Conservation of antiviral activity and improved selectivity in PMEO-DAPym upon pyrimidine to triazine scaffold hopping**

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ABSTRACT

Acyclic nucleoside phosphonates incorporating 2,4-diaminotriazine (DAT) as a 5-aza-analogue of the 2,4-diamino-pyrimidine (DAPym) nucleobase present in PMEO-DAPyms have been synthesized. The lead PMEO-DAT is as inhibitory against HIV, HBV, VZV and MoMSV replication as the parent PMEO-DAPym but neither affects replication of other (herpes) viruses such as HSV-1 and HSV-2 nor triggers expression of chemo/cytokines. A structural rationale is proposed to account for the polymerase selectivity.

Keywords: polymerase inhibitors, antivirals; triazine, phosphonates, scaffold hopping

Abbreviations: DAT, 2,4-diaminotriazine; DAPym, 2,4-diamino-pyrimidine; ACV, acyclovir; HCMV, human cytomegalovirus; HSV, herpes simplex virus; VZV, varicella-zoster virus; ANPs, acyclic nucleoside phosphonates; (R)-PMPA, tenofovir; HBV, hepatitis B virus; DAP, 2,6-diaminopurine; PMEA, adefovir; MoMSV, Moloney murine sarcoma virus

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**Dedicated to the memory of Prof. Antonin Holý.

Acyclic nucleosides are characterized by the substitution of an aliphatic acyclic chain for the furanose sugar ring and some of them have been shown to possess useful antiviral and antitumor activities (Baker and Chu, 2013; De Clercq, 2009; Jordheim et al., 2013; Périgaud et al., 1992). One prominent representative of such kind of drugs is the selective anti-herpetic drug acyclovir (ACV, **1**), widely employed clinically for the treatment of infections caused by herpes simplex and varicella-zoster viruses (HSV and VZV, respectively) (Colla et al., 1983; De Clercq and Field, 2006; Elion et al., 1977).

Acyclic nucleoside phosphonates (ANPs), in particular 2-(phosphonomethoxy)alkyl derivatives of pyrimidines and purines, are also endowed with antiviral and cytostatic activity (De Clercq, 1994; Hockova et al., 2004; Holý, 2002). Representative examples of purine derivatives are the adenine analogues adefovir (PMEA, **2**), active against DNA viruses and retroviruses (De Clercq and Holy, 2005), and also showing pronounced cytostatic activity in well defined tumor models (Hatse et al., 1996; Hatse et al., 1998), and tenofovir ((*R*)-PMPA, **3**) (Fig. 1), which displays pronounced inhibitory activity against human immunodeficiency virus (HIV) and hepatitis B virus (HBV). Of the 2,6-diaminopurine (DAP) analogues of these drugs, PME-DAP (**4**) shows not only inhibition of DNA viruses and retroviruses but also selective antitumor properties (Bobkova et al., 2000; Otova et al., 1999) and (*R*)-PMP-DAP (**5**) is one of the most active anti-HIV compounds (Balzarini et al., 1996; Balzarini et al., 1993). PMEG (**6**) and (*R*)-PMPG (**7**) are the guanine counterparts of **4** and **5**, and they are also potent antiviral and antitumor agents (Balzarini et al., 1997; Franek et al., 1999; Yu et al., 1992). Intriguingly, as a rule, the PME- and PMP-pyrimidine analogues are devoid of antiviral activity in cell culture (Herman et al., 2010).

The presence of the isopolar phosphonomethyl ether group in these ANPs replacing the nucleotide phosphate ester moiety of natural nucleotides prevents their enzymatic degradation (dephosphorylation) and makes it possible to circumvent the first intracellular phosphorylation step required for the activation of nucleosides such as ACV to their respective triphosphates. The ANPs are converted by cellular

kinases into their corresponding diphosphate forms which act as competitive inhibitors of the virus encoded DNA polymerases (Holy et al., 1990; Magee et al., 2008; Mul et al., 1989; Xiong et al., 1997). These ANP-diphosphates, upon incorporation into the elongating DNA as alternative substrates, act as terminators of the growing viral DNA chain.

