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Broad range of inhibiting action of novel camphor-based compound with anti-hemagglutinin activity against influenza viruses *in vitro* and *in vivo*

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ABSTRACT

Influenza virus continues to remain one of the leading human respiratory pathogens causing significant morbidity and mortality around the globe. Due to short-term life cycle and high rate of mutations influenza virus is able to rapidly develop resistance to clinically available antivirals. This makes necessary the search and development of new drugs with different targets and mechanisms of activity. Here we report anti-influenza activity of camphor derivative 1,7,7-trimethylbicyclo[2.2.1]heptan-2-ylidene-aminoethanol (camphecene). In *in vitro* experiments it inhibited influenza viruses A(H1, H1pdm09, H3 and H5 subtypes) and B with EC50's lying in micromolar range. Due to low cytotoxicity it resulted in high selectivity indices (74–661 depending on the virus). This effect did not depend on susceptibility or resistance of the viruses to adamantane derivatives amantadine and rimantadine. The compound appeared the most effective when added at the early stages of viral life cycle (0–2 h p.i.). In direct hemagglutinin inhibition tests camphecene was shown to decrease the activity of HA's of influenza viruses A and B. The activity of camphecene was further confirmed in experiments with influenza virus-infected mice, in which, being used orally by therapeutic schedule (once a day, days 1–5 p.i.) it decreased specific mortality of animals infected with both influenza A and B viruses (highest indices of protection 66.7% and 88.9%, respectively). Taken together, these results are encouraging for further development of camphecene-based drug(s) and for exploration of camphor derivatives as highly prospective group of potential antivirals.

1. Introduction

Influenza virus is one of the human respiratory pathogens of great importance. Due to short-term lifecycle and lack of error-correcting activity of polymerase, influenza virus is able to undergo rapid mutation. Moreover, segmented organization of genome provides a genetic basis for reassortment leading to emergence of novel variants of the virus with pandemic potential. The recent example of this is the 2009 H1N1 pandemic ("swine flu") (http://www.cdc.gov/mmwR/preview/mmwr.html/mm58d0430a2.htm). During inter-pandemic periods, influenza causes annual epidemics (seasonal influenza). Although not as dramatic as pandemics, seasonal influenza is nevertheless responsible for approximately 5–20% morbidity of the population, and 3300–48,600 (average 23,600) lethal outcomes yearly (CDC, 2011).

The most common and effective strategy to combat infection is vaccination. Owing to antigenic drift, influenza viruses can avoid the neutralizing activity of previously developed antibodies thus making them ineffective against next-year infection. Therefore, despite high efficacy of vaccination, its main disadvantage is necessity to annual verification of influenza vaccine strain composition to match circulating viral strains.

Chemotherapy represents another strategy of influenza prevention and treatment. Despite numerous virus-specific components that can be potential targets for drug intervention, only a few drugs are now used in clinical practice. Three classes of compounds are currently used as anti-influenza drugs: adamantane derivatives blocking virus-specific proton channel M2 (Scholtissek et al., 1998; Cady et al., 2010) and two neuraminidase inhibitors: zanamivir (Relenza®) and oseltamivir (Tamiflu®) approved by FDA. Also, FDA has issued an Emergency Use Authorization (EUA) for the clinical use of the unapproved drug Peramivir (Rapiacta®). In addition, laninamivir (Inavir®) was approved for influenza

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treatment in Japan in 2010 and for prophylaxis in 2013 (Ison, 2013). These drugs interfere with the activity of viral neuraminidase. The efficacy of this group of drugs can be achieved only at their early use (48 h or less after onset of clinical symptoms). Nucleoside analogs ribavirin and favipiravir (T-705) exhibit a suppressive effect against almost all RNA-genome human viruses (Lee and Yen, 2012). Meanwhile, being a nucleoside analog, ribavirin possesses numerous side effects, including the reduction of hemoglobin level, neutropenia and pulmonary edema (Torriani et al., 2004).

Variability of influenza virus results, in one hand, in antigenic drift and escape from the adaptive immunity, and, from another hand, to rapid selection of drug-resistant strains. Adamantane resistance among circulating influenza A viruses increased rapidly worldwide in 2003–2004 (CDC, 2011). The resistance is mainly conferred by amino acid substitutions in M2 protein L26F, V27A, S31N and G34E (Furuse et al., 2009; Abed et al., 2005), S31N being the most important and widely distributed. In this regard, one more example should be mentioned where pre-pandemic influenza viruses of H1N1 subtype resistant to oseltamivir, which is effective and internationally accepted anti-influenza drug, emerged and spread worldwide since 2007 to 2009 having resulted in 100% resistance (Samson et al., 2013). These facts pose serious challenges for search and development of novel anti-influenza drugs with broad range and alternative mechanism of activity.

Previously we identified derivatives of camphor as effective inhibitors of influenza virus replication in cell culture against influenza virus A/California/07/09 (H1N1)pdm09 (Sokolova et al., 2013; Sokolova et al., 2014). Further in a framework of this study we discovered that the compound obtained by interaction of camphor and aminoethanol – 1,7,7-trimethylbicyclo[2.2.1]heptan-2-ylide ne-aminoethanol – demonstrates high anti-viral activity together with low toxicity. In addition, it appeared highly soluble in the water. This new imino-derivative of camphor was called Camphecene. In the present work we describe the synthesis, mode and spectrum of anti-influenza activity of this compound and provide evidence for their protective activity in animals.

