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# Nucleoside and Nucleotide Analogues for the Treatment of Herpesvirus Infections: Current Stage and New Prospects in the Field of Acyclic Nucleoside Phosphonates

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## 1. Introduction

Viral infections have accompanied humankind from time immemorial. Herpesvirus infections, especially those caused by HSV-1 and HSV-2, are among the most common viral infections at all. The control and treatment of the virus has changed through history, starting with cauterization with hot iron, recommended in ancient Rome, through the most diverse herbal treatments and naturopathic remedies including miracle diets. Fundamental change in the approach to the whole issue occurred in the second half of the twentieth century with discoveries in the field of nucleic acid chemistry. Explanation of nucleic acid structure initiated not only enormous interest and the subsequent boom of molecular biology and genetics but also the formation of a new field of organic chemistry – chemistry of nucleic acid components: nucleosides, nucleotides and oligonucleotides.

## 2. Chemically modified nucleosides: First generation of antimetabolites of nucleic acids

It was shown that chemically modified nucleosides or nucleobases can work as antimetabolites in the process of nucleic acid metabolism. The word “antimetabolite” means, generally, a chemically modified molecule of a natural metabolite able to influence some enzyme reactions. Antimetabolites can influence processes in cells (neoplasia) as well as processes in cell parasites, e.g. viruses, parasites, and fungi. The first generation of antimetabolites is distinguished by a maximum structural resemblance to natural metabolites. Many nucleoside or nucleobase analogues from this group were found to be effective cytostatics: cytosine arabinoside (Cytarabine, Ara-C), an antileukemic agent used for the treatment of acute myeloid leukemia and non-Hodgkin’s lymphoma, 6-mercaptopurine (Purinethol®, 6-MP) used for the acute lymphoblastic leukemia, 5-fluorouracil (Efudex®, 5-FU), a thymidylate synthase inhibitor used for the treatment of colon, rectal, stomach, skin, breast and pancreatic cancer, and finally, antileukemic agents 5-azacytidine (Vidaza®) and 2'-deoxy-5-azacytidine (decitabine, Dacogene®), so far the most

successful agents approved for therapy of myelodysplastic syndrom. Discoveries of these great molecules came in the sixtieth decade of the last century, a period of the beginning of great development of nucleoside chemistry. This chemistry was substantially supported by the pharmaceutical industry with the intent to finally find an effective medicine against the terror of mankind known as cancer. In these times, viral infections were not in the forefront.

### 3. Acyclic analogues of nucleosides: The second generation of antimetabolites

The situation had changed by the end of the 1960s and at the beginning of the 1970s. The turning point was a large “epidemy” of genital herpes (HSV-2) in the USA, especially widespread due to the promiscuous lifestyle of the time. This sexually transmitted disease became a common problem for the whole society, and a fast solution got priority. The great success came with the synthesis and clinical development of acyclovir (Zovirax) by Burroughs-Wellcome. So far this drug is one of the most frequently used drugs against HSV-1 and HSV-2 infections. Among others, development of acyclovir resulted in the first Nobel Prize targeted to the pharmaceutical industry: in 1988 the Nobel Prize winners for medicine were Gertrude B. Elion and Georg Hitchings from the above mentioned company. Acyclovir, 9-(2-hydroxyethoxymethyl)guanine, similar to the many other antiherpetic agents (penciclovir, ganciclovir and their prodrug forms), belongs to the group of acyclic nucleoside analogues, compounds having the sugar furanose ring substituted with a polyhydroxylic carbon chain (Fig. 1). These compounds represent the so-called second generation of antimetabolites where the structural resemblance to a natural metabolite is only present in some basic aspects and the necessary steric arrangement can be formed by its subsequent contact with a target metabolic enzyme.

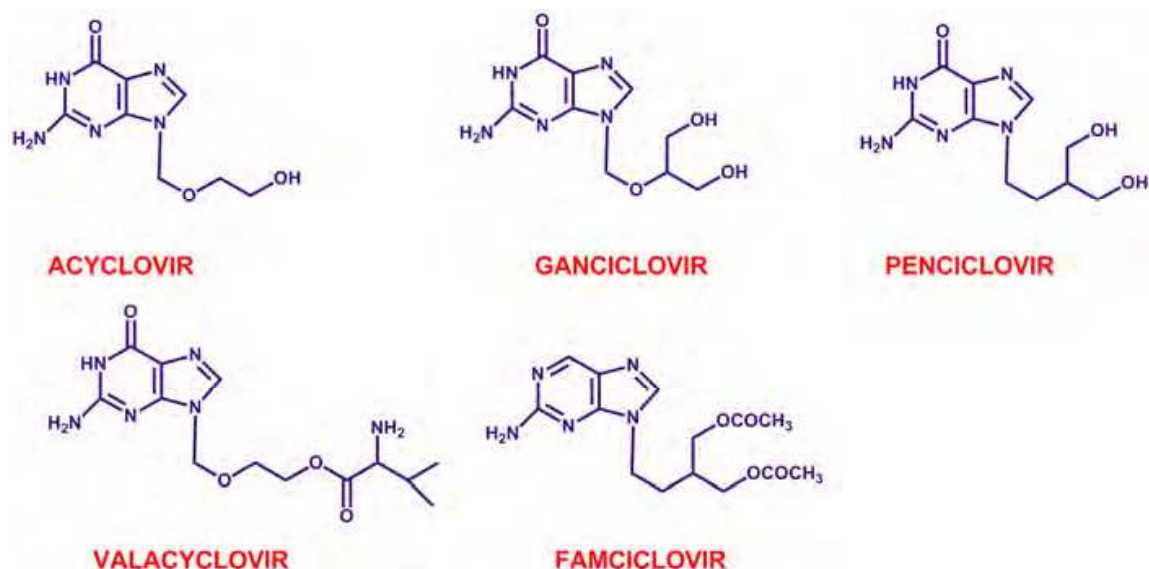


Fig. 1. Structures of the most commonly-used antiherpetic drugs from the family of acyclic nucleoside analogues

Fundamental developments in this field were also made in Prague at the Institute of Organic Chemistry and Biochemistry in the laboratory of Antonin Holy. His synthetic effort and close collaboration with the Belgian virologist Erik De Clercq resulted in many new

acyclic nucleoside analogues whose activity against herpes viruses was confirmed. The most potent agents were found to be *N*<sup>9</sup>-alkyl derivatives of adenine with hydroxyl group(s) on the alkyl chain (*RS*)-3-(adenin-9-yl)-2-hydroxypropanoic acid (AHPA, especially in the form of alkyl esters), D-eritadenine and the broad-spectrum antiviral agent (*S*)-(2,3-dihydroxypropyl)adenine (DHPA) (De Clercq et al. 1978, De Clercq & Holý 1985, Holý et al. 1982). Antiviral potency of these adenosine analogues concerns their inhibition of SAM-dependent methylation reactions via inhibition of S-adenosylhomocysteine (SAH) hydrolase. SAH is the product of S-adenosylmethionine (SAM) mediated transmethylation reactions and it is itself a feedback inhibitor of these reactions. If SAH hydrolase is inhibited, SAH accumulates and thereby all biological processes that require intensive methylations are suppressed. One such situation is the maturation of viral mRNA, i.e., 5'-cap formation and subsequent blocking virus replication. The most effective inhibitor of SAH hydrolase, DHPA became an approved drug for the topical treatment of herpes labialis (HSV-1) in the former Czechoslovakia, marketed under the name Duvira<sup>®</sup> gel (Fig. 2).

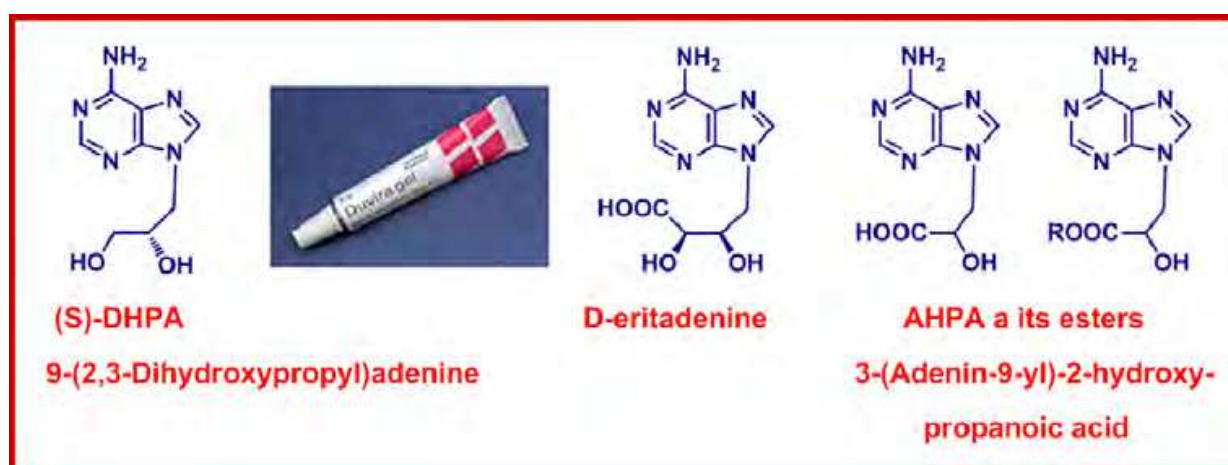


Fig. 2. Aliphatic analogues of adenosine as inhibitors of SAH hydrolase: DHPA ("Duvira gel"), D-eritadenine and AHPA esters

#### 4. Acyclic nucleoside phosphonates

The necessary condition for any antimetabolite to be of use in enzyme reactions is its phosphorylation. Unfortunately, the direct medical application of phosphates of the antimetabolites has no advantage due to their instability. To overcome this problem, in the mid-1980s, our Prague team lead by Antonin Holý came up with the revolutionary idea of preparation of phosphonomethyl derivatives: compounds stabilized by the introduction of a methylene bridge between a phosphonic acid residue and the rest of the molecule. During the short time, acyclic nucleoside phosphonates (ANPs) were revealed as compounds with an extraordinary broad spectrum of biological activities: antiviral, cytostatic, antiparasitic or immunomodulatory. Some of them are commercially available pharmaceuticals effective against serious viral infections (cidofovir, adefovir and tenofovir) (Holý, 2003; De Clercq & Holý, 2005). Several comprehensive reviews on ANPs as antiviral agents have been published recently (Holý & De Clercq, 2010; De Clercq, 2011).

The mentioned compounds represent three different types of ANPs: HPMP derivatives, i.e. (*S*)-[3-hydroxy-2-(phosphonomethoxy)propyl] derivatives (e.g. HPMP, cidofovir), PME

derivatives, i.e. 2-(phosphonomethoxy)ethyl derivatives (e.g. PME<sub>A</sub>, adefovir) and PMP derivatives, i.e. (*R*)-2-(phosphonomethoxy)propyl derivatives (e.g. PMP<sub>A</sub>, tenofovir).

