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Mini-review

Antiviral acyclic nucleoside phosphonates structure activity studies

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Dedicated to Prof. Erik De Clercq on the occasion of reaching the status of Emeritus-Professor at the Katholieke Universiteit Leuven in September 2006.

Abstract

This review concerns acyclic nucleoside phosphonates (ANP) and describes the concept of the design of isopolar and isosteric nucleotide analogues resistant towards degradation by enzymes *in vivo*. It describes the development of research which led to the discovery of several structurally related potent antivirals and ultimately resulted in the development of drugs directed against HIV, HBV and DNA-virus infections in general, namely adefovir, cidofovir and tenofovir. In addition to these "classical compounds" the review describes the present development in the field of ANP, the "open-ring ANP" and discusses the present achievements, concept of prodrug design and application.

Keywords: Acyclic nucleotide analogues; Adefovir; Cidofovir; Tenofovir; Open-ring ANP analogues

Contents

1. Prologue

This review was written with the aim of demonstrating the logical development of the topics which began in 1985, resulted in several important drugs and is still, 20 years later, providing interesting new bioactive compounds. Recently, we have published two extensive reviews on the chemistry and diverse biological aspects of this class (Holy, 2003; De Clercq and Holy, 2005). Interested readers may refer to these reviews for details on the synthesis and biological properties there.

2. Modification of nucleotides at phosphoric acid residue

The majority of biologically active nucleosides modified at the base or sugar moiety are activated by phosphorylation. This process is usually catalyzed by nucleoside kinases and these mononucleotides are transformed by two subsequent phosphorylations to the 5′-triphosphates which serve as alternative substrates for RNA or DNA polymerases, or inhibit the polymerization reactions. On the other hand, nucleic acids are degraded by the catalytic action of nucleolytic enzymes to low-molecular oligonucleotides and finally to nucleosides. These reactions interfere with other complex relations, e.g. action of repair enzymes. *In toto* they affect processes which may be impor-

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tant for efficacy of anticancer drug treatment. It was considered possible to circumvent the need for the enzymatic activation of a bioactive nucleoside, which was in some cases difficult or even impossible, by applying the pre-prepared water-soluble salt of the 5′-phosphate. This was found to be impossible due to the degradation as well as due to the limited transport of such polar compounds to the cell. These reasons led to the need for developing phosphate analogues resistant towards the action of enzymes. We were studying the effects of chemical modification in the phosphoric acid ester residue in nucleotide analogues on selected nucleolytic enzymes. In the course of several years, studies were performed on the enzyme responses (substrate or inhibitory activity) towards regioisomers of nucleoside phosphorous acid esters (phosphites, phosphonates), phosphothioates, alkylphosphonates, and their substituted (hydroxyalkyl, aminoalkyl) derivatives. The following conclusions have been drawn from these experiments: (a) the isopolar and isosteric character of the molecule and the presence of oxygen atom in the vicinity of the phosphorus atom are crucial for optimum recognition by the enzyme and (b) the catabolic stability cannot be achieved solely by chemical modification of the nucleoside moiety in the nucleotide molecule. Briefly, the cleavage must be made impossible by the character of the linkages. The simplest structure which fulfilled these requirements is evidently the system isomeric with phosphoric acid ester, the phosphonomethyl ether of hydroxyl group:

$$–CH_2OP(O)– \sim –O–CH_2P(O)–$$

2.1. Acyclic nucleoside phosphonomethyl ethers

The above analogues of 5′-nucleotides efficiently inhibited 5′-nucleotidases from snake venom and bull semen. We prepared and studied the cytostatic effect of analogous compounds derived from established cytostatic nucleosides (araA, araC, 6-azauridine, 5-bromodeoxyuridine). None of them suppressed cell growth at the concentrations comparable to their parent nucleosides. In 1985, during the study of a reaction which made possible to introduce the phosphonomethyl residue on the hydroxyl group in a *cis*-diol-containing molecule, we used as a model compound the acyclic nucleoside DHPA[1] as a substrate. The reaction product which was a mixture of four isomers, exhibited high activity against all strains of herpesviruses tested, including T-minus mutants of HSV-1. The (2*S*) isomer was solely responsible for the biological activities. General character of this conclusion was recently verified by the regiospecific synthesis of base-modified 2-hydroxy-3-(phosphonomethoxy)propyl isomers of the parent compound (*S*)-HPMPA.[2] Neither the regioisomer, nor the (*R*)-enantiomer of HPMPA has any activity. In addition to its general antiviral activity against essentially all DNA-viruses ever tested (among others, it is active also against adenoviruses), (*S*)-HPMPA differs from all its "natural base" congeners by exhibiting a general activity against intracellular stages of parasites (*Trypanosoma*, *Leishmania*, *Plasmodium*, *Microsporidia*, *Cryptosporidia*). Despite these unique characteristics, it was never developed as a drug.

