beyond Day 4. The virus isolated from subjects 3, 9 and 23 could therefore be resistant to zanamivir but none of the viruses could be cultured (or from other subjects with emergent substitutions), so it is not known what, if any, the effect the substitutions have on susceptibility to zanamivir.

Two of the subjects with emergent substitutions, 19 and 30, had detectable virus at Day 7 and Day 4 respectively but both showed clinical improvement during zanamivir treatment. Although the emergent substitutions were selected during zanamivir treatment, their clinical significance is not known.

4.3. HA sequence analysis

HA resistance amino acid substitutions were detected in 15 subjects, but the majority (12/15) were present at Day 1 and therefore did not arise as a result of zanamivir selection pressure. The five treatment emergent HA substitutions may have arisen due to selection pressure during IVZ treatment or by chance. Two substitutions, A134T and A138S, located in the receptor binding site (RBS) were identified in six subjects. Substitutions in the RBS have been shown to effect susceptibility to NAIs *in vitro* by altering the affinity of the HA to the receptor, thus reducing the need for cleavage by the NA. However, there is limited data describing the role of HA substitutions in mechanisms of resistance *in vivo* and the effect of the HA substitutions observed in this study on susceptibility to zanamivir or their clinical relevance is not clear.

The D222 amino acid is located in the RBS of the HA and amino acid substitutions at this position have been associated with increased virulence of the A/H1N1pdm09 virus (Chan et al., 2011; Kilander et al., 2010). The D222G substitution has been shown to be present at a higher frequency in viruses isolated from patients with severe influenza A/H1N1pdm09 compared to those with mild illness (Chan et al., 2011). In this study, subjects with a D222G substitution had a mean ICU stay of 22.8 days compared to 14.0

Table 9
NGS analysis of influenza A/H1N1pdm09 viruses with NA resistance substitutions.

Subject	Visit	Amino acid summary by NGS	Amino acid substitution by Population sequencing
9	DAY1	96%V106I, 100%N248D, 99%N397K, 98%S442I	V106I, N248D, N397K, S442I
9	PT+5	98%V106I, **26%E119K**, 100%N248D, 93%V264I	Not determined
9	PT+16	99%V106I, **39%E119D**, 20%W190G, 97%N248D, 99%V264I, 99%N397K, 99%S442I	V106I, W190C, N248D, V264I, N397K
14	DAY1	99%V106I, 100%N248D	N248D
14	DAY7	28%Y100H, 99%V106I, **29%L134S**, 100%N248D	V106I, V241I, N248D, N369K
14	DAY10	99%V106I, 99%N248D	V106I, N248D
34	DAY1	99%V81A, 99%V106I, 99%V241I, 98%N248D, 99%N369K	V81A, V106I, V241I, N248D, N369K
34	DAY3	99%V81A, 99%V106I, 99%V241I, 98%N248D, 99%N369K	V81A, V106I, V241I, N248D, N369K, S441S/I
34	PT+5	29%I32K, 20%S52N, 15%V81A, 99%V106I, **71%E119D**, 99%V241I, **7%S247I**, 100%N248D, 98%N369K	V81A, V106I, **E119D/E**, V241I, N248D, N369K
34	PT+9	99%V81A, 99%V106I, 99%V241I, 99%N248D, 99%N369K	V81A, V106I, V241I, N248D, N369K
37	DAY1	98%V106I, 99%V241I, 99%N248D, 90%N369K	V106I, V241I, N248D, N369K, S439S/I
37	DAY3	98%V106I, 98%V241I, 100%N248D, 97%S266L, 41%Y316	Not determined
37	DAY5	99%V106I, 93%V241I, 99%N248D, **90%E277K**, 40%Y316[a], 96%N369K	Not determined
38	DAY1	98%V53F, 99%V106I, 24%V166I, 100%N248D	V53F, V106I, V166I, N248D
38	DAY3	95%V53F, 98%V106I, 20%V166I, **23%D199N**, 100%N248D, 21%R301Q	V53F, V106I, V166I
38	DAY5	98%V53F, 99%V106I, 20%V166I, 100%N248D	V53F, V106I, V166I, N248D
39	DAY1	97%V81A, 95%V106I, 5%I117L, 5%I163M, 5%A181E, 16%S196C, 98%V241I, 99%N248D, 7%Y282D, **5%D294G**, 5%N309Y, 7%G333V, 8%G336C, 7%N344H, 12%G354C, 5%S366I, 6%G370V, 6%N397K, 6%D416N, 5%C421G, 5%S444P	Not determined
39	DAY3	99%V81A, 99%V106I, 6%I193T, 97%V241I, 100%N248D, 96%N369K	V81A, V106I, V241I, N248D, N369K, W437W/L, T438H/T/N/P
39	DAY5	99%V81A, 100%V106I, 100%V241I, 100%N248D, 13%E311V	Not determined

Resistance substitutions in **bold**.
[a] Stop codon.

days for subjects without the substitution and the mean viral titre was higher in ET samples than from corresponding NP samples (Marty et al., 2013). This finding supports the increased virological burden in the lower respiratory tract of the severely ill subjects enrolled in this study.

The treatment emergent amino acid substitutions that were identified in this study have not previously been associated with resistance and because they could not be cultured their effect, if any, on susceptibility to zanamivir could not be determined. Another drawback of this study was that some of the sequencing was of poor quality (many mixtures, particularly at the end of reads), particularly the HA sequences. As a result, there were many emergent substitutions appearing as mixed amino acids, the majority of which were excluded from the analysis because they were either stop codons or were near the start or end of an amplicon sequence. To address the issue of sequence quality, some sequences were repeated at another vendor laboratory. Good quality sequences were obtained from each subject at at-least two timepoints, the Day 1 sample and the last visit with detectable virus.

4.4. Minority species analysis

NGS identified seven amino acid substitutions that were not identified by population sequencing. The majority of the substitutions were not detected at later timepoints confirming that resistant viruses are selected during treatment but are unfit and are quickly overgrown by wild-type viruses. The clinical significance of these substitutions is not clear.

4.5. Summary

Development of resistance is a key factor in reducing the efficacy of antivirals and is of particular concern during treatment of immunocompromised patients. In this study, a significant number of subjects were immunocompromised and there was evidence for resistance selection in a post treatment sample but the resistant variant did not persist in later visit samples. IVZ may be considered as a treatment option for hospitalised patients with influenza particularly when oseltamivir resistant virus is present or suspected.

Acknowledgements

The authors thank the subjects and investigators who participated in this study. Without all of their involvement, this study would not have been possible.

This study was funded by GlaxoSmithKline. All authors are employees of and own shares and stock in GSK. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.antiviral.2016.08.023.

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