#### 4.1 (*S*)-[3-Hydroxy-2-(phosphonomethoxy)propyl] derivatives (HPMP derivatives) – Synthesis and activity of HPMP<sub>A</sub> and its aza/deaza analogues

HPMP derivatives are active against DNA viruses; the activity is bound always to *S* enantiomers. The first described ANP was 9-(*S*)-[3-hydroxy-2-(phosphonomethoxy)propyl]adenine (HPMP<sub>A</sub>), a compound derived from above mentioned DHP<sub>A</sub> by the introduction of a phosphonomethyl group (De Clercq et al., 1986; Holý & Rosenberg, 1987).

Synthesis of (*S*)-HPMP derivatives mostly utilizes basically catalyzed nucleophilic opening of the oxirane ring in (2*S*)-2-[(trityloxy)methyl]oxirane or (*R*)-glycidol butyrate with an appropriate nucleobase, e.g. *N*<sup>4</sup>-benzoylcytosine, *N*<sup>6</sup>-benzoyladenine or 2,6-bis(benzoylamino)purine (Webb et al., 1988; Brodfuehrer et al., 1994). This reaction proceeds regioselectively to *N*<sup>9</sup> position of the purine or *N*<sup>1</sup> position of the pyrimidine base. The intermediary formed 2,3-dihydroxypropyl derivatives are subsequently etherified with dialkyl (diethyl or diisopropyl) ester of tosyloxymethanephosphonate in the presence of sodium hydride. After removal of benzoyl groups and trityl, phosphonic ester groups are deprotected by the treatment with bromotrimethylsilane followed by hydrolysis (Holý, 1993)(Fig. 3).

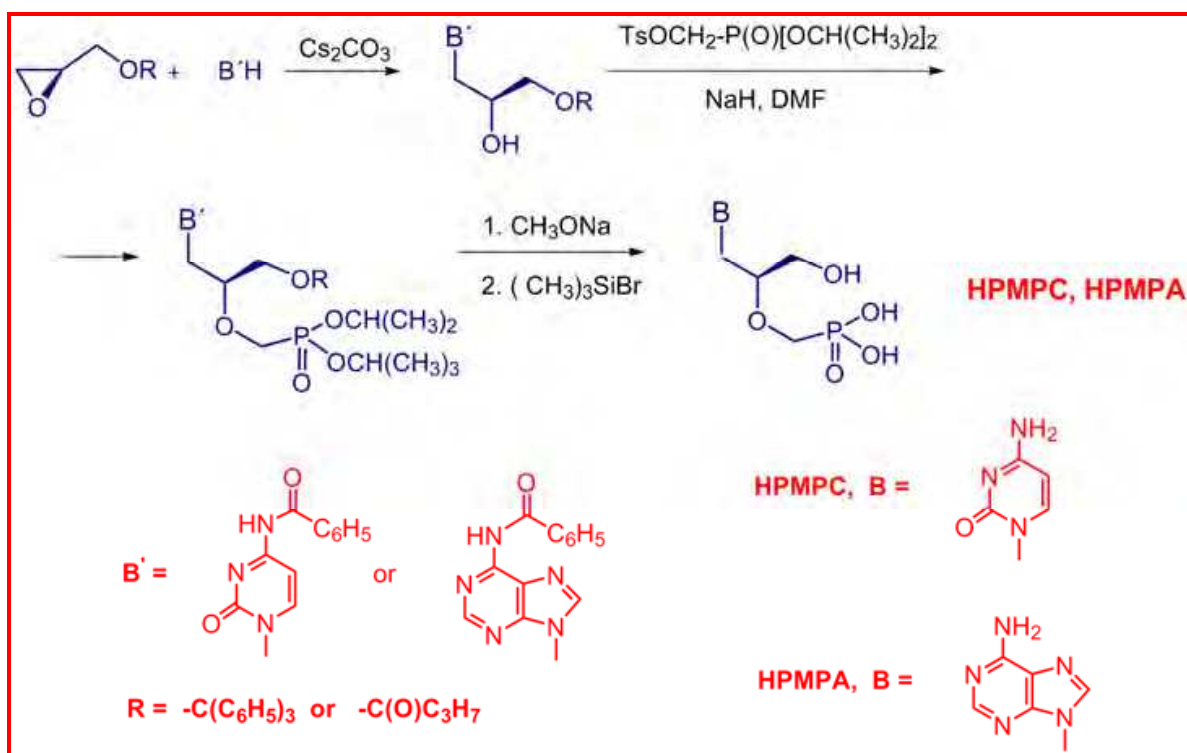


Fig. 3. Synthetic scheme for the preparation of (*S*)-[3-hydroxy-2-(phosphonomethoxy)propyl] derivatives of adenine and cytosine (HPMPC and HPMP<sub>A</sub>) from appropriate oxiranes

Preparation of diisopropyl tosyloxymethanephosphonate consists of the treatment of diisopropyl phosphite with paraformaldehyde and triethyl amine followed by tosylation



(Holý, 1993). Another possibility for the introduction of a phosphonomethyl ether group based on use of diisopropyl bromomethylphosphonate (Göbel et al., 1992) is also recommended (Holý, 2005; Oh & Hong, 2008). Most recently, a novel, very efficient and environmentally friendly synthesis of diisopropyl bromomethylphosphonate (and other dialkylhaloalkylphosphonates in general), has been developed in our Institute using a microwave-assisted Michaelis–Arbuzov reaction of dihaloalkanes. Diisopropyl bromomethylphosphonate thus can be prepared by reaction of dibromomethane with triisopropyl phosphite (Jansa et al., 2011). Syntheses of both agents are depicted in Fig. 4.

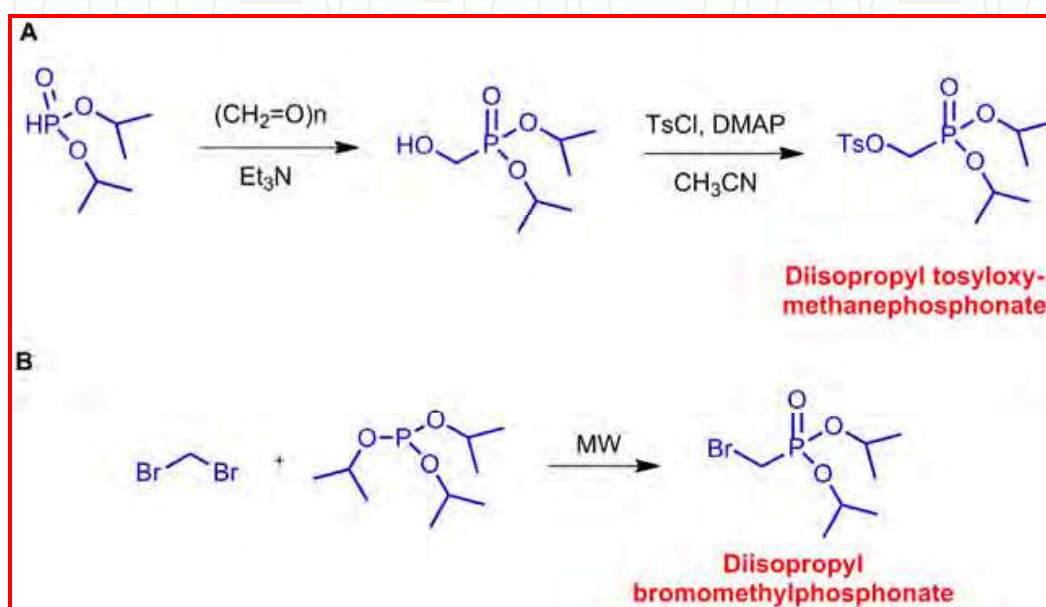


Fig. 4. Syntheses of reagents for an introduction of a phosphonomethyl residue to the molecule of acyclic nucleoside analogues: synthesis of the “Holy’s tosylate”, diisopropyl tosyloxymethanephosphonate and the microwave assisted approach to diisopropyl bromomethylphosphonate by Jansa

(S)-HPMPA is a compound with a broad spectrum of anti DNA-virus activities, so far not clinically developed. It efficiently inhibits herpesviruses (Andrei et al., 1992) including clinical isolates of varicella zoster virus (Andrei et al., 1995), HHV-6 (Reymen et al., 1995) and HHV-8 (Neyts & De Clercq, 1997). HHV-6 is one of the recently discovered members of the betaherpesviridae family. It is involved in pathogenesis of several diseases including the childhood disease exanthema subitum (roseola) (Yamanishi et al. 1988) and lymphoproliferative disorders, and it probably takes part in the progression of chronic fatigue syndrome (Komaroff, 2006). HHV-6 has also been proposed to be a cofactor in the progression of AIDS; high levels of HHV-6 have been found in many AIDS patients. The comparative study of effectiveness of diverse kinds of ANP towards HHV-6 revealed, besides HPMPA, also several other active (S)-HPMP derivatives: (S)-HPMPC, (S)-cHPMPC, (S)-3-deaza-HPMPA, (S)-3-deaza-cHPMPA, (S)-HPMPG, (S)-cHPMPG and (R)-HPMPG, their  $\text{EC}_{50}$  values ranging from 1 to 11  $\mu\text{M}$  and their selectivity index ranging from 6 to 30 (Reymen et al., 1995).

A special attention is now paid to the investigation of HPMPA prodrugs to improve the pharmacokinetic profile of this ANP, especially the development of alkoxyalkyl esters (Beadle et al., 2006). It is based on etherification of the appropriate hydroxy derivate with

alkoxyalkyl tosyloxymethanephosphonate, a phosphonomethyl residue containing an agent having the lipophilic group already preattached. This compound can be prepared in three steps from diethyl tosyloxymethanephosphonate: deprotection of ethyl ester groups with bromotrimethylsilane, transformation to chloridate by the action of oxalyl chloride, and reaction with alkoxyalkanols under basic conditions. The starting material for syntheses of HPMPA esters is (*S*)-9-(3-trityloxy-2-hydroxypropyl)-*N*<sup>6</sup>-trityladenine, an intermediate in preparation of HPMPA according to Webb's method (Webb, 1989).

The whole process is outlined in Figure 5.