The 6-[2-(phosphonomethoxy)alkyloxy]-2,4-diamino pyrimidines such as PMEO-DAPym (**8**) and PMPO-DAPym (**9**) have been described more recently as representatives of a novel and peculiar subclass of ANPs which, in contrast to PME-pyrimidines and PMP-pyrimidines, are markedly active against both HIV and HBV (Balzarini et al., 2002; De Clercq and Holy, 2005; Ying et al., 2005). In these PMEO- or PMPO-ANPs the nucleobase is a 2,4-diaminopyrimidine (DAPym) bonded to an aliphatic alkyloxy phosphonate through an ether linkage to the C-6 position of the pyrimidine base instead of the N-1 or N-9 positions of the pyrimidine or purine nucleobases, respectively (Balzarini et al., 2002; Hockova et al., 2003).

Because of the presence of the amino groups on the pyrimidine ring, PMEO-DAPyms retain the pharmacophore part of a purine for base-pair recognition (Hockova et al., 2003) and can be regarded as open-ring mimics of PME-DAP (**4**) and its guanine equivalent PMEG (**6**). Indeed, it was demonstrated that HIV-1 RT efficiently recognizes and incorporates PMEO-DAPym-pp as a purine but not as a pyrimidine nucleotide analog (Herman et al., 2010).

Several congeners of the O-linked ANPs have been synthesized (Holy et al., 2002). Thus, the amino group at position 4 has been replaced by other groups, the amino at position 2 has been eliminated, substituents have been added at C-5, a second aliphatic side chain has been introduced at the nucleobase and the ether linkage has been replaced by thioether- or amine bonds. These modifications, in most cases, were detrimental for the antiviral activity (Dolakova et al., 2009; Herman et al., 2010; Hockova et al., 2003; Ying et al., 2005). Only those analogues bearing an unsubstituted amino group on both C-2 and C-4, or an amino group at C-2 and an OH at C-4, displayed relevant antiviral activity (Hockova et al., 2003; Krecmerova et al., 2007; Ying et al., 2005).

Aza-analogues of the ANPs have also been described. For example, the 5-aza analogue of (S)-HPMPC (cidofovir, **10**) shows significant antiviral activity specifically against DNA viruses, comparable to or even better than that of the parent compound ((S)-HPMPC) (Krecmerova et al., 2007). However, HPMP-5-aza-C (**11**) is chemically and enzymatically more unstable than HPMPC, due to ring opening between C-6 and N-1 of the triazine (5-aza-cytosine) moiety in aqueous media. The ring-opening reaction is general in all 5-aza-cytosine compounds (Krecmerova et al., 2010).

With the aim to further explore the potential of O-linked ANPs, we pursued the synthesis of O-linked 2,4-diamino-substituted-1,3,5-triazines (DAT) of general structure **A** (PMEO-DAT, Fig. 2) designed as 5-aza analogues of PMEO-DAPym. This synthesis is challenging and has remained elusive as the only examples of triazine congeners of PMEO-DAPym so far described are, to the best of our knowledge, the 4(6)-aryl-substituted compounds **13-16** reported in 2011 (Otmar et al., 2011). These ANPs were presumably devoid of antiviral activity because they lacked the 6-NH₂ group necessary for efficient base pairing. The O-linked-aza-phosphonate-protected compound **12**, although described as a side product in the synthesis of *N*-substituted-5-aza-cytosine ANP derivatives (Krecmerova et al., 2010), was completely degraded under the deprotection conditions with trimethylbromosilane (Krecmerova et al., 2010).

Herein we report the synthesis and cell-based antiviral and cytostatic activities of **two** novel acyclic pyrimidine nucleoside phosphonates of general structure **A** (Fig. 2). One of the PMEO-DAT derivatives proved to be markedly inhibitory against HIV, HBV, VZV, and MoMSV replication in cell culture at non-toxic concentrations. In contrast, it was devoid of activity against other DNA (i.e. HSV, HCMV, vaccinia virus and adenovirus) and RNA viruses (i.e. HCV). Molecular modeling studies were then undertaken to try and provide a structural rationale to these findings.

