Medicinal Chemistry of Phosphonate Prodrugs for Antiviral Therapy

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Abstract

Considerable attention has been focused on the development of phosphonate-containing drugs for application in many therapeutic areas. However phosphonate diacids are deprotonated at physiological pH and thus phosphonate-containing drugs are not ideal for oral administration, an extremely desirable requisite for the treatment of chronic diseases. To overcome this limitation several prodrug structures of biologically active phosphonate analogues have been developed. The rationale behind the design of such agents is to achieve temporary blockade of the free phosphonic functional group until their systemic absorption and delivery, allowing the release of the active drug only once at the target. In this paper, an overview of acyclic and cyclic nucleoside phosphonate prodrugs designed as antiviral agents is presented.

Introduction to acyclic and cyclic nucleoside phosphonate prodrugs

Nucleoside analogues are synthetic compounds that are structurally similar to natural nucleosides, the building blocks of RNA and DNA. Once inside the cell, nucleoside analogues undergo (often three sequential) phosphorylation steps by viral and cellular kinases to generate

most often nucleoside 5′-triphosphates that can act as competitive inhibitors of viral and cellular DNA or RNA polymerases or alternatively can be incorporated into a growing DNA or RNA strands, causing chain termination [1]. However, many nucleoside analogues are not phosphorylated effectively *in vivo,* in particular the first phosphorylation step is often inefficient and rate-limiting step in the conversion to the 5' triphosphate. To circumvent this limitation, nucleotides with a phosphate group already attached to the nucleoside have been designed. However, these nucleotides become potential substrates for phosphatases, leading to removal of the phosphate group. Replacement of the phosphate moiety with an isosteric and isoelectronic phosphonate group, results in a nucleoside phosphonate; chemically and enzymatically much more stable than the corresponding phosphate. Different to the O-P linkage, the CH_2-P-bond, due to its chemical nature, is not susceptible to phosphodiesterase and phosphatase hydrolysis. Enzymatically and chemically stable phosphonate analogues, which mimic the nucleoside monophosphates, can bypass the initial enzymatic phosphorylation and can potentially be more effective antiviral agents. Like a nucleoside monophosphate, a nucleoside phosphonate can be further phosphorylated by cellular nucleotide kinases. The nucleoside phosphonates are classified into major groups: acyclic (ANPs) and cyclic (CNPs) nucleoside phosphonates [2].

ANPs, originally developed by the Holý group in the 1980's, exhibit a broad spectrum of antiviral activities, particularly against DNA viruses and retroviruses [3, 4]. The common structural attribute of ANPs is a nucleobase attached to an aliphatic side chain containing a phosphonomethyl residue. A methylene bridge between the phosphonate moiety and the rest of the molecule excludes the possibility of enzymatic dephosphorylation. The absence of a glycosidic bond in the structure of ANPs further increases their resistance to chemical and biological degradation. Flexibility in the acyclic chain is assumed to allow these compounds,

once diphosphorylated, to adopt a conformation appropriate for interaction with active sites of different target enzymes involved in the biosynthesis of DNA (DNA polymerase for DNA viruses, reverse trascriptase for retroviruses). The diphosphorylated ANPs act as chain terminators of the viral DNA inhibiting viral replication. After the description of the first member of ANPs, (*S*)-9-(3hydroxy-2-phosphonyl-methoxypropyl)adenine ((*S*)-HPMPA, **1**) in 1986 [5], new generations of ANPs were prepared and evaluated for their antiviral activity [6]. The ANPs can be classified, according to their structure, in three different series: 3-hydroxy-2-(phosphonomethoxypropyl) (**HPMP**), 2-(phosphonomethoxypropyl) (Figure 1) (**PME**) and 2-(phosphonomethoxypropyl) (**PMP**) series (Figure 2). The **HPMP** series, characterized by the presence of a hydroxymethyl chain, exhibit (generally as the (*S*)-enantiomer) an antiviral activity against a wide spectrum of DNA viruses encompassing, in particular, adeno-, pox-, polioma-, papilloma- and herpesvirus infections. Among this series, derivatives of adenine (**1**, (*S*)-HPMPA,), cytosine (**3**, (*S*)-HPMPC, Cidofovir, Vistide®, used to treat human cytomegalovirus (HCMV) retinitis in acquired immunodeficiency syndrome (AIDS)) [7], 2,6-diaminopurine (**5**, (*S*)-HPMP-DAP), 2,4-diamino-3-hydroxy pyrimidine (**6**, (*R*)-HPMPO-DAPy) and 5-azacytosine (**7**, (*S*)-HPMP-5-aza-C) were found to be especially potent. In particular, the DAP (2,6-diaminopurine) derivatives show an antiviral potency and activity spectrum comparable to that of their adenine counterparts [8]. Thus, (*S*)-HPMP-DAP (**3**) is equivalent to (*S*)-HPMPA (**1**), i.e. with regard to their activity against poxviruses such as vaccinia [9] and orf virus [10]. Akin to (*S*)-HPMPA (**1**) and (*S*)-HPMPC (**2**), (*R*)-HPMPO-DAPy (**4**) proved to be a potent and selective inhibitor of adenovirus replication *in vitro* [11] and exhibited selective and potent activity against orf virus in both human and ovine cell monolayers and organotypic ovine raft cultures [10]. *In vivo*, (*R*)-HPMPO-DAPy (**4**), similarly to (*S*)-HPMPC (**2**) was shown to lead to healing of