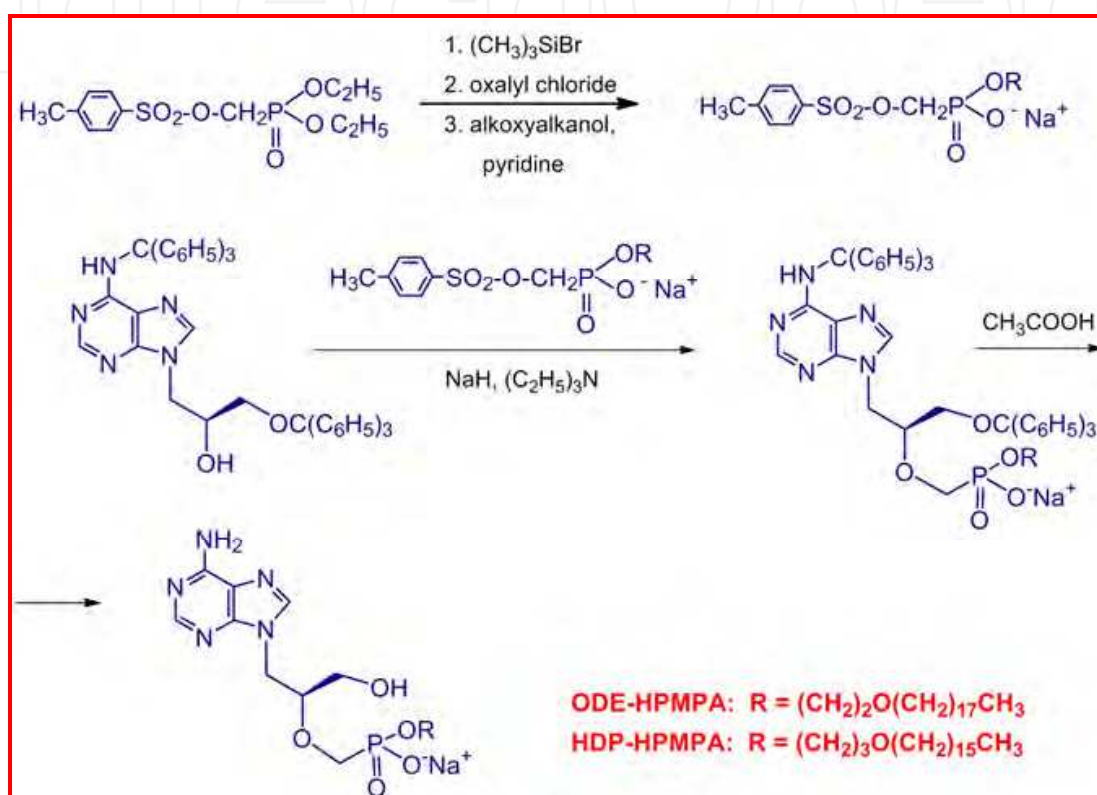


Fig. 5. Synthesis of alkoxyalkyl ester prodrugs of HPMPA.

Excellent anti-DNA virus effects were also found at other HPMP derivatives, e.g. 2,6-diaminopurine counterpart of HPMPA, (*S*)-HPMPDAP. Recently, a detailed investigation of this compound and its ester prodrugs has been performed (Krečmerová et al., 2010a). This research, originally targeted to antipoxvirus agents, also selected, among others, several candidates with excellent antiherpetic effects. Remarkable broad-spectrum antiherpetic effects were found at some base modified ANPs (aza/deaza analogs). Anti-DNA-viral activity was found especially at (*S*)-8-aza-HPMPA and (*S*)-3-deaza-HPMPA.

Syntheses of deazapurines, i.e. purines lacking one nitrogen atom in position 1, 3 or 7 and their nucleosides and ANPs, were developed by Holý and Dvořáková (Dvořáková et al., 1993; Dvořáková & Holý, 1993). Activity of (*S*)-3-deazaHPMPA against herpesviruses is comparable to that of the parent compound (*S*)-HPMPA, especially in case of VZV and CMV (Dvořáková et al., 1990). (*S*)-3-deaza-HPMPA, and its cyclic form, (*S*)-3-deaza-cHPMPA are also highly active against HHV-6 infections (Naesens and De Clercq, 2006). Evaluation of a series of diverse ANPs for activity against HHV-6 in HSB-2 cells showed 3-

deaza-HPMPA as a compound with the highest selectivity index (Reymen et al., 1995). In spite of all the progress in this topic, there is so far no approved drug for the treatment of HHV-6 infections (De Bolle et al., 2005). Moreover there is also another difficulty: no animal model for HHV-6 testing. These facts substantiate further intensive exploration of ANPs, especially 3-deaza-HPMPA and HPMPA, as potential drug candidates for the treatment of HHV-6 infections.

The synthesis of acyclic nucleotide analogues derived from 8-azapurine was first reported in 1996 (Holý et al., 1996). In the HPMP series, only (S)-HPMP-8-azaadenine exhibits remarkable inhibitory potency to all DNA viruses tested. The best data were found for HSV-1, TK<sup>-</sup> HSV, HSV-2, VZV, TK<sup>-</sup> VZV and vaccinia virus. In particular, exquisitely inhibitory effects were found for VZV with MIC 0.2-2 µg/mL. In general, the antiviral potency of HPMP-8-azaA is mostly comparable to (S)-HPMPA. Its 2,6-diaminopurine counterpart, (S)-HPMP-8-aza-DAP is approximately two orders of magnitude less potent of an inhibitor of these viruses than HPMP-8-azaA. Other structural types of 8-azapurine ANPs (PMP and (S)-FPMP derivatives) are not markedly inhibitory to any of the DNA viruses tested. In a series of PME derivatives, only PME-8-azaguanine was inhibitory to HSV-1, the thymidine kinase (TK<sup>-</sup>) deficient HSV-1 strains, HSV-2, CMV, both TK<sup>+</sup> and TK<sup>-</sup> VZV strains and vaccinia virus (Holý et al., 1996). Structures of the most important anti-DNA viral HPMP derivatives are depicted in Fig. 6.

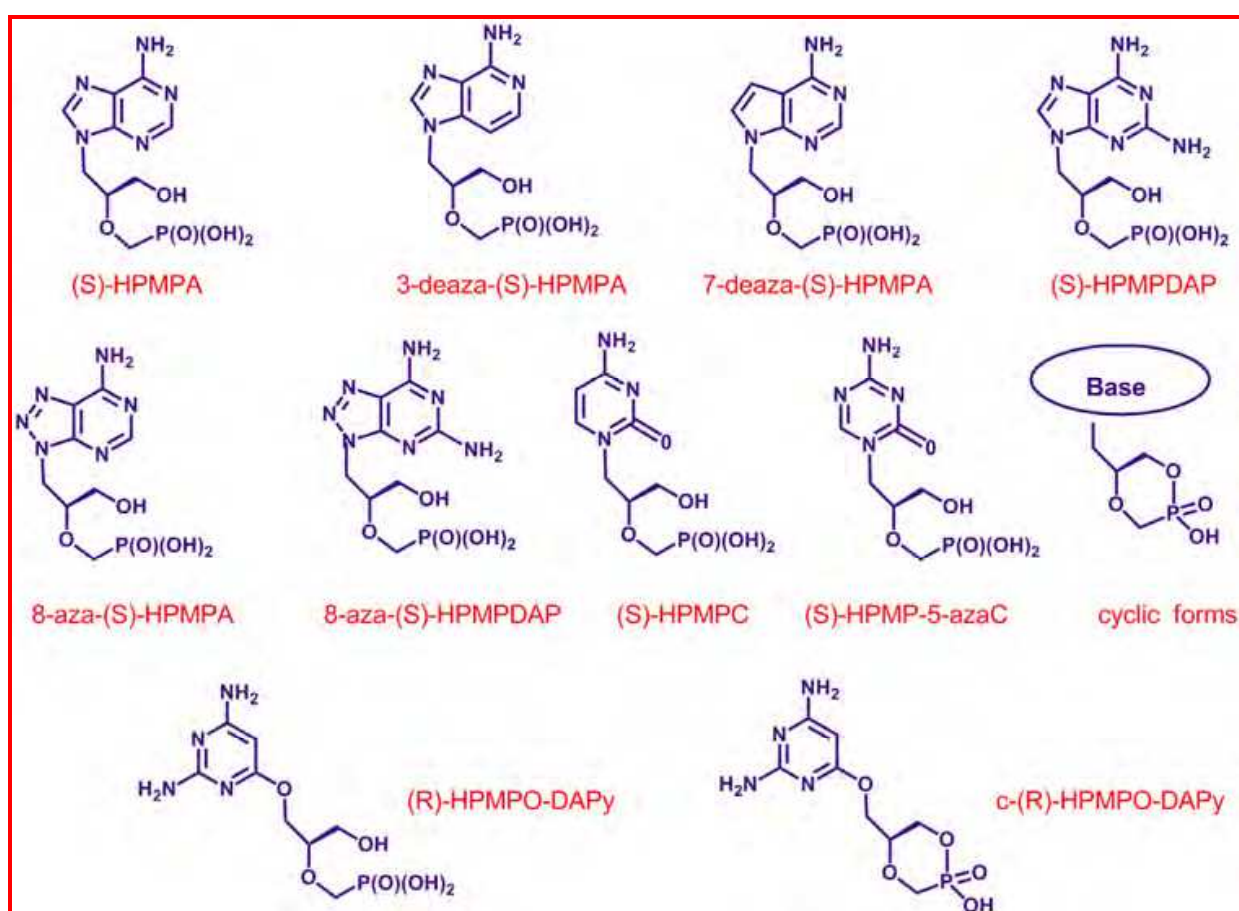


Fig. 6. HPMP derivatives: acyclic nucleoside phosphonates with a broad spectrum of activities against herpes- and other DNA virus infections



#### 4.2 Cidofovir (HPMPC)

Cidofovir, 1-(S)-[3-hydroxy-2-(phosphonomethoxy)propyl]cytosine, is a broad spectrum anti DNA virus agent whose spectrum of activities covers all types of human herpesviruses [HSV-1, HSV-2, varicella-zoster virus (VZV), Epstein-Barr virus, CMV, HHV-6, HHV-7 and HHV-8 – and also thymidine kinase-deficient (TK-) HSV and VZV, and protein kinase-deficient (PK-) CMV variants], and polyoma-, papilloma-, adeno- and poxviruses. The large scale of cidofovir activities has already been a topic of many reviews, e.g. (Hitchcock et al., 1996; Naesens & De Clercq, 1997; De Clercq, 1998; Naesens et al., 1997).

Cidofovir was the first acyclic nucleoside phosphonate utilized in clinical practice: in 1996 this drug was approved by the FDA for the treatment of cytomegalovirus retinitis in AIDS patients. The drug is marketed by Gilead Sciences, Inc. under the commercial name Vistide™ and it is applied to patients in the form of intravenous injections (Fig. 7). CMV retinitis is a systemic infection commonly seen in patients suffering from AIDS. It is most easily characterized by the cloudiness which can appear in the patient's retina. If untreated, the virus will attack retina cells and develop into lesions. These lesions can eventually lead to vision impairment or permanent blindness. Cidofovir suppresses CMV replication by the selective inhibition of viral DNA polymerase and therefore prevention of viral replication and transcription (Wachsman et al., 1996; Lalezari et al., 1997). A serious side-effect of cidofovir (and all other ANPs) is its dose-dependent nephrotoxicity. To surmount this problem, cidofovir must be administered in conjunction with probenecid (Bagnis et al., 1999; Lacy et al., 1998).