Chemical synthesis. Chloro-substituted triazines are not very reactive and substitution of the chlorine atom by alcohols requires strong reaction conditions (Afonso et al., 2006). The synthesis of PMEO-5-aza-DAPym (**19**) was initially attempted following a procedure similar to our described method for the synthesis of amino-1,3,5-triazine methyloxynucleosides (Fernández-Cureses et al., 2015). Treatment of

6-chloro-2,4-diamino-1,3,5-triazine (**17**) with an excess of phosphonate **18** (Naperville et al., 1999), in the presence of sodium hydride under microwave irradiation at 100 °C (Scheme 1) failed to produce the desired phosphonate **19** and **led** to a complex reaction mixture that could not be identified.

The use of different temperatures (100, 110, or 120 °C), bases (KOH) and/or co-solvents (DMF, H₂O, or DMSO) also rendered complex reaction mixtures, from which traces of the desired phosphonate **19** (< 5%) were detected (KOH, DMSO).

Next, we envisaged a second approach based on a reported procedure (Hockova et al., 2004) for the synthesis of acyclic phosphonates of 2,4-diamino-pyrimidines, which involves the etherification of a hydroxyl group, present in 5,6-disubstituted 2,4-diamino-pyrimidines, with 2-(diisopropoxyphosphoryl)methyl tosylate (Holy et al., 2002) in the presence of strong bases (NaH). Thus, we devised the procedure shown in Scheme 2 and used, in our case, 2-(diethoxyphosphoryl)methyl triflate, since it is known that triflates are more reactive than tosylates (Smith, 2001). The key intermediate of this route is the alcohol **20**, which was prepared in good yield (82%) by treatment of 2,4-diamino-6-chloro-1,3,5-triazine (**17**) with excess of ethylene glycol (as reagent and as solvent) in the presence of KOH at 120 °C for 6 h. However, alkylation of **20** with (diethoxyphosphoryl)methyl triflate (Xu et al., 1996) in the presence of NaH (60% or 95%) at different temperatures (from rt to 150 °C), under microwave irradiation or under conventional heating afforded very low yields (< 5%) of **19** together with ammeline (**21**).

To avoid the strong basic conditions that led to complex reaction mixtures and/or very low yields of the target aza-nucleoside we devised a new synthetic procedure (Scheme 3) based on cyanuric chloride (**22**). Thus, reaction of cyanuric chloride (**22**) with the appropriate phosphonates (**18**, **23**) (Naperville et al., 1999; Shankar et al., 2014) in the presence of 2,4,6-triaminopyridine (collidine) in freshly distilled THF for 2 hours at rt, afforded the target triazines **24** (28 %) and **25** (24 %). Subsequent treatment of **24** or **25** with a 2:1 mixture of toluene and 1% ammonium hydroxide solution, in a pressure tube at 80 °C for 5 h, afforded the corresponding DAT derivatives **19** (50 %) and **26** (57%).

Finally, deprotection of **19** and **26** with bromotrimethylsilane followed by hydrolysis gave **27** and **28** in 33% and 37% yields, respectively. It should be noted that it is very important to perform this deprotection step under cold conditions (0 to -10 °C) and carefully neutralize the reaction up to pH ~5 before evaporating the solvent for purification to prevent degradation of the compounds.

Regarding the biological activity, **27** proved to be markedly inhibitory against HIV-1, HIV-2, HBV, VZV, and MoMSV with 50% effective concentrations (EC_{50}) in the same low micromolar range as the parent PMEO-DAPym (Table 1). Both compounds were inactive at 100 μ M against HCMV and VV but **27**, in addition, lacked the modest activity ($EC_{50} \sim 25$ -100 μ M) displayed by PMEO-DAPym on HSV-1 and HSV-2. **27** was moderately cytostatic for proliferating CEM, PC-3, Caki-1 and HEL cell cultures, but not measurably cytotoxic against confluent HEL and 2.2.15 cell cultures (> 100 μ M).