2. Materials and methods

2.1. Compounds

Camphecene – 1,7,7-trimethylbicyclo[2.2.1]heptan-2-ylidene-a minoethanol (Fig. 1) was synthesized in Novosibirsk Institute of Organic Chemistry based on a previous report (Gavrilov et al., 2007). The structures of the purified compound were confirmed by 1H and 13C NMR (Bruker DRX-500). The purity of the prepared compound was determined to be >98%. Rimantadine (1-aminoethyl adamantane, Aldrich Chem. Co., Milw., WI, cat. #39.059-3) and oseltamivir phosphate (Tamiflu, Hoffmann LaRoche, Switzerland) were used as reference compounds.

2.2. Viruses and cells

Influenza viruses A/Puerto Rico/8/34 (H1N1) (A/PR (H1N1)), A/California/07/09 (H1N1)pdm09 (A/Cal (H1N1)pdm09), A/Aichi/2/68 (H3N2), A/mallard/Pennsylvania/10218/84 (H5N2) (A/mallard (H5N2)) and B/Lee/40 were obtained from the collection of viruses of Influenza Research Institute. Prior to experiment, viruses were propagated in the allantoic cavity of 10–12 day old chicken embryos for 48 h at 36 °C (influenza A viruses) or 72 h at 34 °C (influenza B virus). Infectious titer of the virus was determined in MDCK cells (ATCC # CCL-34) in 96-wells plates in alpha-MEM medium with 10% fetal bovine serum.

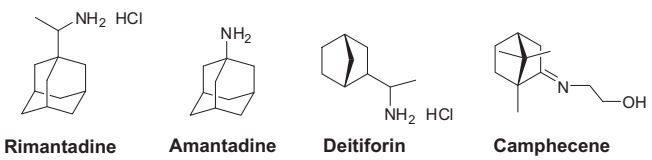

Fig. 1. Chemical structure of cage anti-influenza drugs and camphecene.

2.3. Animals

Inbred female BALB/c mice, 6–8 weeks old, were obtained from the animal breeding facility of Russian Academy of Medicine "Rappolovo" (Rappopolovo, Russia). The mice were quarantined 48 h prior to the experimental manipulation and were fed standard rodent chow and had *ad libitum* access to water. Animal experiments were conducted in accordance with the principles of laboratory animals care (Guide for the Care and Use of Laboratory Animals, National Academy Press, Washington DC, 1996) and approved by the Institutional Ethical Committee.

2.4. Virus titration and virus yield reduction assay

The compounds in appropriate concentrations were dissolved in MEM with 1 μg/mL trypsin and incubated with MDCK cells for 1 h at 36 °C. Each concentration of the compounds was tested in triplicate. The cell culture was then washed twice with phosphate-buffered saline (PBS) and incubated with appropriate viruses (m.o.i. 0.01) for 1 h. The monolayers were washed twice with PBS, and the same compound-containing medium was added. The plates were incubated for 48 h (influenza A) or 72 h (influenza B) at 36 °C in the presence of 5% CO_2. A virus titer in the supernatant was further determined by $TCID_{50}$ assay by MTT test (Mosmann, 1980) after cultivating of the virus in MDCK cells for 48 h (influenza A) or 72 h (Influenza B) at 36 °C in the presence of 5% CO_2. For calculations, virus titer was expressed as percent of the titer in control wells without compounds. The 50% and 90% inhibiting concentrations (IC_{50} and IC_{90}, see below) of the drug, that are, the concentrations at which the virus production decreased two- or ten-fold, correspondingly, and the selectivity index (the ratio of CTD_{50} to IC_{50}) were calculated from the data obtained.

2.5. Time-of-addition experiments

To determine the stage of the viral life cycle that is affected with the compound, cells were seeded into 24-wells plates and incubated with influenza virus A/Puerto Rico/8/34 (H1N1) (m.o.i. 10) for 1 h at 4 °C. After washing of non-absorbed virions for 5 min with MEM, plates were incubated for 8 h at 36 °C at 5% CO_2. The starting point of this incubation was referred as 0 h. Camphecene (final concentration 200 μmol/L) was dissolved in MEM and cells were treated with camphecene for the time periods as following: (−2) – (−1) (before infecting); (−1) – 0 (simultaneously to absorbtion); 0 – 2; 2 – 4; 4 – 6; 6 – 8 h post infection (hpi). The treatment (−2) – 8 hpi was considered as a positive control. In each case after incubation camphecene was removed and cells were washed for 5 min with MEM. After 8 h of growth, the infectious titer of the virus was determined in culture medium and cells as described above.

2.6. Hemolysis assay

The membrane-disrupting activity of viral hemagglutinin was measured according to Maeda and Ohnishi (1980) with slight modifications. Briefly, chicken erythrocytes were washed twice with