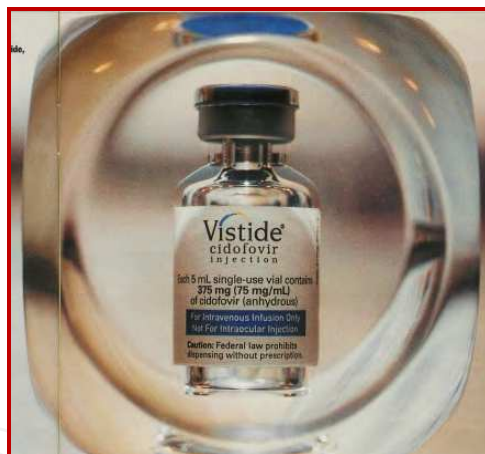


Fig. 7. Vistide™ - cidofovir injections. The first approved acyclic nucleoside phosphonate in clinical practice

At the present time, cidofovir is also important (off label) particularly for the treatment of severe cases of (malignizing) papillomatosis (anogenital, laryngeal) (Calista, 2000; Bielamowicz et al., 2002), progressive multifocal leukoencephalopathy caused by JC virus (Gasnault et al., 2001), adenovirus infections (Legrand et al., 2001) and some rather obscure severe infections caused by poxviruses (vaccinia, orf, molluscum contagiosum) (Bray & Wright, 2003). The attractiveness of cidofovir is dramatically enhanced by its supreme activity against smallpox virus and related monkey pox virus; both of these highly infectious viruses can be easily cultivated and purposely used in a bioterrorist attack. For this purpose, the cidofovir prodrug, hexadecyloxypropyl ester CMX001, is currently clinically investigated (Chimerix, Inc., California). Moreover, CMX001 is also being

developed to treat cytomegalovirus (CMV) and BK virus – potentially deadly diseases for immunocompromised patients (www.chimerix-inc.com).

#### 4.3 5-Azacytosine analogue of cidofovir (HPMP-5-azaC): Higher potency with a lower toxicity

Great success in our search for antiviral compounds came with the discovery of ANPs bearing a triazine ring, especially 5-azacytosine as a base component (Krečmerová et al., 2007a). The 5-azacytosine analogue of cidofovir, i.e. 1-(*S*)-[3-hydroxy-2-(phosphonomethoxy)propyl]-5-azacytosine (HPMP-5-azaC), shows antiviral activities similar - or in some cases higher - compared to cidofovir, better selectivity, and lower toxicity.

Synthesis of (*S*)-HPMP-5-azaC can be performed in two different manners. The first approach is based on nucleophilic opening of an oxirane ring in (2*S*)-2-[(trityloxy)methyl]oxirane with 5-azacytosine under basic conditions, e.g. in a presence of sodium hydroxide. The reaction gives regiospecifically the only N-1 substituted product, 1-[(2*S*)-2-hydroxy-3-(triphenylmethoxy)propyl]-5-azacytosine, in excellent yield. Its further treatment with diisopropyl tosyloxymethylphosphonate in the presence of excess sodium hydride in *N,N*-dimethylformamide gave fully protected phosphonate ester, which was subsequently treated with bromotrimethylsilane followed by hydrolysis. This process leads simultaneously to a removal of trityl group and phosphonate protecting diisopropyl ester groups under formation of the final HPMP-5-azaC (Fig. 8).

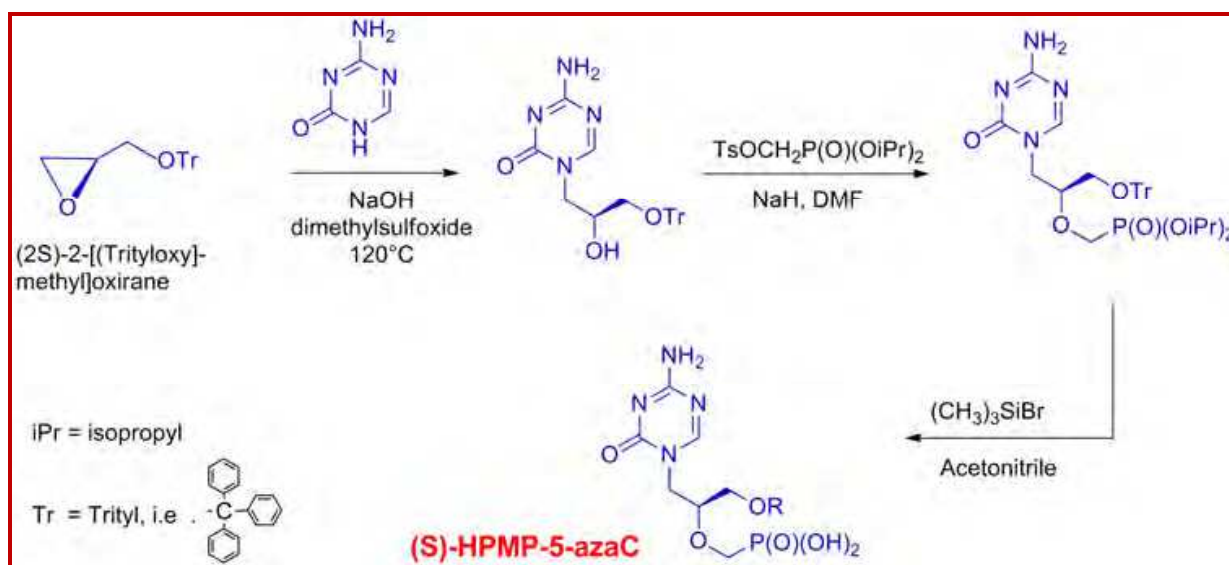


Fig. 8. Synthesis of (*S*)-HPMP-5-azaC from (2*S*)-2-[(trityloxy)methyl]oxirane. The stepwise approach

The second approach is based on alkylation of 5-azacytosine with an appropriate chiral synthon, i.e. the whole aliphatic part activated with a suitable leaving group, usually tosyl or halogen (Fig. 9). Preparation of “(*S*)-HPMP synthon”, i.e. diisopropyl ester of (1*S*)-[2-hydroxy-1-tosyloxymethyl]ethoxy]methylphosphonate followed the protocol originally developed for its racemic form (Hocek et al., 1996). Also in this case, (2*S*)-2-[(trityloxy)methyl]oxirane was selected as a starting material. The oxirane ring was first opened by nucleophilic reaction with sodium benzyloxy-3-

trityloxypentan-2-ol. The next steps are introduction of a phosphonomethyl residue using diisopropyl bromomethanephosphonate, removal of the trityl group with acetic acid, tosylation, and the final deprotection of benzyl group by catalytic hydrogenation. Removal of benzyl group just in this step, i.e. still in a stage of the synthon is necessary. Its removal in some of the following steps would not be possible due to a sensitivity of 5-azacytosine towards catalytic hydrogenation leading to 5,6-dihydro-5-azacytosine derivatives. Diisopropyl (1S)-[2-hydroxy-1-tosyloxymethyl]ethoxy]methylphosphonate (HPMP-synthon) was used for the final condensation with a sodium salt of 5-azacytosine. This condensation proceeds exclusively to form the desired N-1 isomer, i.e. diisopropyl ester of HPMP-5-azaC, accompanied by N-3 and O-isomers in a small amount only. This reaction was revealed as an advantageous approach to HPMP-5-azaC, especially for its larger-scale syntheses (Krečmerová et al., 2010b). The final deprotection of ester groups was performed by the standard procedure with bromotrimethylsilane, followed by hydrolysis.

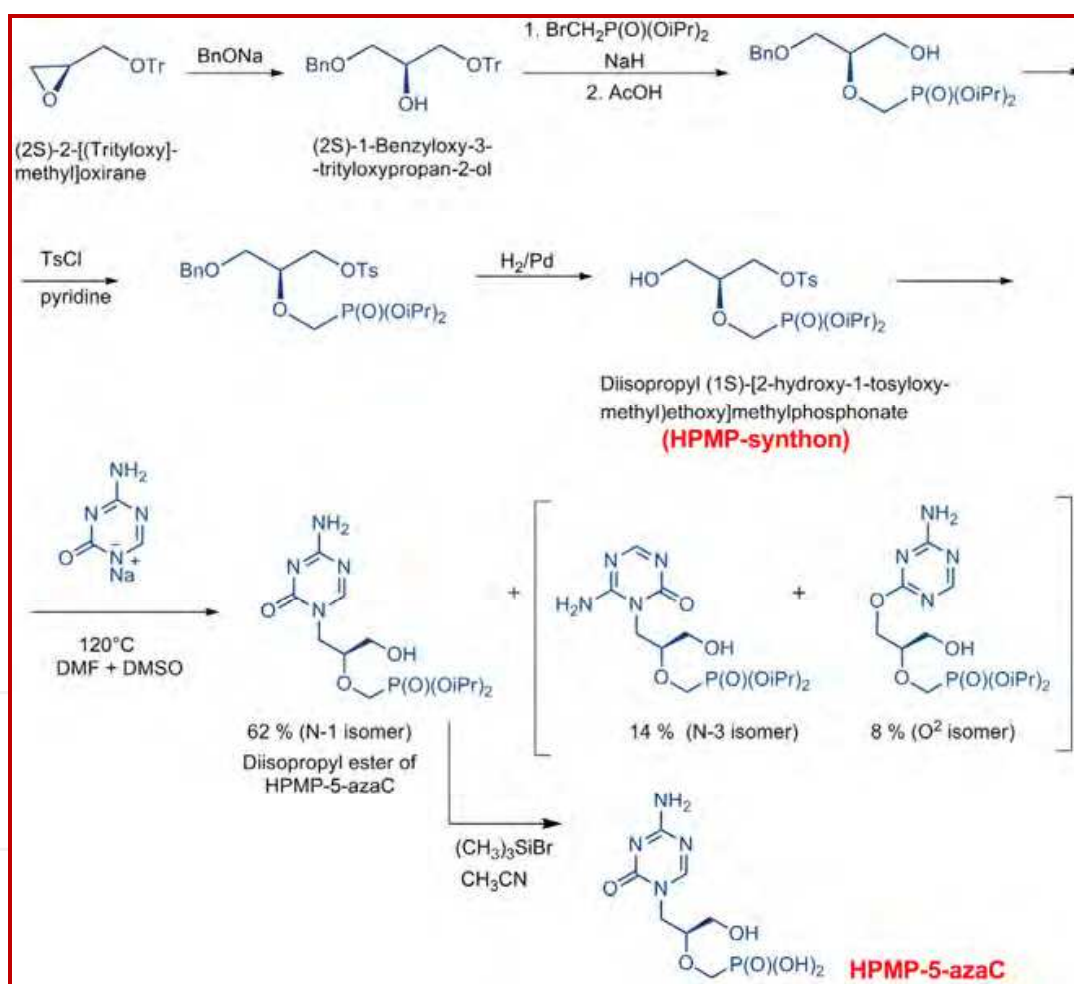


Fig. 9. Synthesis of (S)-HPMP-5-azaC by the synthon approach

HPMP-5-azaC showed potent and selective activity against several DNA viruses, including different herpesviruses (HSV-1, HSV-2, VZV, HCMV and HHV-6), adenovirus (Ad2) and poxvirus (vaccinia virus) with 50% effective concentration (EC<sub>50</sub>) values of 0.71 µg/mL for Ad2, 2.56 µg/mL for vaccinia virus and 0.02-0.6 µg/mL for herpesviruses. The antiviral activity of HPMP-5-azaC was comparable to cidofovir against HSV-1, HSV-2 and vaccinia

virus, or 2 to 7 times more active against VZV, HCMV, HHV-6 and Ad2. HPMP-5-azaC proved to be 2 times less cytotoxic for HEL cells than (S)-HPMPC but 2-fold more toxic for human T-lymphoblast HSB-2 cells. For all these DNA viruses, HPMP-5-azaC showed a 2- to 16-times higher antiviral selectivity index (ratio of  $CC_{50}$  to  $EC_{50}$ ) than (S)-HPMPC (cidofovir) (Krečmerová et al., 2007a).

