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Antiviral activities of 2,6-diaminopurine-based acyclic nucleoside phosphonates against herpesviruses: *In vitro* study results with pseudorabies virus (PrV, SuHV-1)

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A B S T R A C T

Pseudorabies virus (PrV), a causative agent of Aujeszky's disease, is deadly to most mammals with the exception of higher primates and men. This disease causes serious economic loses among farm animals, especially pigs, yet many European countries are today claimed to be Aujeszky's disease free because of the discovery of an efficient vaccination for pigs. In reality, the virus is still present in wild boar. Current vaccines are neither suitable for dogs nor are there anti-PrV drugs approved for veterinary use. Therefore, the disease still represents a high threat, particularly for expensive hunting dogs that can come into close contact with infected boars. Here we report on the anti-PrV activities of a series of synthetic diaminopurine-based acyclic nucleoside phosphonate (DAP-ANP) analogues. Initially, all synthetic DAP-ANPs under investigation are shown to exhibit minimal cytotoxicity by MTT and XTT tests (1–100 μM range). Thereafter *in vitro* infection models are established using PrV virus SuHV-1, optimized on PK-15 and RK-13 cell lines. Out of the six DAP-ANP analogues tested, analogue VI functionalized with a cyclopropyl group on the 6-amino position of the purine ring proves the most effective antiviral DAP-ANP analogue against PrV infection, aided by sufficient hydrophobic character to enhance bioavailability to its cellular target viral DNA-polymerase. Four other DAP-ANP analogues with functional groups introduced to the C2'position are shown ineffective against PrV infection, even with favourable hydrophobic properties. Cidofovir®, a drug approved against various herpesvirus infections, is found to exert only low activity against PrV in these same *in vitro* models.

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1. Introduction

Pseudorabies, also known as "Aujeszky's disease", is an acute, frequently fatal disease caused by PrV, also known as suid herpesvirus 1 (SuHV-1), which belongs to the genus *Varicellovirus*, in the Alphaherpesvirinae subfamily of the family *Herpesviridae* (Pomeranz et al., 2005). Among the most known viruses in the *Varicellovirus* genus are varicellazoster virus (VZV), bovine herpesvirus 1 (BHV-1) and equine herpesvirus 1 (EHV-1) (McGeoch and Cook, 1994). PrV is a pathogen spread mostly among swine and is lethal for almost all mammals except for higher primates and humans. Only wild pigs are able to survive infection and they serve as a natural reservoir of PrV. Dogs, especially hunting dogs, can be infected with this virus by close

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contact with the blood or saliva of shot and wounded wild boar. Consumption of contaminated raw pork or offal is another source of fatal infection in farm and companion dogs (Zhang et al., 2015; Cay and Letellier, 2009). Much has been discovered so far about PrV neurovirulence, neuropathogenesis, the properties of viral proteins involved in those processes, and genome sequences. PrV has also been used as a model herpesvirus to study virus biology and may be used for tracing neural pathways (Pomeranz et al., 2005). While in swine, PrV is transferred to various organs by viremic and lymphatic pathways (Wittmann et al., 1980), there is no evidence of viral replication in the tissues of experimentally infected dogs, except for in tissues of the nervous system. Therefore, the non-neural tissue damage in infected dogs is primarily induced indirectly by PrV. Histological findings in the central nervous systems (CNS) of PrV-infected dogs were found to be restricted to the brainstem (Zhang et al., 2015). Secondary findings of cardiac lesions were made in both the naturally and experimentally PrV-infected dogs (Olson and Miller, 1986). At present only vaccines for swine are available for the prophylaxis of Aujeszky's disease in pig farms. Inactivated vaccines are not effective in dogs and attenuated vaccines are lethal for dogs as well as for farm animals like lambs and sheep (Kong et al., 2013; Van Alstine et al., 1984). Some attempts to develop recombinant protein (rPrV) and DNA vaccines for swine were described in literature. Many of the rPrV vaccine candidates that have been reported in literature albeit they have not been further pursued so are not yet commercially available (Krishnan, 2000; Dong et al., 2014; Kim et al., 2008; Van Rooij et al., 2010). Despite the significant progress made, the efficacy of potentially safe DNA vaccines requires considerable improvement owing to the high mortality rate from the disease in vaccinated animals (Woodland, 2004; Yoon et al., 2006; Fischer et al., 2003).

In the case of chemotherapy, there are no currently approved veterinary drugs against Aujeszky's disease, and there are only limited reports concerning inhibition studies focused on inhibition of PrV proliferation by various preparations. Some natural compounds have been found to be effective *in vitro* against various herpesviruses, but failed against PrV (De Almeida et al., 1998). *In vitro* inhibition of PrV replication was demonstrated for combination of Acyclovir and Ribavirin (Pancheva, 1991). The antiviral effects of lithium chloride and natural compound diammonium glycyrrhizinate were demonstrated *in vitro* on VERO cells (Sui et al., 2010). Several nucleotide based drugs like bromovinyl deoxyuridine, acyclovir and 2′-nor-2′-deoxyguanosine have been studied as *in vitro* inhibitors and in mice models *in vivo*. Mice survival was most prolonged post administration of 2′-nor-2′-deoxyguanosine (Field, 1985).

