



## Short communication

## Sensitivity of influenza viruses to zanamivir and oseltamivir: A study performed on viruses circulating in France prior to the introduction of neuraminidase inhibitors in clinical practice

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### Abstract

Influenza virus neuraminidase inhibitors (NAIs) were introduced in clinical practice in various parts of the world since 1999 but were only scarcely distributed in France. Prior to the generalization of zanamivir and oseltamivir utilization in our country, we decided to test a large panel of influenza strains to establish the baseline sensitivity of these viruses to anti-neuraminidase drugs, based upon a fluorometric neuraminidase enzymatic test. Our study was performed on clinical samples collected by practitioners of the GROG network (Groupe Régional d'Observation de la Grippe) in the south of France during the 2002–2003 influenza season. Out of 355 isolates tested in the fluorometric neuraminidase activity assay, 267 isolates could be included in inhibition assay against anti-neuraminidase drugs. Differences in  $IC_{50}$  range were found according to the subtype and the anti-neuraminidase drug. Influenza B and A/H1N1 viruses appeared to be more sensitive to zanamivir than to oseltamivir (mean B  $IC_{50}$  values: 4.19 nM versus 13 nM; mean H1N1  $IC_{50}$  values: 0.92 nM versus 1.34 nM), while A/H1N2 and A/H3N2 viruses were more sensitive to oseltamivir than to zanamivir (mean H3N2  $IC_{50}$  values: 0.67 nM versus 2.28 nM; mean H1N2  $IC_{50}$  values: 0.9 nM versus 3.09 nM). Out of 128 N2 carrying isolates, 10 isolates had zanamivir or oseltamivir  $IC_{50}$  values in upper limits compared to their respective data range. Sequencing of the neuraminidase of these outliers N2 highlighted several mutations, but none of them were associated with resistance to neuraminidase inhibitors.

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Influenza viruses contain two glycoproteins on their surface, hemagglutinin (HA) and neuraminidase (NA), which recognize sialic acid on host cell glycoconjugates. HA binds to terminal sialic acid groups on cell surface glycoconjugates leading to attachment and subsequent penetration of the virus into cells (Wiley and Skehel, 1987), while NA possesses an enzymatic activity that removes sialic acid from glycoconjugates, facilitating the release of progeny virions from infected cells and preventing the aggregation of progeny virions (Griffin et al., 1983; Liu et al., 1995). The emergence of annual influenza epidemics and sporadic influenza pandemics are mainly a consequence of rapid rates of evolution in the genes that encode HA and NA, the two major antigenic

determinants of influenza virus. These antigenic variations – drift/shift – of influenza viruses produce new virus strains that may not be recognized by antibodies to earlier influenza strains. In order to avoid failure of immune protection in vaccinated people, the influenza vaccine is updated on a yearly basis to keep up with changes in influenza viruses.

Antiviral agents can be useful during epidemics and pandemics. New drug design strategies allowed the development and subsequent licensing of two anti-influenza A and B drugs, zanamivir (von Itzstein et al., 1993) and oseltamivir (Li et al., 1998) that inhibit virus replication in vitro and in vivo. These drugs act by inhibiting neuraminidase activity, preventing release of progeny virions budding out from cell surface. Neuraminidase was chosen as a suitable drug target because NA plays a major role in influenza virus propagation, and the amino acids residues of the active site interacting directly

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with the substrate or surrounding the central active site of the enzyme are strictly conserved (Burmeister et al., 1991; Taylor and von Itzstein, 1994).