In contrast to cidofovir, HPMP-5-azaC has more complicated metabolic profile, and, similar to other N-1 substituted 5-azacytosine derivatives (riboside, 2'-deoxyriboside, arabinoside), it decomposes in alkaline conditions (Dračínský et al., 2008). The first step is a reversible ring opening of the sym-triazine to the N-formylguanidine derivative which can close back to the cyclic structure. This hydrolytic reaction is slow and reaches equilibrium within several days. However, the reversible ring-opening hydrolysis is accompanied by irreversible deformylation reaction of the intermediary formyl derivative that gives rise to antivirally inactive 2-[[[(2S)-3-hydroxy-2-(phosphonomethoxy)propyl]carbamoyl]guanidine. Among these decomposition products (Fig. 10), the N-formylguanidine derivative, which can close back to the cyclic structure, showed activity with equivalent  $EC_{50}$  values to those obtained for the original compound HPMP-5-azaC (VZV and HCMV) or at 3- to 25-fold higher  $EC_{50}$ 's for HSV-1, HSV-2, HHV-6, Ad2 and vaccinia virus. In contrast, the final decomposition product, the carbamoylguanidine derivative, is antivirally inactive (Krečmerová et al., 2007a).

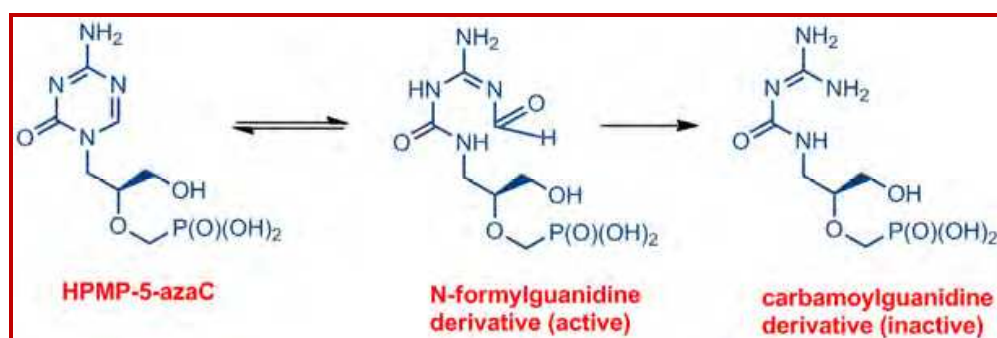


Fig. 10. The course of decomposition of (S)-HPMP-5-azaC in alkaline conditions

Investigation of the intracellular metabolism of HPMP-5-azaC revealed its phosphorylation to mono- and diphosphate (60 fold higher than cidofovir) and deaminated uracil product (HPMP-5-azaU) as a minor component (6%). HPMP-5-azaC also showed about 45-fold higher incorporation into cellular DNA than cidofovir. In general, HPMP-5-azaC has a favorable metabolic profile that is characterized by low sensitivity to catabolic deamination and high efficiency for phosphorylation and DNA incorporation (Naesens et al., 2008).

Discovery of the unique antiviral activity of HPMP-5-azaC resulted in the necessity to also prepare some kinds of prodrugs: the cyclic form of 1-(S)-[3-hydroxy-2-(phosphonomethoxy)propyl]-5-azacytosine (cHPMP-5-azaC) and its esters. Transformation of HPMP-5-azaC to its cyclic form was realized by the action of dicyclohexylcarbodiimide (DCC) and *N,N*-dicyclohexyl-4-morpholinocarboxamide. To assess the role of ester structure in antiviral activity, four diverse ester types were synthesized: alkyl (octadecyl), alkenyl (erucyl, i.e. (Z)-docos-13-enyl), pivaloyloxymethyl (POM) and alkoxyalkyl (2-(hexadecyloxy)ethyl, HDE). The most successful esterification method was the reaction of tetrabutylammonium salt of cHPMP-5-azaC with alkyl bromides or with chloromethyl pivalate, respectively (Fig. 11) (Krečmerová et al., 2007b).



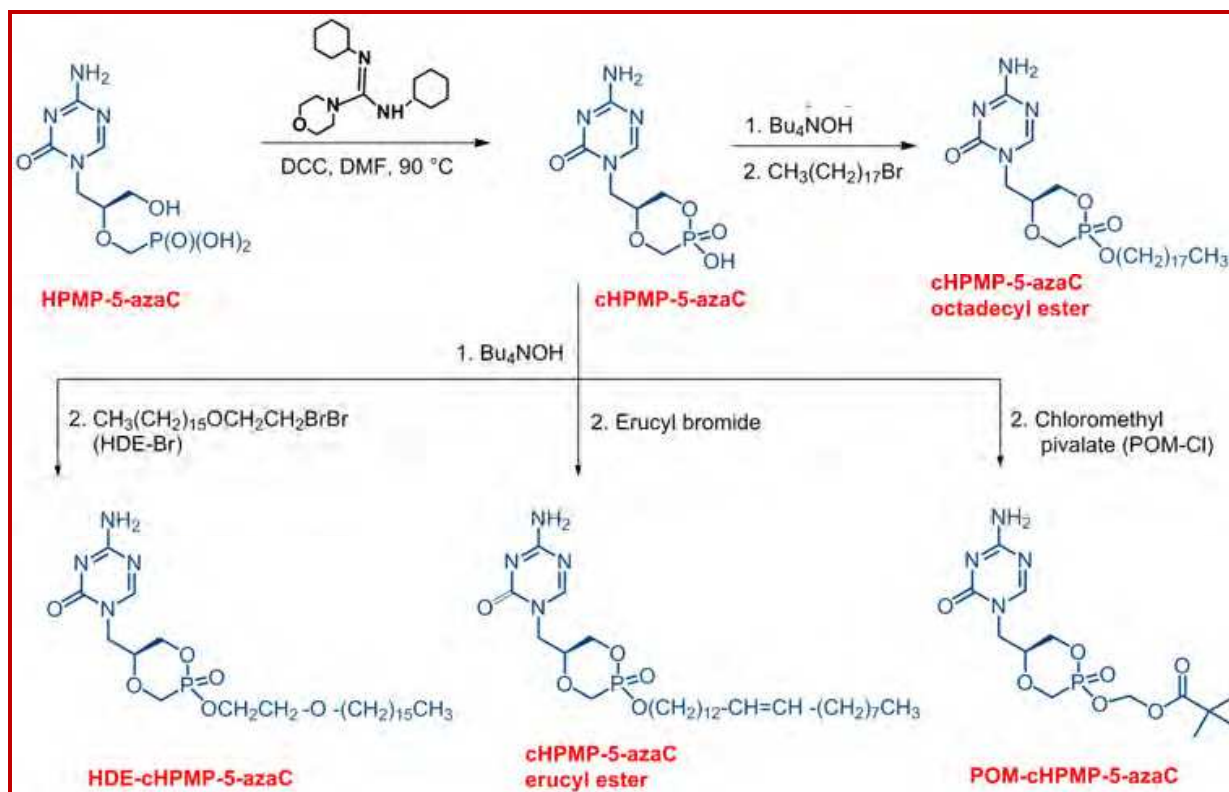


Fig. 11. Synthesis of ester prodrugs derived from the cyclic (S)-HPMP-5-azaC

cHPMP-5-azaC was able to inhibit the replication of poxviruses (vaccinia virus), and different herpesviruses, including herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2), thymidine kinase-deficient HSV-1 [acyclovir resistant (ACV<sup>r</sup>)], varicella-zoster virus (VZV), human cytomegalovirus (HCMV) and human herpesvirus 6 (HHV-6) with EC<sub>50</sub> values in the range of 0.06 to 3.1 µg/ml. cHPMP-5-azaC did not affect cell morphology or cell growth (measured on HEL cells). This resulted in selectivity indices (ratio of CC<sub>50</sub> to EC<sub>50</sub>) varying from >47 (vaccinia virus) to >1500 (HCMV). The potency of the new triazine analogues HPMP-5-azaC and cHPMP-5-azaC was comparable to that of HPMP-5-azaC and cHPMP-5-azaC; however, HPMP-5-azaC and cHPMP-5-azaC proved to be approximately 2 times less cytostatic for HEL cells than HPMP-5-azaC and cHPMP-5-azaC, resulting in a superior selectivity.

When the different ester prodrugs, i.e. octadecyl, (erucyl), alkoxyalkyl (HDE) and pivaloyloxymethyl (POM), were evaluated, the HDE emerged as the most active one, with EC<sub>50</sub> values in the range of 0.003 to 0.008 µg/mL for HSV, ≤0.0008 to ≤0.0014 µg/mL for VZV, ≤0.00014 to ≤0.00038 µg/mL (HCMV), 0.008 to 0.037 µg/mL for HHV-6 and 0.037 µg/mL for vaccinia virus. This resulted in 58- (vaccinia virus), 100- (mean for HSV strains), 123- (mean for VZV strains) to 250-fold (mean for HCMV strain) increase in antiviral activity when compared to cHPMP-5-azaC. Not only an improvement in the antiviral activity, but also an increase in selectivity, was observed for HDE-cHPMP-5-azaC. Thus, selectivity indices for HDE-cHPMP-5-azaC were found 43 (HHV-6A), 70 (HHV-6B), 173 (vaccinia virus), 1160 (HSV), ≥5800 (VZV) and ≥24600 (HCMV), as compared to 74 (HHV-6A), 11 (HHV-6B), >47 (vaccinia virus), >180 (HSV), >740 (VZV) and >1540 (HCMV) for the free cHPMP-5-azaC.

Although a marked increase in antiviral potency was noted for the octadecyl ester compared to free cHPMP-5-azaC against HCMV (EC<sub>50</sub> = 0.0014-0.0037 µg/mL), only a slight increase in activity against VZV or a decrease in potency against HSV was observed. Despite the fact

that the octadecyl ester did not affect cell growth up to a concentration of 100 µg/mL, it produced an alteration of cell morphology at a concentration  $\geq 20$  µg/mL.

The esterification of cHPMP-5-azaC to the erucyl prodrug resulted in a loss of activity against vaccinia virus and no improvement or slight decrease in activity against HSV and VZV. The pivaloyloxymethyl ester has a good potency against HCMV ( $EC_{50} < 0.032$  µg/mL), but generally, its  $EC_{50}$  values are equivalent to those of the free cHPMP-5-azaC.

Progress in the investigation of HPMP-5-azaC and its derivatives is currently still under way. The compound finished its preclinical stage of investigation; its eventual clinical development can be supported by the very promising results in *in vivo* models of poxvirus and herpesvirus infections, as well as by our recent progress on the development of new types of ester prodrugs. These results can substantially improve oral bioavailability of the compound and pharmacological properties in general. HPMP-5-azaC also has a potent activity and selectivity against polyomaviruses: murine polyoma virus, primate simian virus 40 strains and BK virus in human primary renal cells. These findings highlight HPMP-5-azaC and its derivatives as potential drug candidates against polyomavirus associated nephropathies in kidney transplant patients, a diagnosis so far with no FDA approved treatment (Topalis et al., 2011).