Molecular modeling. The hydrogen-bonding potential of the triazine in **27** is, in principle, similar to that of PMEO-DAPym and other related DAP or DAPym derivatives. On the other hand, the distinct molecular electrostatic potentials (MEP) calculated for the planar moieties of **27** and PMEO-DAPym (Fig. 3) reflect differences in their respective charge distributions that can be translated into distinct interactions not only with the template base but also with surrounding protein residues and (possibly) water molecules in the DNA polymerase active site. In this regard it must be borne in mind that the plasticity of this cavity, whose shape is defined by both the enzyme wall and the template strand, depends on the protein architecture, which varies among the different families and depends on the particular amino acid composition of its structural elements.

Given that the viral DNA polymerases represent the eventual targets for the antiviral action of these compounds, the distinct selectivity profile between DAT and DAPym must point to subtle differences that exist in the active sites of these enzymes in HSV, VZV, HIV, HBV, and MSV. Since the activity assays on HSV, VZV, and HCMV have been performed in the same confluent HEL cell cultures no differences can be expected in the metabolic activation of the test compounds to their diphosphate forms.

It is remarkable that **28**, which contains an additional methylene in the acyclic (methoxypropoxy) chain, was devoid of antiviral activity when evaluated against HIV-1, HIV-2, and MSV. This points to the methoxyethoxy moiety as the optimal linker between the phosphonate and the nucleobase.

PMEO-DAPym has been recently shown to trigger a notable expression of a wide variety of chemo/cytokines including the CCR5 agonists RANTES, MIP-1 α and MIP-1 β , upon short exposure to PBMC cultures (Balzarini et al., 2013). When the DAT derivative **27** was added to PBMC cultures at 100, 20 or 4 μ M concentrations, no significant induction of chemo/cytokines could be observed. It should be noticed that these immunomodulatory effects of PMEODAPym are directly triggered by the compound itself, and likely without the need for prior metabolic conversion to its diphosphate metabolite (Tuske et al., 2004). Therefore, although the structural requirements within the class of PMEODAPym compounds to efficiently trigger chemo/cytokine induction are still unclear, it seems that the triazine skeleton is not recognized by the cellular target(s) that efficiently interact(s) with the DAPym derivative.

A previously reported molecular model of PMEODAPym-pp bound at the DNA polymerase active site of HIV-1 RT in complex with a template-primer double helix showed that the binding mode of this ligand is likely to be very similar to that experimentally shown for tenofovir diphosphate (TNV-pp) (Tuske et al., 2004) and to equally allow for Watson-Crick hydrogen bonding to a thymine base because the pyrimidine ring in PMEODAPym mimics an open, incomplete DAP ring (Herman et al., 2010). We show here that the same is true for **27**, the lead compound of this novel series of triazine derivatives, and extend these results to HBV RT (Suppl. Fig. 1). A distinct feature of the RT complexes involving **27** is that the extra ring nitrogen is in close proximity (~ 4 Å) and pointing to the aromatic side chain of the residue that discriminates against ribonucleotides (Tyr115 in HIV-1 RT and Phe88 in HBV RT) and the "steric gate" residue (Gln151 in HIV-1 RT and Met171 in HBV RT). In the case of VZV DNA polymerase, this same nitrogen atom also points towards the ribonucleotide discriminating residue (Tyr687, po-

sitionally equivalent to Tyr722 in HSV-1 DNA polymerase) and also towards Tyr782, which occupies a position equivalent to that of Tyr567 in RB69 DNA polymerase (RB69pol) (Suppl. Fig. 2).