Introduction of a new antiviral drug in clinical practice requires suitable tools to monitor antiviral susceptibility to take account of the probable emergence of drug-resistant variants. Viruses resistant to zanamivir and oseltamivir carboxylate have been generated in vitro, enabling the characterization of three mechanisms involved in the resistance: substitution in the NA, in HA or in both glycoproteins. NA variants can be found after in vitro or in vivo studies (see review by Gubareva, 2004), NA mutations such as R292K, E119G, and H274Y have been selected after several passages of influenza viruses in cell culture in the presence of either zanamivir or oseltamivir (Tai et al., 1998; Barnett et al., 2000; Gubareva et al., 2001). Other NA variants like R152K were isolated from patients (Gubareva et al., 1998). Neuraminidase inhibitor (NAI)-resistant mutants can be detected in the NA inhibition assays. Mutations in HA led to a reduced efficiency of virus binding to sialic acids and then to a decrease in dependence of virus on NA function (Staschke et al., 1995; McKimm-Breschkin et al., 1996; Gubareva et al., 1996). Such resistant variants can be detected in cell based assays using MDCK SIAT1 cells which have been transfected for over-expressing Sia  $\alpha$  2–6 receptors (Matrosovich et al., 2003), and by the analysis of HA gene sequences. Another mechanism of resistance involves substitutions in the conserved residues in the NA enzyme active site with compensatory mutations on the HA glycoprotein (McKimm-Breschkin et al., 1996; Gubareva et al., 1996). Virus replication in MDCK cells of resistant variants with NA mutations is reduced, and can be partly rescued by HA compensatory mutations (McKimm-Breschkin et al., 1998). However, most of these viruses showed reduced infectivity and virulence in animal models (Tai et al., 1998; Blick et al., 1998), suggesting that this type of viruses would be severely compromised with a very low clinical impact in man (Carr et al., 2002).

The present study on sensitivity of influenza viruses to zanamivir and oseltamivir was initiated prior to the generalization of the use of anti-neuraminidase compounds in the French population. For this aim, 355 clinical isolates obtained by culture of throat and nasal swabs on MDCK cells (Lina et al., 1996), were provided by the GROG network for further analysis in fluorometric neuraminidase test. Out of 355 isolates, 267 viruses were found positive for NA activity and were subsequently introduced in fluorometric NA inhibition test (Tisdale, 2000). Such a test, contrary to plaque reduction assay, was shown uniformly sensitive independently of the cell substrate used for growing virus isolates (Abed et al., 2002; Barnett et al., 2000). The fluorometric NA inhibition test restricted the detection of potential resistant viruses to those viruses mutated in the NA gene. Indeed, the accurate detection of HA-mediated resistant viruses was not considered due to the lack of reliable cell culture based assay when starting this study. Recently, Matrosovich et al. (2003) reported an improvement in the sensitivity of human

Table 1

Neuraminidase activity (nmol/h/ml) of influenza isolates in the fluorometric assay

	N isolates	Mean	Max	Min	S.D.
B	80	962.13	3076	179	616.55
A/H3N2	128	658.54	2485	71	407.98
A/H1N2	50	764.1	2267	216	415.58
A/H1N1	97	32.6	31	0	39.8
A/H1N1 <sup>a</sup>	9	14.3	31	0	12.2
A/H1N1 <sup>a</sup> + BSA 5 mg/ml	9	64.2	107.5	43.5	19.4

<sup>a</sup> Nine isolates out of 97 A/H1N1 isolates were chosen at random and analyzed in parallel with and without addition of BSA in the virus harvest.

influenza A and B viruses to the neuraminidase inhibitor oseltamivir carboxylate, when using MDCK cells transfected for over-expressing Sia  $\alpha$  2–6 virus receptors. The fluorometric NA test that we used in this study was a modification of the technique previously described by Potier et al. (1979).

Briefly, serial two-fold dilutions of reference strains and isolates (HA titer  $\geq 16$ ) in 32.5 mM MES (2-(*N*-morpholino) ethanesulfonic acid, sodium salt, Mw 217.2, Sigma–Aldrich) pH 5.8, 4 mM CaCl<sub>2</sub> buffer were prepared in a 96-well flat bottom plates. Plates were gently shaken on a mechanical vibrator after the addition of a 100  $\mu$ M working solution of 2'-(4-methylumbelliferyl)- $\alpha$ -D-*N*-acetylneuraminic acid, sodium salt (MUN, MW 489.4, Sigma–Aldrich, St. Louis, MO) prepared in 32.5 mM MES pH 5.8, 4 mM CaCl<sub>2</sub> buffer and were then incubated for 1 h at 37 °C. The reaction was stopped by adding 150  $\mu$ l per well of 50 mM glycine buffer pH 10.4. Fluorescence was measured in a DYNEX, MRX fluorometer with an excitation wavelength of 355 nm and an emission wavelength of 460 nm. Both reference sensitive and reference resistant control viruses were included in each assay. These controls were kindly provided by Glaxo Smith Kline (B/Beijing/1/87) and Roche (A/Sydney/05/97). Resistant viruses have a specific mutation in the NA gene, position E119G (Barnett et al., 2000) or R292K (Gubareva et al., 1997; McKimm-Breschkin et al., 2003), respectively.