Recently, a new largescale method for synthesis of antiretroviral agent 9-[2-(*R*)-(phosphonomethoxy) propyl]-2,6-diaminopurine, (*R*)-PMPDAP, was developed (Krecmerova et al., 2013) and used for synthesis of analogues of diaminopurine-based acyclic nucleoside phosphonate (DAP-ANP) (Holy et al., 1999; Jansa et al., 2012). We tested these compound *in vitro* various herpesviruses, especially strains resistant against established antiviral drugs, including PrV which is of importance in veterinary medicine. Two of these DAP-ANP analogues were found to be highly active against PrV in model *in vitro* cell culture studies, while Cidofovir®, a marketed drug active against many viruses including herpes, adeno, polyoma, papilloma, poxviruses and retroviruses (De Clercq, 1998; De Clercq, 2002), only exerted low anti PrV activity. This is the first study focused on the antiviral effects of DAP-ANP analogues against PrV.

Table 1
Molecular formulae and weight of chosen antivirotics.

Compound	Molecular formula	Molecular weight (Da)
I	$C_8H_{13}N_6O_4P$	288
II	$C_9H_{12}N_6O_5PNa_2$	362
III	$C_{10}H_{16}N_6O_5P$	318
IV	$C_{10}H_{14}N_5O_4P$	302
V	$C_7H_{10}N_4O_5PF_3$	365
VI	$C_{11}H_{17}N_6O_4P$	328
CIDOFOVIR	$C_8H_{14}N_3O_6P$	279

Note: I: 9-[2-(phosphonomethoxy) ethyl]-2,6-diaminopurine; II: (*S*)-9-[2-hydroxy-3-(phosphonomethoxy) propyl]-2,6-diaminopurine; III: (*R*)-9-[3-hydroxy-2-(phosphonomethoxy) propyl]-2,6-diaminopurine; IV: (*R*)-9-[2-(phosphonomethoxy) propyl]-2,6-diaminopurine; V: (*R,S*)-9-[3,3,3-trifluoro-2-(phosphonomethoxy) propyl]-2,6-diaminopurine; VI: *N* 6-cyclopropyl-9-[2-(phosphonomethoxy) ethyl]-2,6-diaminopurine.

2. Materials and methods

2.1. Syntheses and structure of nucleotide analogues

A series of 6 DAP-ANP analogues (Table 1 and Fig. 1) differing in their hydrophobicity and substitution in the side chain were synthesized according to previously reported methods (Krecmerova et al., 2013; Holy et al., 1999; Jansa et al., 2012; Holy et al., 2001; Jansa et al., 2011). Cidofovir® (active compound (*S*)-HPMPC); (*S*)-1-[3-hydroxy-2-(phosphonylmethoxypropyl)]cytosine as well as its inactive (*R*)-isomer were kindly gifted by prof. Antonín Holý.

2.2. Chromatographic analyses HPLC–MS/MS and hydrophobicity of tested compounds expressed as capacity factor

Sample analyses were performed by using high performance liquid chromatography in tandem with mass spectrometry. An Agilent 1200 chromatographic system (Agilent Technologies, Germany), consisting of binary pump, vacuum degasser, auto sampler, UV detector and thermostat column compartment, was used. Separation of modified nucleotides was carried out using Zorbax Eclipse Plus, 2.1 × 150 mm, 3.5 μm particle size column (Agilent Technologies, USA) under isocratic conditions. Mobile phase contained 0.8% of methanol and 0.1% of formic acid in water. The flow rate of the mobile phase was 0.25 ml/min, the column temperature was set at 40 °C. UV detector was used for determination of nucleotides capacity factors. A triple quadrupole mass spectrometer Agilent 6410 Triple Quad LC/MS (Agilent Technologies, USA) with an electrospray interface (ESI) was used for quantification of tested compounds in cells. The mass spectrometer was operated in the positive ion mode. Multiple reaction monitoring (MRM) with the mass transitions *m/z* 329.1–191.2, 216.9, 247.0 and 281.2 was used. Relative hydrophobicity of tested compounds was expressed as capacity factor *k'* to compare their potential to penetrate cell membranes.

2.3. In vitro *testing—tissue culture*

MDCK, MDBK, PK-15, RK-13, VERO cell lines were tested to select appropriate cell line for *in vitro* assays. Cell line PK-15 and RK-13 were selected as the best model with respect to virus multiplication and sensitivity for XTT/MTT cytotoxicity tests. The data obtained on these cell lines gave long term coherent results. The cultures were grown in Dulbeco's minimal essential medium (DMEM/High Glucose, HyClone, Thermo Scientific) supplemented with antibiotics, fungizone and 10% foetal bovine serum (FBS). The cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2 (HERAcell 150i CO2 incubator, Thermo Scientific, Germany).

2.4. In vitro *testing —virus strain*

SuHV-1CAPM V-166 isolate was obtained from the Collection of Pathogenic Microorganisms (Veterinary Research Institute, Brno) and was used throughout these experiments. This isolate was

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