Despite the fact that TNV is an adenine mimic, deviations from canonical base pairing have been noticed for TNV-pp both when it is non-covalently bound at the nucleotide-binding site and upon its incorporation into the terminated primer that leads to the dead-end complex (Tuske et al., 2004). In this respect, we note (i) that abundant evidence exists nowadays supporting the view that strict Watson–Crick hydrogen-bonding is not an absolute requirement for efficient synthesis by DNA polymerases (Egli, 2012; Xia and Konigsberg, 2014) and (ii) that similar non-canonical arrangements occurring frequently in the nascent base-pair site (NBS) of low-fidelity, error-prone DNA polymerases can also be observed in high-fidelity DNA polymerases such as RB69pol upon incorporation of well-defined mutations (Xia and Konigsberg, 2014). One important outcome of these studies is the well-founded proposal that electrostatic interactions involving the incoming dNTP, the penultimate base pair, and the hydration shell surrounding the incoming dNTP modulate nucleotide insertion efficiency and base selectivity. We believe the same arguments can be used to account for the distinct sensitivity of different RNA- and DNA-dependent DNA polymerases to several antiviral drugs including those shown here. Consequently, the ability of **27** to inhibit the DNA polymerase activities of the viruses shown in Table 1 may well depend on the same structural features that allow this class of enzymes to tolerate incorrect matching of incoming dNTPs at the NBS.

Differences in MEP such as those shown in Fig. 3 will most likely translate into different matching patterns with template bases and stacking interactions that will be tolerated (and lead to a stable complex) or not depending on whether the remaining part of the molecule can simultaneously be accommodated in a very restricted conformation. This selectivity filter, which strongly depends on amino acid composition in different protein domains, has indeed been shown to be responsive to subangstrom increments in size and polarity of the incoming dNTP, as demonstrated in vitro by the differential efficiency of insertion of a series of gradually expanded dTTP analogs by DNA Pol I (Klenow fragment, exonuclease-

deficient) (Xia et al., 2013). Furthermore, incorporation of incorrect dNTPs by the high-fidelity B family RB69pol is greatly favored in variants containing L561A/S565G/Y567A and L415A/L561A/S565G/Y567A substitutions, and use of the latter in X-ray crystallographic work has allowed the capture of ternary complexes involving all 12 possible mismatches (Xia et al., 2013). This elegant work has clearly shown not only that an incorrect incoming dNTP that deviates from normal Watson-Crick hydrogen-bonding can stack as well as the correct dNTP onto the penultimate base-pair of the primer but also that it maintains virtually identical conformations of the sugar moiety and the triphosphate tail. As a result, this accommodation entails an enlargement of the NBS and a repositioning of the template base facing the mismatch which may tilt or shift relative to the correct nucleotide or fail to stack altogether, as in the case of purine-purine mismatches. We believe these findings are in very nice correspondence to what has been observed in the complexes of HIV-1 reverse transcriptase (RT) with DNA/RNA incorporating TNV (Tuske et al., 2004) and could provide an explanation for the different sensitivities to **27** and PMEO-DAPym of the herpesviridae DNA polymerases. In this regard it is interesting to note that the highly conserved tyrosine (Tyr567 in RB69pol, Suppl. Fig. 2) is followed by some other residues that do vary between HSV and VZV.

Taking all of these results together, our hypothesis is that the greater the ability to discriminate between correct and mismatched bases in the active site, the lesser the sensitivity of the polymerase to the type of agents studied here. The structural similarities between HIV-1, HBV and MoMSV RTs convincingly account for the inhibition of these enzymes by **27** on the basis of similar findings reported for TNV and PMEO-DAPym. On the other hand, the striking findings that (i) VZV, but not HSV-1, HSV-2 or HCMV, is also sensitive to **27** and (ii) this selectivity is even more pronounced for **27** than for PMEO-DAPym (Table 1) point to the need for additional structural studies because the NBS of these four DNA polymerases is made up of identical residues. This means that the basis for selectivity needs to be sought elsewhere, possibly in the intervening variable regions between the highly conserved amino acids necessary for nucleotide binding and catalysis (Suppl. Fig. 2).