Relative fluorescent units (RFUs) of samples were measured and quantification of NA activity was deduced from comparison of sample RFUs values with a standard curve established by using 4-methylumbelliferone (4-Mu) sodium salt. Contrary to influenza viruses A/H3N2, A/H1N2, and B, A/H1N1 isolates showed very low NA activity when using standard infection medium (Table 1). Addition of 5 mg/ml BSA to the medium on time of virus harvest was shown to enhance and stabilize NA N1 activity. Such a technical modification was implemented for the 2003–2004 isolates. Due to the lack of NA activity A/H1N1 isolates from the 2002 to 2003 season, 88 isolates could not be introduced in the NA inhibition test against NA inhibitors. In the perspective of a continuous monitoring the sensitivity of influenza isolates to anti-neuraminidase drugs, an interesting approach would be to use MDCK SIAT1 cells for isolating viruses from clinical swabs. Indeed, Matrosovich et al. (2003) who established this modified MDCK cell line showed that over-expression of Sia

Table 2a  
Influenza A and B IC<sub>50</sub> upper limit

Subtype	A/H3N2 (N=128)	A/H1N2 (N=50)	A/H1N1 (N=9)	B (N=80)
Zanamivir (IC <sub>50</sub> (nM))				
Mean	2.28	3.09	0.92	4.19
Max	5.97	6.70	1.09	11.90
Upper limit	5.6	6.4	1.2	13.4
Oseltamivir carboxylate (IC <sub>50</sub> (nM))				
Mean	0.67	0.9	1.34	12.99
Max	4.46	2.38	1.62	30.20
Upper limit	2.1	2.2	1.7	33.5

Mean values, maximum data, upper limit value (mean + 2.5 S.D.) for each subtype and for both zanamivir and oseltamivir carboxylate.

α 2–6 virus receptors was responsible for an increased binding of clinical isolates when compared with MDCK cells. From these results it is possible to expect a better replication of influenza isolates and, as a consequence, a higher NA activity, and an increase in the number of isolates entering in the enzymatic inhibition test with anti-neuraminidase drugs.

The neuraminidase activity inhibition assay was performed using a standardized dose of virus (10 nmol/h/ml). This virus dose was incubated with 10-fold dilutions of neuraminidase inhibitors prepared in 32.5 mM MES pH 5.8, 4 mM CaCl<sub>2</sub> buffer (from 30,000 to 0.003 nM) and incubated for 15 min at 37 °C. The reaction was started by adding MUN substrate. Plates were incubated for 1 h at 37 °C. The reaction was stopped by adding 150 μl of 50 mM glycine buffer pH 10.4 per well. Fluorescence was immediately quantified by using a DYNEX, MRX fluorometer. IC<sub>50</sub> values of antiviral drugs were calculated according to the concentration required for reducing NA activity by 50%. Baseline sensitivity of N1, N2 and B viruses were calculated from the mean IC<sub>50</sub> values of non-outliers strains (Table 2a). IC<sub>50</sub> values showed differences according to subtypes and NAI (Fig. 1). Viruses with N2 NA were shown to be more sensitive to oseltamivir carboxylate than to zanamivir inversely to N1 NA viruses and

B viruses. These results are in agreement with those reported previously (McKimm-Breschkin et al., 2003; Wetherall et al., 2003; Hurt et al., 2004).

A total of 80 influenza B isolates and 9 A/H1N1 isolates were introduced in the neuraminidase inhibition test. All of them were found sensitive to zanamivir and oseltamivir carboxylate as well. Out of 178 N2 carrying viruses, only 10 plotted at the upper limit (>mean + 2.5 S.D. values) and concerned either zanamivir or oseltamivir carboxylate IC<sub>50</sub> values (Tables 2a and 2b). Two A/H1N2 isolates and six A/H3N2 isolates showed zanamivir IC<sub>50</sub> values in the upper limit, corresponding to an average 1.5-fold reduction of sensitivity, while one A/H1N2 isolate and two A/H3N2 isolates showed oseltamivir carboxylate IC<sub>50</sub> values in the upper limit, corresponding to a three-fold and five-fold reduction in sensitivity, respectively.