#### 4.4 (S)-HPMPDAP and its prodrugs – Synthesis of ester prodrugs in HPMP series

In our recent collaborative project with the Rega Institute in Belgium, we have performed a very detailed study of the antiviral efficacy of (S)-HPMPDAP against various DNA viruses, including poxviruses [i.e. vaccinia virus (VACV)] and herpesviruses [i.e. herpes simplex virus type 1 (HSV-1)] and type 2 (HSV-2), thymidine kinase-deficient HSV-1 (acyclovir-resistant, ACV<sup>r</sup>), varicella-zoster virus (VZV), human cytomegalovirus (HCMV), and human herpesvirus 6 (HHV-6)]. In the case of HSV-1, HSV-1 ACV<sup>r</sup>, HSV-2, and VZV,  $EC_{50}$  values for (S)-HPMPDAP and its cyclic form and (S)-HPMPC were similar. In contrast, (S)-HPMPC was more inhibitory towards HCMV than (S)-HPMPDAP or its cyclic form (Krečmerová et al., 2010a).

(S)-HPMPDAP, similar to other acyclic nucleoside phosphonates, represents a compound with a low bioavailability caused by the high polarity of a phosphonic group; its utilization in a prodrug form is thus highly desirable (Fig. 12).

ANPs lacking a free hydroxyl group in a side chain (PME and PMP derivatives) are mostly developed in a form of neutral bis(esters) or bis(amidates). In contrast to them, no prodrugs are commercially available in the case of HPMP derivatives, compounds having a free hydroxyl group in a side chain. Synthesis of their diesters is problematic due to a formation of corresponding cyclic phosphonates. So far, the most promising prodrugs seem to be alkoxyalkyl monoesters (Kern et al., 2002). One representative of this group, hexadecyloxypropyl ester of cidofovir (CMX001) is currently developed as antipox virus agent in clinical Phase II ([www.chimerix-inc.com](http://www.chimerix-inc.com)).

The alkoxyalkyl ester prodrugs of (S)-HPMPDAP, i.e. hexadecyloxypropyl, octadecyloxyethyl and hexadecyloxyethyl derivatives, emerged as the most potent and selective compounds against VACV with  $EC_{50}$  values consistently in the range of 0.00074–0.0012 µM. Although the alkoxyalkyl ester prodrugs proved more toxic than the parent compound (S)-HPMPDAP, either for HEL cell morphology or HEL cell growth, they were more selective than (S)-HPMPDAP, with selectivity indices [ratio =  $CC_{50} / EC_{50}$ ] higher than 10,000 compared to >625 for (S)-HPMPDAP. The alkoxyalkyl ester prodrugs of the cyclic form of (S)-HPMPDAP (both *cis* and *trans* isomers) inhibited VACV replication with  $EC_{50}$

values that were 10-fold (ODE ester, *trans*), 48-fold (HDP ester), 100-fold (ODE ester *cis*) and 100- to 200-fold (HDE, *cis* and *trans*) lower than that of cyclic(S)-HPMPDAP. The EC<sub>50</sub> values obtained for the alkoxyalkyl ester prodrugs of cyclic(S)-HPMPDAP were about 3- to 70-fold higher against vaccinia virus than the corresponding monoester prodrugs of (S)-HPMPDAP, and they had selectivity indices of 1,200 to 8,000.

The alkoxyalkyl ester prodrugs of (S)-HPMPDAP and its cyclic form also proved remarkably potent and selective against HSV-1, HSV-2, HSV-1 ACV<sub>r</sub>, VZV, and HCMV, with EC<sub>50</sub> values in the range of 0.007-0.0008 μM. Lower activities of the alkoxyalkyl ester prodrugs of cyclic(S)-HPMPDAP than of the corresponding alkoxyalkyl ester prodrugs of (S)-HPMPDAP were also observed against HSV and VZV. The alkoxyalkyl ester prodrugs also showed potent activity against HCMV, with EC<sub>50</sub> values at least 225-fold lower than those for the corresponding parent compound. The alkoxyalkyl ester prodrugs proved rather toxic against the lymphoblast HSB-2 cells; and the only compounds, the HDP derivatives of (S)-HPMPDAP and cyclic(S)-HPMPDAP, respectively, showed selective activity against HHV-6 at non-toxic concentrations.

Although the POM esters (both monoester and *cis* and *trans* isomers of the cyclic form), 2,2,2-(trifluoro)ethyl esters, the butylsalicylyl derivatives, and prodrugs based on peptidomimetics proved less active than the alkoxyalkyl ester prodrugs, they appeared to be less cytotoxic and cytostatic. The POM derivate of cyclic(S)-HPMPDAP, either the diastereoisomeric mixture or the *trans* isomer, was able to inhibit VACV replication with EC<sub>50</sub> values similar to those observed with the parent compounds, while 2,2,2-(trifluoro)ethyl esters, the butylsalicylyl derivatives, and peptidomimetic esters showed EC<sub>50</sub> values 4- to 16-fold higher than the parent compounds. The POM, 2,2,2-(trifluoro)ethyl, butylsalicylyl derivatives, and prodrugs based on peptidomimetics also proved active against HSV, VZV, and HCMV, with EC<sub>50</sub>'s similar or 10-fold higher than those of the parent drugs. Both types of POM esters (cyclic and monoester) also displayed potent and selective activity against HHV-6. The EC<sub>50</sub> values and the selectivity indices for these compounds against HHV-6 were equivalent to those observed for (S)-HPMPDAP.

As mentioned above, despite the great experience in development of phosphonate and phosphate prodrugs, preparation of prodrugs in HPMP series remained long problematic (Hecker & Erion, 2008). In our recent work on HPMPDAP prodrugs, we managed to work out conditions for the selective transformation of a free HPMP derivative to the corresponding POM monoester in one reaction step. Reaction can be achieved by the action of chloromethyl pivalate in DMF in the presence of *N,N*-dicyclohexyl-4-morpholinecarboxamidine; the reaction conditions were optimized so that no cyclization or formation of decomposition products occurred (Krečmerová et al., 2010a). This procedure was also found generally useful for preparation of other POM HPMP monoesters (e.g. HPMP-C, HPMP-5-azaC, HPMPA). Preparation of cyclic HPMP POM esters can be performed by the action of chloromethyl pivalate and a tetrabutyl ammonium salt of an appropriate cyclic phosphonate (Fig. 13).

Besides procedures based on alkylation of tetrabutylammonium salt of the cyclic phosphonate, we focused on development of new approaches based on activation of a phosphonic acid residue with hexafluorophosphate coupling agents - PyBOP, PyBroP or HATU. Such reagents are currently used as coupling agents, especially in peptide chemistry (Han & Kim, 2004). This approach proved successful, e.g. in preparation of 2,2,2-(trifluoro)ethyl esters, so far known only in PME series (Sekyia et al., 2002; Kamiya et al.,



2002). Also in this case, the desired 2,2,2-(trifluoroethyl) monoester could be prepared from a free phosphonic acid (HPMPDAP) in one reaction step without any protection (Fig. 14).

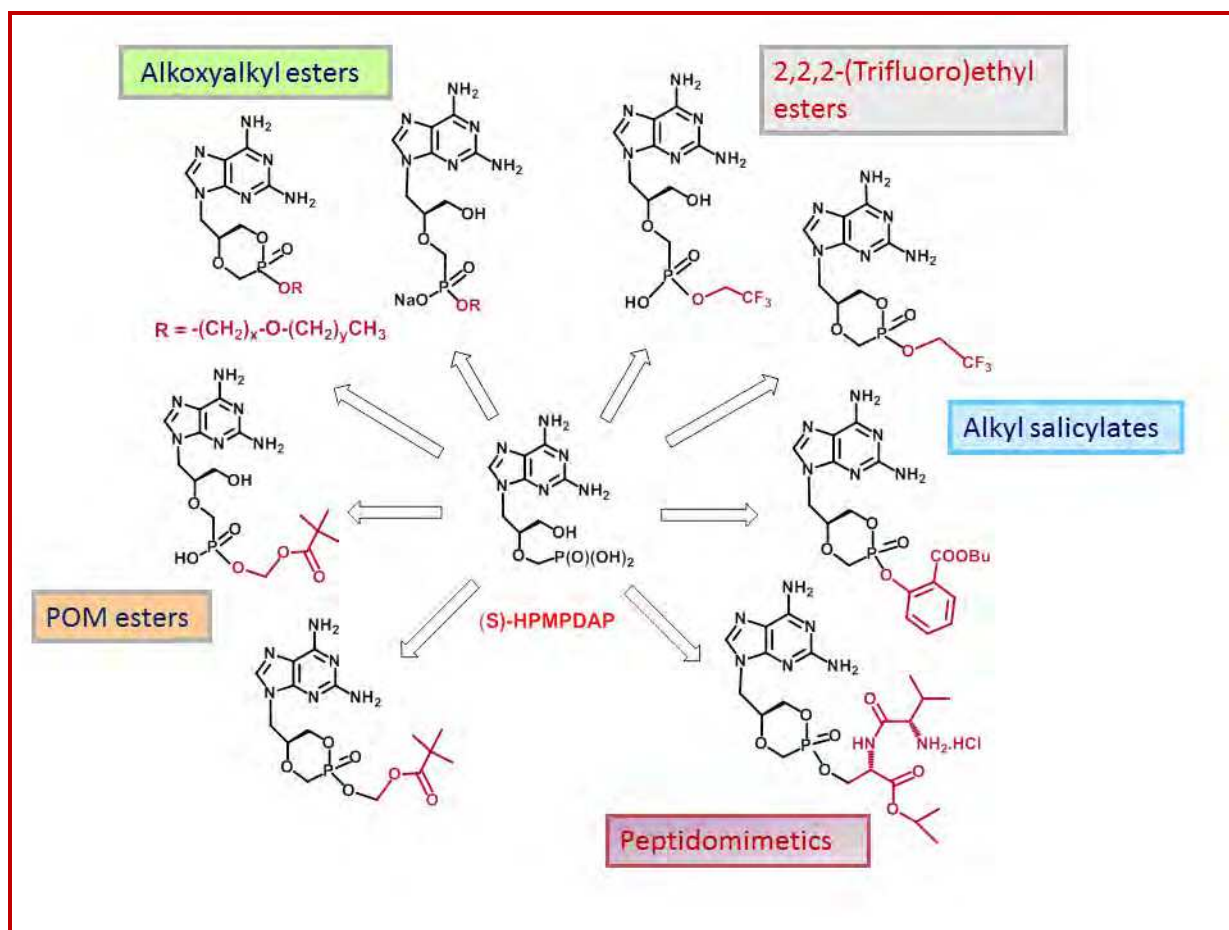


Fig. 12. Structurally diverse types of (S)-HPMPDAP prodrugs prepared for investigation of their anti-poxvirus and anti-herpesvirus activities

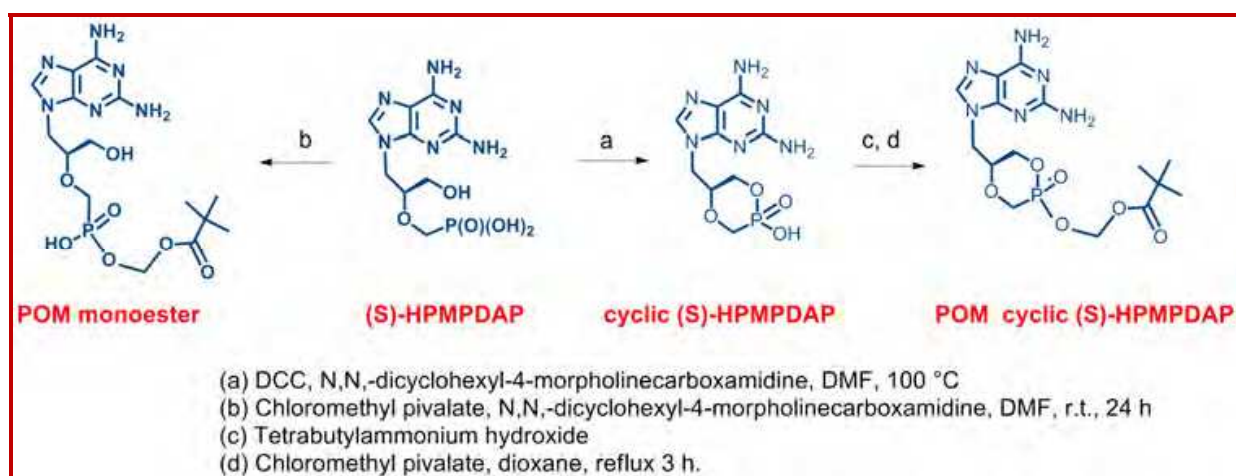


Fig. 13. Synthesis of POM esters of (S)-HPMPDAP. The reactions were successfully applied also for HPMPDC, HPMP-5-azaC and HPMPA.