The chemical structure of acyclic nucleoside phosphonates (ANP) is characterized by several structural parameters. Thus, their structure activity relationships must be simplified in order to be analyzed. In (*S*)-HPMP compounds, change of adenine to hypoxanthine, or thiooxopurine (6-sulfanylpurine) is counterproductive, as well as substitution of the 6-amino group by one or two alkyl, alkenyl, etc. groups. Essentially inactive are also 8-substituted (*S*)-HPMPA derivatives (bromo, amino, sulfanyl, hydroxy, methyl). In adenine, position 2 is not prone to substitution either: compounds bearing alkyl, hydroxy, alkoxy, sulfanyl or alkylsulfanyl group in this position are inactive. Contrary to the negative effect of the above effect of substituents, the introduction of an amino function to the position C2 of the adenine nucleus substantially increases the antiviral activity of the molecule. 2-Amino-(*S*)-HPMPA (HPMPDAP) is very active compound. It is at best demonstrated by the powerful inhibition of papillomaviruses and poxviruses, including, e.g. the rare orf virus. The absolute configuration in HPMPDAP and/or HPMPG is not as important as it is in the case of adenine derivative HPMPA. This observation is interpreted by the requirements of the first-step in the ANP activation sequence, the nucleotide kinase conversion to ANP monophosphate; this enzyme does not sufficiently differentiate between the enantiomers in the substrate activity of the kinase catalyzed phosphate transfer reaction. The enhancement of activity by introducing the amino function is a general phenomenon. It turns the inactive purine to active 2-aminopurine derivative and hypoxanthine to guanine with an extreme activity in the HPMP series.[3] It applies not only for antiviral activity, but also for anticancer efficacy. In general, the order of activity is G > DAP > A > 2-aminopurine.

It was considered interesting to find a reasonable explanation for the specific influence of amino group upon the antiviral efficacy of ANP. In order to distinguish between the effect of the basicity of the group and its involvement in the heteroaromatic π-electron distribution, we have prepared the ANP substituted at the positions 2 and 6 by the strongly basic primary aminomethyl function replacing amino group(s). Ultimately, the replacement was made by an even stronger base—the guanidino function. None of these replacements gave positive response; all these compounds are inactive as antivirals (Cesnek et al., 2005). Thus, the effect of the amino group appears to relate to its participation in the heteroaromatic electron system in the π-electron system of the purine ring. It is easy to understand, why the

[1] DHPA, *O*-(22,3-dihydroxypropyl)adenine, an acyclic nucleoside analogue which inhibits several minus stranded RNA viruses. Its (*S*)-form acts as methylation inhibitor *via* (*S*)-adenosylhomocysteine hydrolase inhibition.

[2] *Nomenclature*: PME, 2-(phosphonomethoxy)ethyl; HPMP, 3-hydroxy-2-(phosphonomethoxy)propyl; FPMP, 3-fluoro-2-(phosphonomethoxy)propyl; PMP, 2-(phosphonomethoxypropyl); (*R*) and (*S*) reflects absolute configuration according to Kahn–Prelog–Ingold rule.

[3] In general, all guanine containing ANP derivatives are strongly active antivirals or cytostatics. However, they are all very toxic to the host cells. This toxicity is interpreted as a consequence of strong inhibition of purine nucleoside phosphorylase as proven by direct experiments *in vitro*. This process leads to a disturbance of nucleoside level in the cell pool and the subsequently increased toxicity caused by an increased level of purine nucleosides or bases.

6-amidino compounds, which are easily available by ammonolysis of 6-cyanopurine derivatives, fail to give antivirally active compounds.