NAs of isolates exhibiting IC<sub>50</sub> values in the upper limit range were subsequently sequenced. RNA was extracted from cell culture supernatants by using QIAamp viral RNA kit (QIAGEN, Valencia, CA). Amplified products with specific neuraminidase gene primers (forward primer N2Fp1 5' GTGAAGATGAATCCAATCAA 3', N2Fp15 5' TACAGAAATTGTCAAAGCCG 3' and reverse primer N2Rp2 5' GCGAAAGCTATATAGGC 3', N2Rp4 5' ACAAACGCATTCCGACTCCTG 3' (Eurogentec, Liege, Belgium)) were purified from dNTPs and primers in excess using the DNA Clean & Concentrator 5 kit (Zymo Research Inc., USA) and the GFX™ PCR DNA and Gel band purification Kit (Amersham Biosciences Europe GmbH, Orsay, France). Sequencing was performed in both directions with 120 pmol purified template and 10 pmol of the primers used in the PCR. Cycle sequencing was carried out with fluorescent-labeled dideoxy-chain terminators and the reagents in the BigDye sequencing kit (Applied Biosystems, Foster City, CA). All 10 N2 neuraminidase outliers exhibited several mutations apart from antigenic sites and apart from phylogenetically informative regions (PIRs) (Fanning et al., 2000). None of these N2 neuraminidases

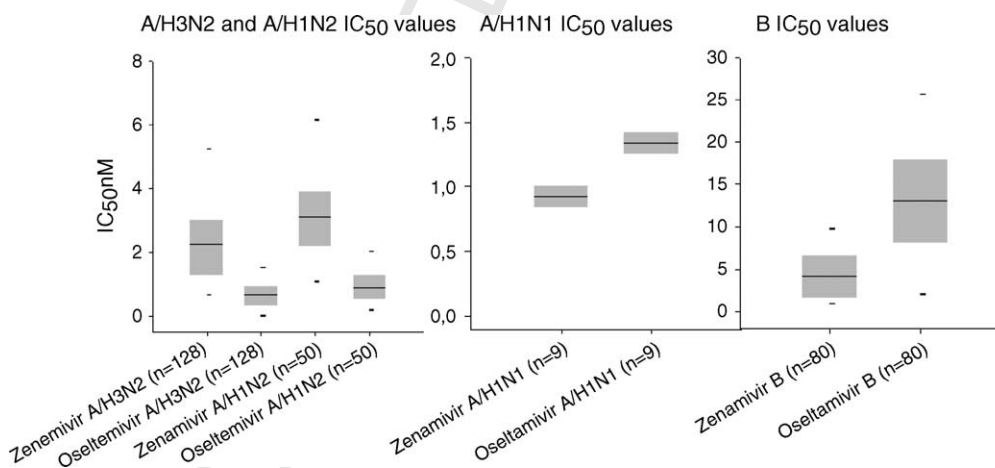


Fig. 1. Box plots of zanamivir and oseltamivir carboxylate IC<sub>50</sub> values for A/H3N2, A/H1N2, A/H1N1 and B isolates obtained in the fluorometric neuraminidase inhibition test. The horizontal mark on either side of the box represent the 5% and 95% confidence limits. The box stretches from the lower hinge (25th percentile) to the upper hinge (75th percentile). The mean is shown as a line across the box.

