Virology 524 (2018) 69–77

Contents lists available at ScienceDirect

Virology

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

Selection of influenza virus resistant to the novel camphor-based antiviral camphecene results in loss of pathogenicity

Vladimir V. Zarubaev[a,*], Ekaterina A. Pushkina[b], Sophia S. Borisevich[c], Anastasia V. Galochkina[a], Angelica V. Garshinina[a], Anna A. Shtro[d], Anna A. Egorova[d], Anastasia S. Sokolova[e], Sergey L. Khursan[c], Olga I. Yarovaya[e,f], Nariman F. Salakhutdinov[e,f]

[a] Pasteur Institute of Epidemiology and Microbiology, 14 Mira St., St. Petersburg 197101, Russia
[b] St. Petersburg State University, 7/9 Universitetskaya nab., St. Petersburg 199034, Russia
[c] Ufa Institute of Chemistry, 71 Prospekt Oktyabrya, Ufa 450054, Russia
[d] Smorodintsev Research Institute of Influenza, 15/17 Prof. Popova St., St. Petersburg 197376, Russia
[e] N.N. Vorozhtsov Novosibirsk Institute of Organic Chemistry, Siberian Branch, Russian Academy of Sciences, 9 Lavrentjev Avenue, Novosibirsk 630090, Russia
[f] Novosibirsk State University, 2 Pirogova St., Novosibirsk 630090, Russia

ARTICLE INFO

Keywords:
Influenza virus
Hemagglutinin
Antivirals
Camphecene
Drug resistance
Pathogenicity

ABSTRACT

Due to the ability of influenza virus to develop drug resistance, the search for novel antivirals is an important goal of medical science and health care systems. We assessed the ability of the influenza virus to develop resistance to the hemagglutinin inhibitor camphecene and characterized laboratory-selected resistant strains. We showed by electron microscopy that camphecene decreases the number of virions fusing their envelopes with endosomal membranes. A 160-fold decrease in virus susceptibility was observed after six passages in cells. This was associated with the emergence of a V458L mutation in the HA2 subunit of HA and with a decrease in viral pathogenicity. Molecular modeling predicts that this substitution results in a more stable HA molecule compared to wild-type HA; and an altered camphecene-binding site. Therefore, despite the relatively rapid development of resistance, camphecene remains promising as a potential antiviral due to the low pathogenicity of resistant viruses that may arise.

1. Introduction

The influenza virus is one of the most important human viral respiratory pathogens, and it is responsible for yearly epidemics with high morbidity, including from 250,000 to 500,000 estimated fatalities per year (Fiore et al., 2008). Its segmented genomic organization allows for reassortment, resulting in the emergence of antigenically novel strains causing pandemics. The most recent pandemic occurred in 2009 with the introduction of the H1N1pdm09 virus ("swine flu") to the human population (Webster and Govorkova, 2014).

Due to the fact that influenza RNA polymerase lacks error-correcting activity, the rate of mutation in viral progeny is very high (Sanjuán et al., 2010). This leads to rapid evolution of the virus, allowing it to escape from antigen-specific immune responses (antigenic drift). For this reason, the antigenic properties of circulating influenza isolates must be continuously monitored, and the strain composition of influenza vaccines must be revisited annually to ensure that they correspond to the results of monitoring. In addition, selective pressure due to the treatment of influenza infections with antivirals further results in the rapid development of drug-resistant strains.

Anti-influenza drugs fall into four categories which differ according to their viral targets. The first category is adamantine derivatives. These compounds, amantadine (1-aminoadamantane) and rimantadine (α-methyl-1-adamantylamine hydrochloride), interfere with the virus-specific M2 proton channel, thus inhibiting acidification of the virion core and the fusion of viral and lysosome membranes (Scholtissek et al., 1998; Cady et al., 2010).