It would have been complicated to perform all these studies with the HPMP compounds. Most of the SAR-studies concerning the base modifications were made on simpler molecules of so-called PME compounds, i.e. *N*9-[2-(phosphonomethoxy)ethyl] derivatives. PME-derivatives are active both against retroviruses and HBV as well as (to a lesser extent than the HPMP compounds) against DNA-viruses. The SAR-studies concerning base modification and substitution in PME-derivatives are consistent with the investigations made on compounds with other side chains. This applies also to *N*6-substituted derivatives of adenine and 2,6-diaminopurine. Among the large series of compounds of this type there are quite a few potentially interesting compounds, some of them with an extreme activity against varicella zoster virus and/or cytomegaloviruses, others active against herpesviruses in general. We have selected, taking in account also their parallel antineoplastic activity *in vitro*, the following six compounds for additional investigation: *N*6-methyl, *N*6-dimethyl, *N*6-cyclopropyl, *N*6-allyl, *N*6-(3,3,3-trifluoroethyl) and *N*6-(2-dimethylaminoethyl) derivatives of PMEDAP. It is interesting to note that at least some though probably not all of them are prodrugs of PMEG. It certainly applies to the cyclopropyl derivative which was shown to furnish *in vitro* and *in vivo* the triphosphate analogue PMEGpp. The enzyme which catalyzes this conversion is similar to AMP deaminase. It is a so far unidentified nucleotide deaminase identical with the enzyme which catalyzes the conversion of the *N*6-cyclopropyl-2,6-diaminopurine base in the 5′-nucleotide analogue of another nucleoside antiviral, abacavir. This metabolite is by the action of this enzyme converted to the appropriate *N*9-substituted guanine derivative. We have been recently able to show that this enzyme is *N*6-methyladenylate/*N*6-methyl-2-deoxyadenylate deaminase; in cellular metabolism, it is evidently triggering the catabolic sequence of methylated oligonucleotides in RNA (Schinkmanova et al., 2006). It should be mentioned that the neutral prodrug of *N*6-cyclopropyl-PMEDAP is an excellent candidate for a drug against papillomaviruses.

An anti-HBV activity of 9-[2-amino-6-(4-methoxyphenylsulfanyl)]purine was reported in the literature. This fact is rather exceptional, as the corresponding 6- (or 2-) sulfanyl, alkylsulfanyl, hydroxy or alkoxy derivatives do not exhibit any antiviral or cytostatic activity.

We have also investigated PMEDAP derivatives substituted at position 6 with an amino derivative bearing an acid group (carboxylate, phosphate, phosphonate, sulfate, sulfonate). We are interested in the amino acid derivatives with an amino group linked to the 6-position. In addition to beta alanine or GABA, it was considered interesting to study the effect of absolute configuration at the remote asymmetry center of the amino acid. Though this study is not yet finished, it is evident that the antiviral effect of the individual enantiomers of some compounds is quantitatively different. This observation further supports the hypothesis on the active role of the position N6 in the interaction with the target enzyme(s) of virus life cycle.

An important alternative to the substitution of the purine bases is their modification. We have exhaustively studied the effect of mutual CH– ∼ –N= interchange. The effect of such a structural alteration at the position 2 is negative: the 2-azaadenine ANP's are inactive. While in the 8-azapurine series the N9-isomers retain the activity, their fluorescent N8-regioisomers are not active. In the opposite case, the antiviral activity is lost in all series tested. Replacement of N1 by CH results in the loss of activity, while the same change at the position N3 gives rise to antivirally active compounds with – in the most cases – preserved activity. In fact, 3-deaza-(*S*)-HPMPA retains even the antiparasitic activity of the adenine congener. Thus, the position N3 is evidently less important for the interaction of the heterocyclic base moiety with the enzymes compared to the position N1 (N1 is also the protonation site in adenine derivatives).

2.2. Pyrimidine derivatives

To our knowledge, there is only one important ANP molecule derived from "natural" pyrimidine base: the cytosine derivative cidofovir [(*S*)-HPMPC] which was originally approved for CMV keratitis in AIDS patients (Vistide™), but was later shown to act against essentially all DNA-viruses. Therefore, it was frequently used as a "drug of the last hope" in the often obscure DNA-virus infections occurring in AIDS patients. Fortunately, the treatment was mostly successful. Its most popular potential applications in the eyes of public might be smallpox and/or monkeypox, both related to bioterrorism. Though an aerosol of the drug was perfectly efficient in curing the monkeypox lung infection in macaques (Stittelaar et al., 2006) there is an effort to develop its (lipophilic) prodrugs (*vide infra*).

