Antiviral Research 85 (2010) 381–388

Contents lists available at ScienceDirect

Antiviral Research

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

Host cell selection of influenza neuraminidase variants: Implications for drug resistance monitoring in A(H1N1) viruses☆

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ARTICLE INFO

Article history:
Received 4 August 2009
Received in revised form 21 October 2009
Accepted 9 November 2009

Keywords:
Cell culture
Antiviral resistance
Tamiflu
Relenza
Peramivir
A-315675

ABSTRACT

The neuraminidase inhibitors (NAIs), oseltamivir and zanamivir, are essential for treatment and prevention of influenza A and B infections. Oseltamivir resistance among influenza A (H1N1) viruses rapidly emerged and spread globally during the 2007–2008 and 2008–2009 influenza seasons. Approximately 20% and 90% of viruses tested for NAI susceptibility at CDC during these seasons, respectively, were resistant to oseltamivir (IC_{50} ~100–3000 time > those of sensitive viruses), based on the chemiluminescent NA inhibition assay. Pyrosequencing analysis confirmed H274Y mutation (H275Y in N1 numbering) in the neuraminidase (NA) gene of oseltamivir-resistant viruses. Full NA sequence analysis of a subset of oseltamivir-resistant and sensitive virus isolates from both seasons ($n = 725$) showed that 53 (7.3%) had mutations at residue D151 (D → E/G/N), while 9 (1.2%) had mutations at Q136 (Q → K) and 2 (0.3%) had mutations at both residues. Viruses with very high IC_{50} for oseltamivir and peramivir, and elevated IC_{50} for zanamivir, had H274Y in addition to mutations at D151 and/or Q136, residues which can potentially confer NAI resistance based on recent N1 NA crystal structure data. Mutations at D151 without H274Y, did not elevate IC_{50} for any tested NAI, however, Q136K alone significantly reduced susceptibility to zanamivir (36-fold), peramivir (80-fold) and A-315675 (114-fold) but not oseltamivir. Mutations at D151 and Q136 were present only in MDCK grown viruses but not in matching original clinical specimens ($n = 33$) which were available for testing, suggesting that these variants were the result of cell culture selection or they were present in very low proportions. Our findings provide evidence that propagation of influenza virus outside its natural host may lead to selection of virus variants with mutations in the NA that affect sensitivity to NAIs and thus poses implications for drug resistance monitoring and diagnostics.

Published by Elsevier B.V.

1. Introduction

Antiviral drugs occupy an important niche in the management of influenza infections (Moscona, 2008; Abed et al., 2006; Moscona, 2005). They directly target influenza viruses and are effective for treatment when administered early in the course of infection, or for prophylaxis when used soon after exposure (Moscona, 2008). Two classes of antiviral agents are currently licensed for the control of influenza infections: M2 ion channel blockers and neuraminidase inhibitors (NAIs). The M2 blockers (amantadine and rimantadine) are effective against influenza A viruses, but not B viruses (Hayden, 1996). However, the effectiveness of this class of drugs has greatly been compromised by the rapid emergence of resistance among influenza A (H3N2) subtype and among some A (H1N1) viruses circulating in certain geographic areas (Bright et al., 2005; CDC, 2008a; Deyde et al., 2007). So far, all the recently emerged 2009 pandemic H1N1 viruses tested for adamantane resistance at CDC have shown resistance to the drug (CDC, 2009; Dawood et al., 2009; Garten et al., 2009).

Oseltamivir and zanamivir are currently the only drugs approved for use against type A and type B influenza infections (Moscona, 2005; Moscona, 2008), including the novel 2009 pandemic H1N1 viruses (CDC, 2009; Dawood et al., 2009). Oseltamivir is administered orally while zanamivir is inhaled (Smith et al., 2002; Colman, 2005) and therefore not recommended in certain patients including those who are severely ill, young children and the elderly (Freund et al., 1999; Medeiros et al., 2007; Hedrick et al., 2000; Diggory et al., 2001). The availability of an intravenous formulation of zanamivir could be beneficial for such patients, especially in

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0166-3542/$ – see front matter. Published by Elsevier B.V.
doi:10.1016/j.antiviral.2009.11.005

instances of oseltamivir resistance (Moscona, 2008). The investigational NAI peramivir, though not yet licensed (Sidwell and Smee, 2002; Babu et al., 2000), is currently undergoing clinical trials utilizing an intravenous or intramuscular formulation.

Emergence of NAI-resistant viruses has previously been low (Hatakeyama et al., 2007; Ward et al., 2005; Whitley et al., 2001) though studies of virus variants collected from young children following oseltamivir treatment revealed 18% resistance (Kiso et al., 2004). However, during the 2007–2008 influenza season (viruses collected October 01, 2007 to September 30, 2008), the emergence and transmission of H274Y oseltamivir-resistant influenza A (H1N1) viruses was simultaneously detected in several countries globally (Dharan et al., 2009; Hauge et al., 2009; Meijer et al., 2009; Sheu et al., 2008; CDC, 2008a,b; Lackenby et al., 2008a; Besselaar et al., 2008). Similar trends were observed during the 2008–2009 season (viruses collected October 01, 2008 to September 30, 2009), with many countries reporting up to 100% oseltamivir resistance in influenza A (H1N1) viruses http://www.who.int/csr/disease/influenza/h1n1_table/en/index.html and http://www.cdc.gov/flu/weekly/. It is therefore critical to enhance surveillance on NAI susceptibility of influenza viruses circulating in different parts of the world, expecially those carrying the N1 enzyme (pandemic H1N1, avian H5N1 and others), since NAIs are currently the only effective antivirals for treatment and chemoprophylaxis of both seasonal and pandemic influenza infections.