The second category contains three neuraminidase inhibitors (NAIs) that have received FDA approval, oseltamivir (Tamiflu®), zanamivir (Relenza®), and peramivir (Rapivab®) (Ison, 2013). Another NAI, laninamivir octanoate (Inavir®), is now used in Japan for the prophylaxis and treatment of influenza (Ikematsu et al., 2015). All NAIs interfere with the activity of viral neuraminidase, which is essential for the release of progeny virus particles from the cell membrane.

The third category contains the nucleoside analog favipiravir (T-705, Avigan®). It causes a suppressive effect against a wide range of

* Corresponding author.
E-mail address: zarubaev@gmail.com (V.V. Zarubaev).

https://doi.org/10.1016/j.virol.2018.08.011
Received 9 April 2018; Received in revised form 8 August 2018; Accepted 14 August 2018

human viruses by inducing significant mutagenesis in the viral genome which is, in turn, lethal to the virus (Lee and Yen, 2012). Favipiravir is approved for influenza treatment in Japan. The fourth category features Arbidol (umifenovir), which is a drug that was first developed in Russia and which is currently used in both Russia and China for influenza prevention and treatment (Pécheur et al., 2016).

Influenza's variability results in two effects. On one hand, antigenic drift can lead to escape from adaptive immunity. On the other hand, drug resistant strains can become rapidly selected (Petrova and Russell, 2018). In 2003–2004, adamantane resistance among circulating influenza A viruses rapidly increased worldwide (Hurt, 2014). For this reason, adamantanes are not currently recommended for the treatment of influenza caused by contemporary viruses (CDC, 2018). Resistance is mainly conferred by amino acid substitutions in the M2 protein, i.e. L26F, V27A, S31N, and G34E (Abed et al., 2005).

In this context, another event should be mentioned. Oseltamivir is an effective and internationally accepted anti-influenza drug. From 2007 to 2009, however, pre-pandemic H1N1 subtype influenza viruses resistant to it emerged and spread, resulting in almost 100% resistance (Samson et al., 2013). Fortunately, the pandemic H1N1pdm09 virus (of swine origin) appeared. It is oseltamivir-susceptible and has displaced all of the previously circulating oseltamivir-resistant strains (Hussain, 2017). These facts demonstrate the serious challenges encountered when searching for and developing novel anti-influenza drugs with broad spectrum or alternative mechanisms of action.

Previously, we identified derivatives of camphor as effective inhibitors of influenza virus replication in cell culture (Sokolova at al, 2013, 2014, 2017). Camphecene ((+)-1,7,7-trimethylbicyclo[2.2.1] heptan-2-ylidene-aminoethanol) is one of the most effective of these compounds (Sokolova et al., 2015). It is most effective when added to cell culture in the early stages of the viral life cycle (0–2 h p.i.) (Zarubaev et al., 2015). By fusion inhibition assay, camphecene was shown to decrease the HA activity of influenza A and B viruses. Camphecene activity was further confirmed in experiments with influenza virus-infected mice. Later, we developed and validated a method for the quantification of camphecene in whole rat blood, using dried blood spots and LC-MS/MS, and determined the main pharmacokinetic parameters of the compound after intravenous administration (Rogachev et al., 2016). In the present work, we describe the selection of a camphecene-resistant variant of the influenza virus, as well as elaborate on its pathogenicity in animals and on its amino acid substitutions as compared to the parent virus.

2. Materials and methods

2.1. Compounds

Camphecene ((+)-1,7,7-trimethylbicyclo[2.2.1]heptan-2-ylidene-aminoethanol) was synthesized from (+)-camphor and ethanolamine according to previously described procedures (Sokolova et al., 2015).

2.2. Viruses and cells

The A/Puerto Rico/8/34 (H1N1) influenza virus was obtained from the collection of viruses of Smorodintsev Research Institute of Influenza. In preparation for experimentation, the virus was propagated in the allantoic cavities of 9–11 day old chicken embryos for 48 h at 36 °C. The infectious titer of the virus was determined in MDCK cells (ATCC # CCL-34) using 96-well plates.