The substitution variability is in this case still more limited than in adenine derivatives (*vide supra*). Neither any C-5, nor N4 substituents are permissible. On the other hand, the modification to azapyrimidine (triazine) ring system gives rise to interesting compounds: while the 6-azapyrimidine analogs of both PMEC and HPMPC are antivirally inactive, the *sym*-triazine (5-azacytosine) analogue of (*S*)-HPMPC is in its general character and efficacy comparable with cidofovir, or even better. It remains to be seen whether this compound could find a clinical application, the more that 5-azacytosine and its nucleosides are clinically used as antineoplastics. It cannot remain unnoted that the major and characteristic part of the triazine structure, the –N1–C4(NH_2)–N5– has its counterpart in the –N1–C2(NH_2)–N3– grouping of 2,6-diaminopurine ANP derivatives.

This structural fragment is evidently typical for the heterocyclic part of the biologically active ANP molecule. It is present also in the so-called open-ring ANP (Holy et al., 2002) which are considered to be the "second generation ANP" (De Clercq et al., 2005). These compounds are derivatives of 2,4-diaminopyrimidine or 2-amino-4-hydroxypyrimidine substituted at the position 6 by the ANP chain linked to the heterocycle *via* an ether bridge. They can be simultaneously looked at as analogues of 2,6-diaminopurine with a degraded imidazole ring and nitrogen atom N9 replaced by O6-oxygen bearing the ANP chain. It is interesting to note that the biological (anti-

DNA-viral and/or antiretroviral) activity of these compounds, if any, is qualitatively identical with those of their "classical" counterparts. Substitution of these pyrimidines at the position 5 by chloro, bromo, methyl or cyano group significantly enhances the antiviral activity (Hockova et al., 2003; Ying et al., 2005).

2.3. The influence of the chain structure

Considering the presumption of the necessary proximity of the phosphorus and oxygen atoms, three parameters remain to be analyzed: (a) the distance between these two atoms; (b) the distance between the linking atom of the base and the oxygen atom mentioned; (c) the localization and nature of the chain substituents. The distance between P and O is maximum one bond: solely the phosphonomethyl ethers are active. The homologous 2-[2-(phosphonoethoxy)ethyl]ethers are already inactive. The distance between the nitrogen atom of the heterocycle and the ether oxygen bearing the phosphonomethyl substituent is also critical. It can be solely two carbon atom bonds long; the 3-(phosphonomethoxy)propyl as well as 2-hydroxy-3-(phosphonomethoxy)propyl derivatives of ANP bioactive bases (adenine, guanine, 2,6-diaminopurine) are inactive. In this case, none of pyrimidine bases (uracil, thymine, cytosine) gave any compound of interest either.

The data on the effect of substitution at the position alpha to the base are also scarce; the existing information suggests that any substitution at this position is banned. The possibilities offered by the position beta are broader: the unsubstituted derivatives are the above-mentioned PME compounds; in addition to the already mentioned adefovir (PMEA), the most important compounds of this series from the point of view of antiviral activity are PMEDAP, the open chain nucleotide analogue (PMEODAPy), the *N*6-cyclopropyl-PMEDAP and several others *N*6-substituted PMEDAP derivatives (*vide supra*). PMEDAP and its *N*6-substituted derivatives also exhibit antineoplastic activity which was examined both *in vitro* and *in vivo*. PMEA also has antiinflammatory properties. The guanine derivative PMEG is extremely active both as an antiviral and as cytostatic, but the safety margins of this compound are very narrow for practical use. PMEG could be potentially useful for papillomavirus. However, for this indication it was replaced in the clinical trials by its prodrug *N*6-cyclopropyl-PMEDAP which has better pharmacological parameters. The extreme activity of the cytosine derivative HPMPC (cidofovir) has no parallel in this series, nor are uracil or thymine congeners active as antivirals. PMEA (adefovir) is the active principle formed from its diester (adefovir dipivoxil) which is approved for use in the therapy of chronic lamivudin-resistant hepatitis B (Hepsera™). Introduction of a methyl group to the position beta of the chain gives rise to two enantiomeric series of so-called PMP [2-(phosphonomethoxy)propyl] derivatives[4]: only the (*R*)-enantiomer is active against HIV and MSV, whereas the (*S*)-enantiomer is inert (Balzarini et al., 1993). There is essentially no activity against DNA-viruses. While cytosine, uracil or thymine derivatives are totally inactive as antivirals, adenine, 2,6-diaminopurine, guanine and *N*6-substituted PMPDAP compounds have a great potential; specifically (*R*)-PMPDAP is a very powerful antiretroviral. Purine aza and deaza congeners follow the same pattern of SAR as the PME-derivatives. Also among the open-ring compounds, the introduction of PMP substituent has exactly the expected consequence: in the PMPO series (PMPODAPy), the strong antiretroviral activity is also clearly enantiospecific. The N6-substituent effect is present, but not overwhelming, at least not in the antiviral activity. However, numerous *N*6-substituted adenine or 2,6-diaminopurine ANP also exhibit immunomodulatory activity which affects the action of cytokines and chemokines. The activity of NO-synthase in resting macrophages is dramatically stimulated even in the absence of interferon alpha which is otherwise required for the process to proceed (Zidek et al., 2003a). *N*6-Cycloalkyl-(*R*)-PMPDAP derivatives as the preferred group of effectors, with the cyclopentyl derivative as the most active compound (Zidek et al., 2003b).