Zanamivir, unlike oseltamivir, has been prescribed less often due to current limitations of its use (CDC, 2008b), therefore emergence of resistance to this drug has been rare. Previously, the only reported zanamivir resistance was in an influenza B virus with R152K mutation in the NA which was isolated from a zanamivir-treated immunocompromised patient (Gubareva et al., 1998), and recently a novel Q136K mutation (Hurt et al., 2009) was detected in the NA of influenza A (H1N1) isolates which conferred resistance to zanamivir and peramivir, but had no effect on oseltamivir susceptibility. In addition, zanamivir-resistant mutants have been generated *in vitro*, including E119G, D, A and R292K in influenza N2 (Gubareva et al., 1996, 1997), E119G and R292K in influenza N9 (McKimm-Breschkin et al., 1998; Blick et al., 1995) and E119G, D in influenza B (Cheam et al., 2004; Barnett et al., 1999; Staschke et al., 1995).

Molecular markers of resistance to NAIs are currently less well established compared to M2 blockers; hence resistance to this newer class of drugs is not well characterized. Presently, detection of NAI-resistant viruses in surveillance laboratories is conducted using either the chemiluminescent or the fluorescent NA inhibition (NI) assay where generated IC_{50} values (drug concentration needed to inhibit 50% of NA enzyme activity) of test viruses are compared with those of sensitive control viruses. Elevated IC_{50} values alone are however, not sufficient criteria for defining NAI resistance and should be combined with detection of known molecular markers of resistance by conventional sequencing (McKimm-Breschkin et al., 2003; Monto et al., 2006; Sheu et al., 2008) or pyrosequencing (Lackenby et al., 2008b; Deyde et al., 2009; Deyde and Gubareva, 2009). The clinical relevance of the resistance detected using the NA inhibition assay has not been fully evaluated; nevertheless, it is essential to monitor changes in the NA and their possible effect on virus susceptibility to existing NAIs.

Assessment of drug susceptibility in NA inhibition assays requires the use of cell grown viruses. In the present study we detected a fraction of seasonal human A (H1N1) viruses collected during 2007–2008 and 2008–2009 influenza seasons (October 01, 2007 to September 30, 2009) that carried mutations in the NA not seen in matching clinical specimens, suggesting that even in the absence of drug pressure, propagation of viruses outside of natural host (in MDCK cells) can lead to selection of NA variants with altered susceptibility to NAIs (Hurt et al., 2009). Mechanisms driving such a selective process are not clearly understood, but the phenomenon poses serious implications for the detection of antiviral resistance.

2. Materials and methods

2.1. Viruses and cells

Influenza virus isolates and matching clinical specimens collected during 2007–2008 and 2008–2009 seasons were submitted to the World Health Organization Collaborating Center for Surveillance, Epidemiology and Control of Influenza at the CDC in Atlanta, GA, USA. Virus isolates were propagated further in Madin-Darby canine kidney (MDCK) cells (ATCC, Manassas, VA) at CDC. Reference viruses A/Georgia/17/2006 (H1N1) and A/Georgia/20/2006 (H1N1) representative of sensitive and oseltamivir-resistant A(H1N1) viruses, respectively, were also propagated in MDCK cells.

2.2. NA inhibitors

Zanamivir was supplied by GlaxoSmithKline (Uxbridge, UK), while oseltamivir carboxylate, the active compound of the ethyl ester prodrug oseltamivir phosphate, was supplied by Hoffmann-La Roche (Basel, Switzerland). Additional investigative inhibitors were used in the study including peramivir (BioCryst Pharmaceuticals, Birmingham, AL) and A-315675 (Abbott Laboratories, Abbott Park, IL).

2.3. NA inhibition assays

Susceptibility of viruses to NAIs was assessed using chemiluminescent NI assay (Buxton et al., 2000). The chemiluminescent NI assay uses a 1,2-dioxetane derivative of sialic acid as substrate and was performed using the NAStar™ Kit (Applied Biosystems, Foster City, CA) as described in detail (Sheu et al., 2008).

2.4. Statistical analysis

Calculation of 50% inhibitory concentration (IC_{50}) values and curve-fitting were performed by Robosage Version 7.31 software (GlaxoSmithKline, inhouse program), an add-in for MS Excel (Microsoft Corp., Redmond, WA) using the equation $y = V_{max} \times \{1 - [x/(K + x)]\}$ as previously described by Sheu et al., 2008; McKimm-Breschkin et al., 2003, where V_{max} is the maximum rate of metabolism, x is the inhibitor concentration, y is the response being inhibited and K is the IC_{50} for the inhibition curve (that is, $y = 50\%\ V_{max}$ when $x = K$).

2.5. Pyrosequencing

Viral RNAs were extracted from 100 μl of viral cell culture supernatant using the QIAamp® Viral RNA Mini Kit (Qiagen, Valencia, CA) according to manufacturer's instructions, and RT-PCR performed with Qiagen™ One-Step RT-PCR Kit (Qiagen, Valencia, CA). Pyrosequencing to detect molecular markers of resistance at codons 136, 151 and 274 in NA were performed on the PSQ96MA platform (Biotage AB, Uppsala, Sweden) as previously described (Deyde et al., 2009). Pyrosequence data consisting of 45–60 nucleotide reads were quantified and background corrected using PSQ96MA version 2.0.2 software (Biotage AB, Uppsala, Sweden). Sequences were aligned and analyzed using DNAStar analysis programs (DNAStar, Madison, WI, USA).