It can be concluded that we have designed and synthesized an acyclic 2,4-diaminotriazine nucleoside phosphonate (**27**, PMEO-DAT) that is as inhibitory against HIV, HBV, VZV, and MoMSV as the parent 2,4-diaminopyrimidine (PMEO-DAPym) derivative, but more antivirally selective because it does not affect replication of other (herpes)viruses such as HSV-1 and HSV-2. Like PMEO-DAPym, **27** was devoid of activity against other DNA viruses such as HCMV, VV and adenovirus, and RNA viruses such as HCV. In contrast, however, **27** does not induce production of chemo/cytokines when added to PBMC cultures.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at ...

Suppl. Figures 1, 2 and 3. Chemical Methods including: General procedures and Chemical Synthesis of compounds **19**, **20**, **24**, **25**, **26**, **27** and **28**. NMR data of all compounds. Cytostatic activity, anti-MSV assay and Bio-Plex cytokine assays. Molecular modeling methods.

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Table 1. Antiviral activity of compound **27** and PMEO-DAPym in cell culture

Virus strain	EC ₅₀ ^a (μM)		CC ₅₀ ^b (μM)		MCC ^c (μM)	
	Compound 27	PMEO-DAPym	Compound 27	PMEO-DAPym	Compound 27	PMEO-DAPym
HIV-1 (CEM)	2.4 ± 1.8	2.7 ± 1.2	55 ± 7 (CEM)	63 ^e	-	-
HIV-2 (CEM)	3.7 ± 0.6	1.9 ± 0.6			-	-
HBV (2.2.15)	3.1 ± 0.5	0.3 ± 0.2 ^d	-	379 ^d	> 100 (2.2.15)	-
HCV (Huh-5.2)	> 33	-	-	-	> 33 (Huh-5-2)	-
HSV-1 (HEL)	> 100	25 ^e	49 ± 0 (HEL)	-	> 100 (HEL)	> 100
HSV-2 (HEL)	> 100	91 ^e				
HCMV/AD-169 (HEL)	> 100	> 100 ^e				
HCMV/Davis (HEL)	> 100	> 100 ^e				
VZV/OKA (HEL)	3.1 ± 1.9	8.6 ^e				
VZV/07-1 (HEL)	7.9 ± 0.1	4.2 ^e				
Adenovirus-2 (HEL)	> 100	> 100				
VSV (HEL)	> 100	> 100				
INF-A (MDCK)	> 100	> 100	-	-	> 100	-
INF-B (MDCK)	> 100	> 100	-	-	> 100	-
MSV (C3H)	0.35 ± 0.16	0.29 ± 0.01	-	-	> 100 (C3H)	> 100

^a50%-Effective concentration

^b50%-

^c50%-

^dData obtained in HepAD38 and HepAD79 cell cultures and taken from reference (Ying et al., 2005).

^eData taken from references (Holy et al., 2002) and (Balzarini et al., 2004).

Figures and Schemes

Fig. 1. Structures of acyclic nucleosides

Fig. 2. Structures of HPMPC (**10**), HPMP-5-aza-C (**11**), PMEO derivatives (**12-16**) and target ANPs (PMEO-DAT)

Fig. 3. Negative regions (red isocontours) of the molecular electrostatic potentials (MEP) calculated on the optimized geometries of the heterocyclic planar moieties of PMEO-DAPym (left) and **27** (right). Note that significant differences exist not only for the nitrogen replacing the ring carbon but also for both the ring sp^2 and exocyclic amino sp^3 nitrogens.

Scheme 1. Reagents and conditions: NaH or KOH, 100 to 120° C, 30 min to 12 h

Scheme 2. Reagents and conditions: (i) ethylene glycol, KOH, 120°C, 6h;

(ii) $\text{CF}_3\text{SO}_3\text{CH}_2\text{P}(\text{O})(\text{OEt})_2$, NaH, rt to 150°C, 30 min to 12 h.

Scheme 3. Reagents and conditions: (i) 2,4,6-triaminopyridine, THF, rt, 2h;

(ii) toluene/1% NH_4OH (2/1), 80°C, 5 h, pressure tube; (iii) Me_3SiBr , acetonitrile, 0°C to rt, 2h