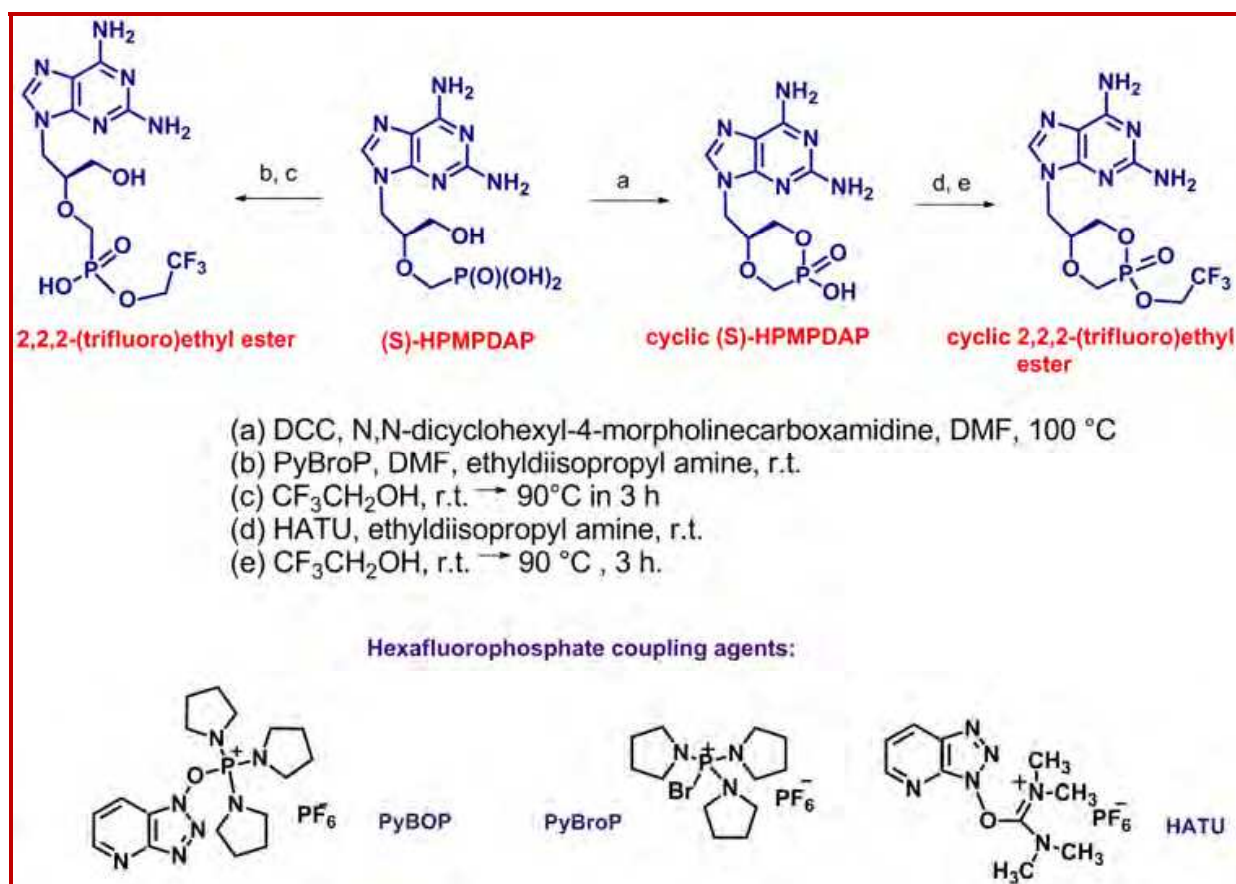


Fig. 14. Structures of some hexafluorophosphate coupling agents and their utilization in synthesis of 2,2,2-(trifluoro)ethyl ester prodrugs of (S)-HPMPDAP

Also, synthesis of other types of HPMP prodrugs can be simplified using the above-mentioned coupling agents, e.g. synthesis of salicyl esters or peptidomimetic prodrugs ((Krečmerová et al., 2010a; Peterson et al., 2011).

#### 4.5 PME derivatives, open-ring analogues and other structures

In a (phosphonomethoxy)ethyl (PME) series, special attention should be paid to the adenine derivative 9-(2-phosphonomethoxyethyl)adenine (PMEA), 2,6-diaminopurine derivative (PMEDAP) and its N<sup>6</sup>-substituted analogues. The activity of PME-derivatives against DNA viruses is generally lower compared to their counterparts in the HPMP-series (Holý, 2003). The most active compound is 9-(2-phosphonomethoxyethyl)-2,6-diaminopurine (PMEDAP). PME-derivatives of adenine and 2,6-diaminopurine reveal remarkable activity against HHV-6 *in vitro* (Reymen et al., 1995; Manichanh et al., 2000). PMEDAP is also highly effective against HCMV; it inhibits the expression of HCMV late antigens.

N<sup>6</sup>-substitution on the 6-amino group of PMEDAP by one or two alkyl, aryl or cycloalkyl group lead to the compounds with pronounced activities against herpesviruses, especially cytomegalovirus, VZV and EBV (Snoeck et al., 1997; Meerbach et al., 1998). Despite its excellent antiviral parameters, PMEDAP has never been systematically investigated as an antiviral agent. Its main clinical potential lies in its cytostatic effects.

During the past decade, we have also systematically investigated a novel group of base modified ANP, the “open-ring” analogues (Fig. 15). They are characterized by an aliphatic

chain (PME-, PMP and/or HPMP-group) linked to the position 6 of 2,4-diaminopyrimidine (DAPy) *via* the oxygen atom. These compounds can be thought of as mimics of the appropriate 2,6-diaminopurine derivatives with an open imidazole ring. Their antiviral activity is essentially identical to that of the parent compounds, including their enantiomeric specificity (De Clercq et al., 2005). The 6-[2-(phosphonomethoxy)ethoxy]-2,4-diaminopyrimidine (PMEO-DAPy) and its 5-substituted derivatives offer antiviral potential similar to adefovir, mostly against retroviruses and HBV. (R)-HPMPO-DAPy has antiviral potential similar to cidofovir (herpes-, adeno, pox-, polyoma and papillomaviruses) (De Clercq, 2011).

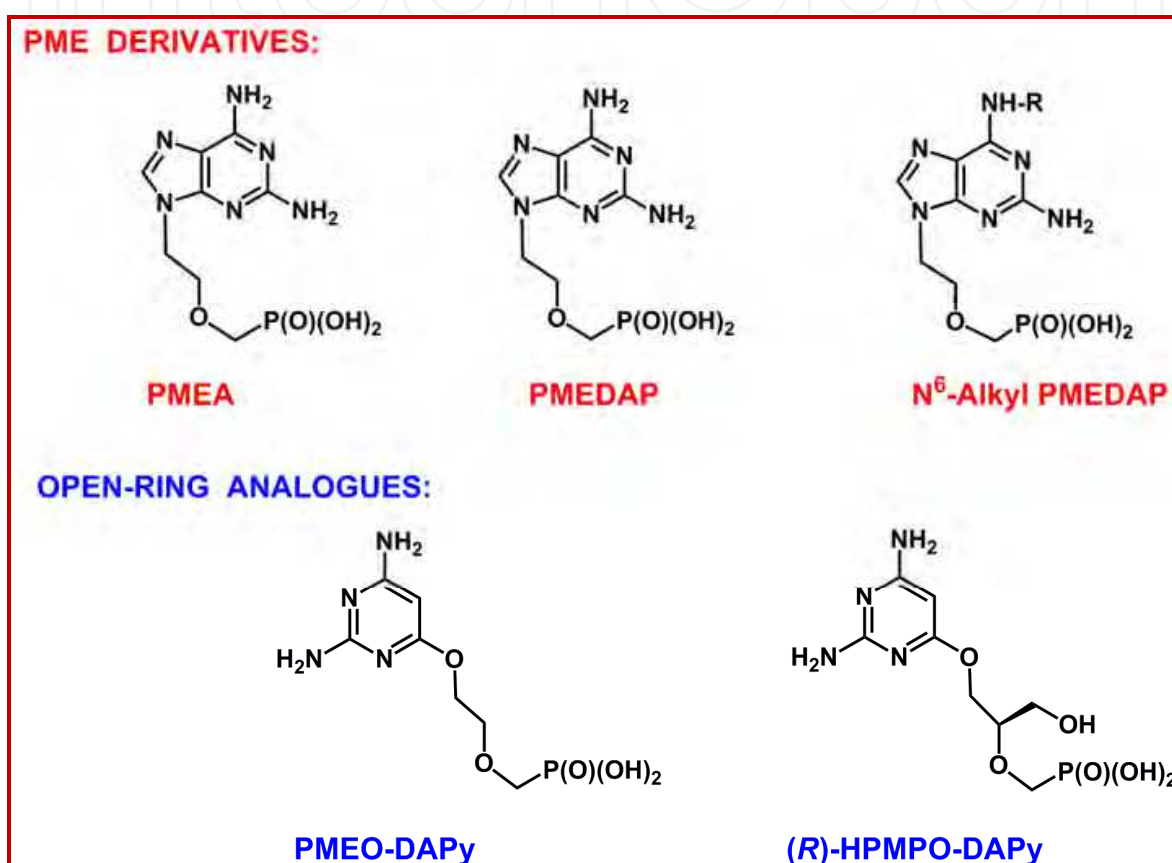


Fig. 15. Structures of selected (phosphonomethoxy)ethyl (PME) derivatives and “open-ring” analogues

## 5. Animal herpesviruses

The last chapter should be devoted to a special part of virology – animal viruses (Giguère et al., 2006). Their study is important from a veterinary viewpoint in general but many of them can cause diseases which are economically devastating. There is a wide variety of different herpesviruses with different biological characteristics. In animals the most important herpesviruses belong to the Alphaherpesviridae.