The lipophilic prodrug of (*R*)-PMPA (tenofovir disoproxil fumarate) was approved as an oral formulation for AIDS treatment (Viread™). Its toxicity is very low. Due to its long half-time the drug can be applied in one pill a day regimen. It is active also against HBV. In a combination pill with another antiretroviral nucleoside analogue, emtricitabine, it is marketed for therapy of AIDS (Truvada™).

Replacement of methyl group at the position beta of the chain by ethyl, propyl, isopropyl or cyclopropyl, benzyl or phenyl group in the above purine PMP derivatives completely suppresses the antiviral activity. The introduction of hydroxymethyl group results in the above-mentioned enantiomeric series of HPMP derivatives. Replacement of hydroxyl group in these compounds by fluorine affords 3-fluoro-2-(phosphonomethoxy)propyl (FPMP) derivatives which, similarly as the PMP-derivatives, are specifically active against retroviruses (Balzarini et al., 1991). Animal experiments gave good therapeutic effect in cats infected with FIV, with low toxicity (Hartmann et al., 1998). The FPMP derivatives deserve much more attention than they received so far. We have recently developed a method for direct conversion of HPMP to FPMP series (Votruba et al., 2005).

Replacement of hydroxyl group in the HPMP derivatives by azido, amino, mono- and disubstituted amino, morfolino and trimethylammonium group did not result in compounds with even the slightest antiviral effect (Dvorakova et al., 1996). There are some papers on other chain-modified analogues (for reference see Holy, 2003). However, they do not seem to open any broad field of inquiry.

3. Lipophilic ANP prodrugs

Polar character of ANP is not suitable for transport over the cell membrane. It is a serious obstacle for certain applications and would disqualify the drug, e.g. for oral application. Several chemical principles were used to design oral prodrugs of

[4] Due to the altered preference of the substituent order, the formal designation of absolute configuration is reversed: absolute configuration of (*R*)-PMP corresponds to that of (*S*)-HPMP configuration.

ANP. The most important ones are diester, esteramidates and diamidates. Classical pivoxil group from penicillin chemistry was used for adefovir, more recent isoproxil group for tenofovir. Other important diester type prodrugs are the SATE compounds, and the present most important category are the alkoxyalkyl (e.g. C16OC3) ester, the esteramidates (e.g. phenyl ester of ANP alkyl D-alaninates) and bis(amidates) [e.g. ANP bis(alkyl D-alaninates)]. In esteramidates the presence of a new assymetry centre at the phosphorus atom might cause difficulties in the approval process and the practical separation on industrial scale will not be easy.

All these prodrugs efficiently increase the blood plasma level of the drug. Some of the mono-(alkoxyalkyl) ester are reportedly several orders of magnitude more active under the *in vitro* conditions compared to the free phosphonate molecules (for reference see Holy, 2003). It can be disputed whether there is a need to enhance the transportability of ANPs through the cellular membrane. In such a case the dosage required to achieve the same intracellular concentration of the drug would be lower and the toxicity (mainly nefrotoxicity caused by the interaction of ANP dianions with proximal tubules) might substantially decrease. The ANP formed inside the cell from the prodrug would persist there. Owing to the acute toxicity and genotoxicity of some ANP (Otova et al., 1997) it might not be very desirable to get them to all sorts of somatic cells. It applies particularly to HPMP derivatives which might incorporate into the DNA chain and remain there over the whole mitotic cycle. And we know that their mechanism of action is not limited to the antimetabolic principle. Interference of ANP or their anabolites with cytokines and chemokines and possibly with protein kinases, on telomerase (Hajek et al., 2005) and/or on tubulin (Matulová, unpublished data), as well as their effect on purine receptor system Zidek et al. (2004)—all that suggests the targeted transport application as a method of choice rather than a simple prodrug mode of enhancing ANP transport in general.

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