2.3. Animals

Female BALB/c mice, 6–8 weeks old, were obtained from the Russian Academy of Medicine's Rappolovo animal breeding facility (Rappolovo, Russia), and they were quarantined for one week prior to experimental manipulation. Mice were fed standard rodent feed and were provided unlimited access to water. Animal experiments were conducted in accordance with the principles of proper laboratory animal care (*Guide for the Care and Use of Laboratory Animals*, National Academy Press, Washington DC, 1996) and were approved by the Institutional Ethical Committee.

2.4. Virus resistance assay

Camphecene in serial concentrations (500–1.5 μM) was incubated with MDCK cells for 1 h at 36 °C. The cell culture was then inoculated with influenza virus (moi 0.01) for 1 h. Unbound virus was washed off with MEM and a fresh portion of camphecene was added. The plates were then incubated for 24 h at 36 °C in the presence of 5% CO_2. The virus titer in the culture medium was determined in a separate passage after 48 h of cultivation. To detect the virus, the medium (100 μl) was placed into the wells of a round-bottom plate, and an equal amount of a 1% suspension of chicken erythrocytes in saline was added. The reaction was evaluated after 60 min of incubation at room temperature. Each concentration of the compounds was tested in triplicate. Virus titers were plotted against the logarithm of concentration, and the IC_{50} values for each virus were calculated using GraphPad Prism software.

2.5. In vitro selection and analysis of resistant mutants

In order to study the development of resistance to camphecene, the A/Puerto Rico/8/34 (H1N1) (PR8) influenza virus was serially passaged in MDCK cells in the presence of increasing concentrations of the compound resulting in CF+ virus. Cells were infected with the virus and incubated for 3–5 days at 36 °C with 5% CO_2 until a cytopathic effect was observed. The culture supernatants were centrifuged, and aliquots were used for sequential selection. Two initial passages were performed in the presence of 100 μM camphecene followed by four passages at 200 μM. The control virus (CF-) was serially passaged the same number of times in MDCK cells in the absence of the antiviral agent.

After the passaging procedure, we had three viruses: the initial PR8 that was not passaged; CF-; and CF (two wells each). Culture media from two parallel wells of these viruses were mixed and viruses were plaque-purified in MDCK cells. Three clones of each virus were propagated in chicken embryos in the absence or presence of 200 μM camphecene. Viral RNA was extracted using an RNAzol® kit (Gibco BRL). After reverse transcription, cDNA was amplified using four pairs of primers (Table 1).

The cycling conditions were as follows: (i) denaturation (3 min at 95 °C), (ii) 20 cycles of 95 °C for 30 s, 60 °C for 30 s, 72 °C for 45 s and (iii) 30 cycles of 95 °C for 30 s, 50 °C for 30 s, 72 °C for 60 s with final elongation at 72 °C for 7 min. PCR products were sequenced using an ABI PRISM 3100-Avant Genetic Analyzer (Applied Biosystems, USA) using the BigDye Terminator Cycle Sequencing Kit and ABI PRISM 3100-Avant Data Collection software provided by manufacturer. All sequences were compared with the amino acid sequence of A/Puerto Rico/8/34 virus (access code P03452).

Table 1
Primers used for sequencing of the hemagglutinin genes of influenza viruses.

Pair	Name	Type	Sequence (5′→3′)
1	PR8-HA-22F	forward	GGGAAAATAAAAACAACCAAAATG
	PR8-HA-586R	reverse	ATGAATACCCCACAGTACAAGGAC
2	PR8-HA-410F	forward	AGCTCATGGCCCAACCAC
	PR8-HA-1024R	reverse	GGATTGAATGGACGGAGTGTT
3	PR8-HA-795F	forward	CACCAATGTATGCTTTCGCAC
	PR8-HA-1314R	reverse	GCATTATATGTCCAAATGTCCAGA
4	PR8-HA-1022F	forward	TCCAGAGGTCTATTTGGAGCC
	PR8-HA-1712R	reverse	TTCTGAAATTCTAATCTCAGATGCA