Pseudorabies virus causing Aujeszky's disease in pigs is now extensively studied as a model for basic processes during lytic herpesvirus infection and for unravelling molecular mechanisms of herpesvirus neurotropism. The disease is caused by porcine herpesvirus 1, also called pseudorabies virus (PRV) or suid herpesvirus-1 (SHV-1). PRV is considered to be

the most economically important viral disease of swine in areas where hog cholera has been eradicated ("Hog cholera" is an alternative name of classical swine fever (CSF), an infectious disease caused by a pestivirus CSFV from the family *Flaviviridae*) (Fenner et al., 1993). Pseudorabies is endemic in most parts of the world. Pigs are the only natural host for the Aujeszky's virus. Clinical signs in pigs vary depending on the age of the pigs involved. In neonatal pigs, the incubation period is 2-4 days, and signs of central nervous system disease (shivering, inco-ordination and hind leg weakness) are seen. In weaned pigs, respiratory disease is the predominant problem. Sneezing, coughing and laboured breathing is accompanied by fever and weight loss. Mortality rates tend to decrease as the age of the affected pigs rises. Clinical signs can be present for 6-10 days. In uncomplicated cases, the animals often recover. (Lee & Wilson, 1979). Other domestic and wild mammals, such as cattle, sheep, goats, cats, dogs and raccoons (Thawley & Wright, 1982), are also susceptible to this virus. For these hosts, the disease is usually fatal and no effective treatment is available. The main symptoms in dogs include intense itching, jaw and pharyngeal paralysis, howling, and death. In cats, the disease is so rapidly fatal that there are usually no symptoms. Death usually occurs within 48 hours. Vaccines are available only for swine.

Bovine herpesvirus 1 causes several diseases in cattle: infectious bovine rhinotracheitis (IBR), infectious pustular vulvovaginitis (IPV), balanoposthitis, conjunctivitis, abortion, encephalomyelitis, and mastitis. The respiratory form is most common. The viral infection itself is not life-threatening but predisposes to secondary bacterial pneumonia, which may result in death. (Jones & Chowdhury, 2007, 2010). Immunization with modified live or inactivated virus vaccine generally provides adequate protection against clinical disease.

The avian herpesvirus 1 (also called Gallid herpesvirus 1, GaHV-1) is phylogenetically different from both abovementioned viruses and serves to underline similarity and diversity within the Alphaherpesviridae. The virus is a causative agent of laryngotracheitis in poultry. (Hidalgo, 2003). A vaccine is available but it does not prevent latent infections.

Herpesvirus infections are one of the main reasons of animal abortions (Chénier et al., 2004; Smith, 1997). Abortion or neonatal diseases may follow infection with not only alpha-herpesviruses but also beta and gamma-herpesviruses. The alpha-herpesvirus, equine herpesvirus-1 (EHV-1), causes single or epizootic abortions or neonatal deaths in horses, and the closely related virus EHV-4 causes sporadic equine abortions. In cattle, the alpha-herpesviruses, bovine herpesvirus-1 (infectious bovine rhinotracheitis virus) and bovine herpesvirus-5 (bovine encephalitis virus), and a gamma-herpesvirus, bovine herpesvirus-4, have all been implicated as causes of abortion. In pigs, suid herpesvirus-1 (SHV-1: pseudorabies virus), an alpha-herpesvirus, and SHV-2 (porcine cytomegalovirus), a beta-herpesvirus, each cause abortion or neonatal piglet losses. Caprine herpesvirus-1, canine herpesvirus and feline herpesvirus-1, all alpha-herpesviruses, cause abortions or neonatal deaths in goats, dogs and cats, respectively.

### **5.1 Investigation of antiviral activity of acyclic nucleoside phosphonates against animal herpesviruses**

Many years of lasting research on acyclic nucleoside phosphonates (ANPs) as exceptional drugs and/or drug candidates against human viral infections gave an impetus for their study as antivirals in veterinary medicine. The well-researched candidate is cidofovir, active against equine herpes virus type 1, bovine herpesvirus type 1, feline herpesvirus type 1 and caprine herpesvirus type 1. Remarkable effects against animal herpesviruses were also

observed at HPMPA and finally at PMEDAP, 9-(phosphonomethoxypropyl)-2,6-diaminopurine, a compound mostly investigated for its cytostatic effects.

Recently, several extensive studies have been paid to the treatment of caprine herpesvirus 1 (CpHV-1) infections by cidofovir.

Caprine herpesvirus 1 (CpHV-1) is responsible for recidivous genital disease in adult goats, characterized by confluent vesicles evolving to ulcers and crusts on the vulvar rima and vaginal mucosa (Tempesta et al., 1999). Several biological similarities exist between CpHV-1 and the human genital herpesvirus type 2, such as the preferential tropism for the genital tract, the vesicular ulcerative nature of the topical lesions, and the tendency to become latent in the sacral ganglia (Lafferty et al., 1987; Tempesta et al., 1999). This makes the genital CpHV-1 infection of goats a reliable animal model for comparative studies. In described experiments, goats were infected experimentally by the vaginal route with CpHV-1 and then treated topically at different time intervals after infection. The administration of 1% cidofovir cream onto vaginal mucosa was able to prevent the onset of genital lesions and to decrease significantly the titers of the virus shed by the infected animals, notably in the groups treated shortly after infection (24 and 48 h). The efficacy of cidofovir against caprine herpesvirus infection was higher when the treatment was started shortly after infection than when lesions were already present and advanced (Tempesta et al., 2007, 2008). Concerning other drugs effective against CpHV-1, the attention of researchers has been focused in recent years also on the study of acyclovir and ganciclovir.

In horses, two herpesvirus infections mostly occur: equine herpesviruses 1 (EHV-1) and 4 (EHV-4) (Patel & Heldens, 2005). Although both viruses may cause febrile rhinopneumonitis, EHV-1 is the main cause of abortions, paresis and neonatal foal deaths. The lesion central to these three conditions is necrotising vasculitis and thrombosis resulting from lytic infection of endothelial cells lining blood capillaries. The initiation of infection in these lesions is likely to be by reactivated EHV-1 from latently infected leukocytes. However, host factors responsible for reactivation remain poorly understood. Although vaccines are available, they are not fully protective and outbreaks of disease may occur in vaccinated herds. Therefore, there is an urgent need for effective antiviral treatment.

It was found that ganciclovir is the most potent compound and therefore a valuable candidate for the treatment.

Ganciclovir displays the best overall activity against EHV-1-infection in vitro, without affecting cell viability. However, due to the high cost price, there is no direct clinical application possible. Therefore, acyclovir seems to be a more valuable candidate for antiviral therapy against EHV-1. As acyclovir has been patented since 1997 and generic alternatives are available, it seems attractive to use this drug for the treatment of horses during an outbreak (Wilkins et al., 2005). However, the bioavailability of orally administered acyclovir is a serious restriction (Bentz et al., 2006). Bioavailability of only 2.8%, resulting in low plasma concentrations, is inadequate to expect any clinically relevant antiviral efficacy. The oral prodrug of acyclovir, valacyclovir seems to be more effective due to its higher bioavailability (Ormrod & Goa, 2000). Recently, a large study comparing the effect of several antiviral drugs - acyclovir, ganciclovir, cidofovir, adefovir, 9-(2-phosphonylmethoxyethyl)-2,6-diaminopurine (PMEDAP) and foscarnet against three abortigenic (94P247, 97P70 and 99P96) and three neuropathogenic isolates (97P82, 99P136 and 03P37) of EHV-1 was performed (Garré, B. et al., 2007). Ganciclovir was most potent in reducing plaque number, followed by PMEDAP and acyclovir. Adefovir and cidofovir were



less effective and foscarnet was the least effective compound. There were no differences detected for acyclovir, ganciclovir, adefovir and PMEDAP between the abortigenic and neuropathogenic isolates. Although cidofovir is not highly efficient in reducing plaque number, it is able to significantly reduce plaque size at very low concentrations. Cidofovir was 40-fold more effective in reducing plaque size than in reducing plaque number (Garré, B. et al., 2007).

A similar comparative study was also performed for feline herpesvirus 1 (FHV-1) is a common cause of respiratory and ocular disease in cats. Especially in young kittens that have not yet reached the age of vaccination, but have already lost maternal immunity, severe disease may occur. In the study, the efficacy of six antiviral drugs - acyclovir, ganciclovir, cidofovir, foscarnet, adefovir and 9-(2-phosphonylmethoxyethyl)-2, 6-diaminopurine (PMEDAP), against FHV-1 was compared in Crandell-Rees feline kidney (CRFK) cells using reduction in plaque number and plaque size as parameters. The capacity to reduce the number of plaques was most pronounced for ganciclovir, PMEDAP and cidofovir. All antiviral drugs were able to significantly reduce plaque size when compared with the untreated control. As observed for the reduction in plaque number, ganciclovir, PMEDAP and cidofovir were most potent in reducing plaque size. Adefovir and foscarnet were intermediately potent. The most remarkable effect was observed for cidofovir and ganciclovir. None of the products were toxic for CRFK cells at antiviral concentrations (van der Meulen, K. et al., 2006).

## 6. Conclusion

For a long time, viral infections remained a major medical problem worldwide due to a lack of therapy, prevention, or vaccination strategy, and also due to the rapid development of resistance. Four decades of intensive research in the field of nucleic acid component chemistry brought the fundamental changes in the treatment of most viral infections. Discovery of acyclic nucleoside phosphonates and their systematic investigation resulted in marketed drugs effective so far against fatal infections (AIDS, HBV, CMV infections in immunocompromised patients, etc.). The fundamental research in the field resulted in many excellent active structures so far waiting for clinical investigation, e.g. “open-ring” analogues, HPMP-5-azaC, HPMPDAP, and many other structures described in the text. Investigation of these compounds still remains a significant challenge for the pharmaceutical industry.

## 7. Acknowledgment

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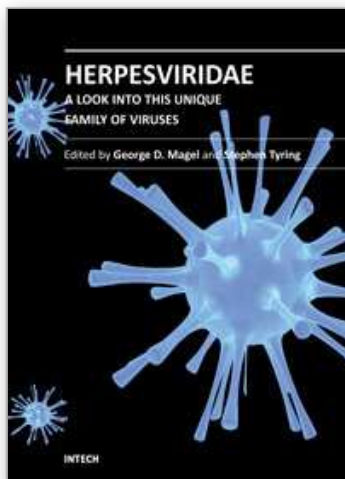
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## **Herpesviridae - A Look Into This Unique Family of Viruses**

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In order to fully understand the nature of viruses, it is important to look at them from both, their basic science and clinical, standpoints. Our goal with this book was to dissect Herpesviridae into its biological properties and clinical significance in order to provide a logical, as well as practical, approach to understanding and treating the various conditions caused by this unique family of viruses. In addition to their up-to-date and extensive text, each chapter is laced with a variety of diagrams, tables, charts, and images, aimed at helping us achieve our goal. We hope that this book will serve as a reference tool for clinicians of various specialties worldwide.

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