Table 2b  
Influenza A and B IC<sub>50</sub> upper limit

Virus strains	Subtype	NA activity (nmol/h/ml)	Zanamivir IC <sub>50</sub> (nM) <sup>a</sup>	Zanamivir isolate/Sydney <sup>b</sup>	Zanamivir isolate/mean IC <sub>50</sub> <sup>c</sup>		Oseltamivir carboxylate IC <sub>50</sub> (nM) <sup>a</sup>	Oseltamivir carboxylate isolate/Sydney <sup>b</sup>	Oseltamivir carboxylate isolate/mean IC <sub>50</sub> <sup>c</sup>	
					A/H3N2	A/H1N2			A/H3N2	A/H1N2
A/Lyon/523/03	H3N2	321	5.72	1.4	2.5		4.09	5.2	6.1	
A/Lyon-CHU/8698/03	H3N2	193	2.3	0.6	1		4.46	5.7	6.7	
A/Lyon-PAH/1014/03	H3N2	857	5.91	1.5	2.6		0.42	0.5	0.6	
A/Lyon-PAH/1070/03	H3N2	377	5.75	1.4	2.5		0.33	0.4	0.5	
A/Lyon/771/03	H3N2	159	5.75	1.4	2.5		0.76	1	1.1	
A/Poitiers/1194/03	H3N2	686	5.97	1.5	2.6		1	1.3	1.5	
A/Lyon/1300/03	H3N2	639	5.59	1.4	2.5		1.43	1.8	2.7	
A/Lyon-CHU/8633/03	H1N2	927	6.51	1.6		2.1	0.66	0.8		0.7
A/Poitiers/1355/03	H1N2	1293	3.02	0.8		1	2.38	3		2.6
A/Lyon/1281/03	H1N2	383	6.7	1.7		2.2	0.88	1.1		1
A/Sydney/05/97	H3N2	221.9	3.95	1	1.73	1.28	0.79	1	1.18	0.9
R292K	H3N2	52.5	14.07	3.6	6.2	4.55	13	16916	19946	14849
B/Beijing/1/87 wt	B	437	8.39				11.64			
B/Beijing/1/87 R	B	44.2	14000				277			

<sup>a</sup> Selecting of isolates as outliers when IC<sub>50</sub> values were near or above the upper limit value with at least one inhibitor. IC<sub>50</sub> values are the average of at least three determinations for each virus-antiviral combination.

<sup>b</sup> Fold reduction of isolates' sensitivity to NA inhibitors: isolates/A/Sydney/05/97 IC<sub>50</sub> ratio.

<sup>c</sup> Fold reduction of isolates' sensitivity to NA inhibitors compared to the mean IC<sub>50</sub> for the same subtype: isolates/mean IC<sub>50</sub> ratio.

Table 3  
NA mutations of outlier isolates

Virus strains	Subtype	Amino acids mutations										
		A18S	L23F	C42F	<b>E119G</b>	R143V	<b>R152K</b>	E199K	<b>H274Y</b>	<b>R292K</b>	S332F	K431N
A/Lyon/523/03	H3N2	A18S	L23F	C42F		R143V						
A/Lyon-CHU/8698/03	H3N2	A18S	L23F	C42F		R143V						
A/Lyon-PAH/1014/03	H3N2									S332F		
A/Lyon-PAH/1070/03	H3N2					R143V						
A/Lyon/771/03	H3N2							E199K				K431N
A/Poitiers/1194/03	H3N2									S332F		
A/Lyon/1300/03	H3N2					R143V		E199K				
A/Lyon-CHU/8633/03	H1N2							E199K				K431N
A/Poitiers/1355/03	H1N2							E199K				K431N
A/Lyon/1281/03	H1N2							E199K				K431N
A/Sydney/05/97	H3N2											
R292K	H3N2									R292K		
B/Beijing/1/87 wt	B											
B/Beijing/1/87 R	B				<b>E119G</b>							

Amino acids mutations in bold correspond to reference in vitro NA mutations.

with reduced zanamivir or oseltamivir carboxylate sensitivity, revealed variations on amino acids residues previously correlated with a consistent pattern of altered sensitivity to these inhibitors (Table 3). Nevertheless, while no variation was shared by all 10 NAs, some of them (E199K, K431N and R143V) were associated with four to five viruses in the outlier range. In addition, three mutations, A18S, L23F, and C42F were found in two viruses exhibiting a five-fold reduction in NA sensitivity to oseltamivir carboxylate. The reduction in NA sensitivity of these isolates to anti-neuraminidase drugs was very low when compared with the in vivo (Kiso et al., 2004) or in vitro resistant strains.

The use of the fluorometric NA test allowed us to establish the baseline sensitivity of N1, N2, and B influenza virus neuraminidases to neuraminidase inhibitors. Such a baseline was found dependent on both NA type and subtype and NA inhibitors. The reduction in NA sensitivity of the N2 isolates we ranged as outlier viruses, was similar to values previously reported by McSharry et al. (2004) and Hurt et al. (2004). These N2 outliers could not be considered as resistant viruses when considering, first, the limited reduction in NA sensitivity to the inhibitors and, second, the existence of mutations on NA which did not belong to those currently associated with influenza resistance to zanamivir and oseltamivir inhibitors (E119G, R152K, H274Y, and R292K).

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