cutaneous vaccinia lesions in athymic-nude mice (which corresponds to an experimental model infection for disseminated vaccinia in immunosuppressed patients inadvertently vaccinated with live smallpox vaccine) [9]. The antiviral activity of (*S*)-HPMP-5-aza-C (**7**) was also shown to be comparable to that of the reference drug (*S*)-HPMPC (**3**) against herpes viruses (HSV-1, HSV-2) and vaccinia virus (VV), or 2-7-fold more active against varicella zoster virus (VZV), HCMV, human herpes virus type-6 (HHV-6), and adenovirus type-2 (Ad2). For all these DNA viruses, (*S*)-HPMP-5-aza-C (**7**) showed a 2-16-fold higher antiviral selectivity index (ratio of CC_{50} to EC_{50}) than (*S*)-HPMPC (**3**) [12]. In an effort to increase the cell permeability of HPMP-based nucleoside investigators have also generate a cyclic version of ANPs, forming an internal phosphonate ester bond with the hydroxymethyl chain (Figure 1). As a representative example, cyclic (*S*)-HPMPC (**4**) has been shown to have some advantages respect to (*S*)-HPMPC such as reduced nephrotoxicity due to diminished uptake in the renal proximal tubular cells [13]. Researchers have identified a cellular cCMP phosphodiesterase that can converts cHPMPC to (*S*)-HPMPC *in vivo* [14].

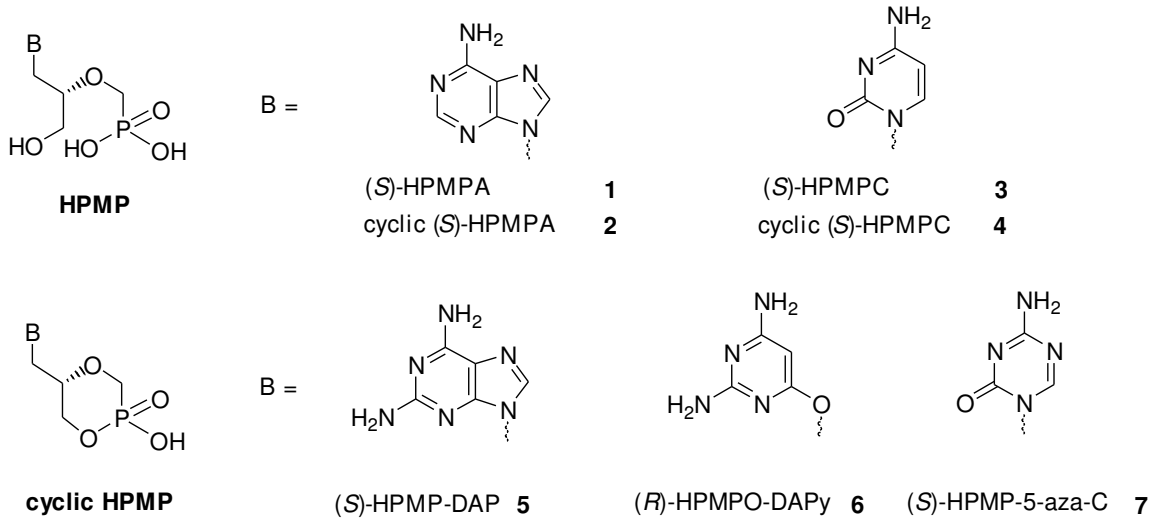


Figure 1. Structures of acyclic nucleoside phosphonate **HPMP** and **cyclic HPMP** series.

The **PME** and **PMP** series of ANPs, which lack respectively the hydroxymethyl and hydroxyl groups in their acyclic side chain, are based on PMEA (**8**) and (*R*)-PMPA (**9**) structures. The **PME** structures unlike the **HPMP** and **PMP** series do not contain a chiral carbon center and thus do not exist as (*R*) and (*S*) enantiomers. The **PMP** series exerts its antiviral activity mostly as the (*R*)-enantiomer. These nucleotides and their analogues have shown potent activity against retro- and hepadnaviruses. PME-DAP (**11**) was found to be more active as an anti-retrovirus agent than PMEA (**8**), but also more toxic, so that its therapeutic index, based on its *in vivo* activity against murine sarcoma virus (MSV), was equivalent to that of PMEA (**8**) [15]. It was reported with even higher anti-retrovirus potency than (*R*)-PMPA (**9**) and has proved active against (both wild-type and lamivudine resistant) hepatitis B virus (HBV) with a potency comparable to that of (*R*)-PMPA (**9**) [16, 17]. "*O*-linked" ANPs such as 2,4-diamino-6-[2-(phosphonomethoxy)ethoxy]pyrimidine (**12**, PMEO-DAPy) and 2,4-diamino-6-(*R*)-[2-(phosphonomethoxy)propoxy]-pyrimidine (**13**, (*R*)-PMPO-DAPy) were found to inhibit the replication of herpesviruses (HSV-1, HSV-2, VZV) as well as retroviruses such as human imunodeficency viruses type 1 and 2 (HIV-1, HIV-2) [18]. In particular, the antiretroviral activity of PMEO-DAPy (**12**) and (*R*)-PMPO-DAPy (**13**) appeared interesting, as this was comparable to that of PMEA (**8**) and (*R*)-PMPA (**9**) and was also observed *in vivo* [19].

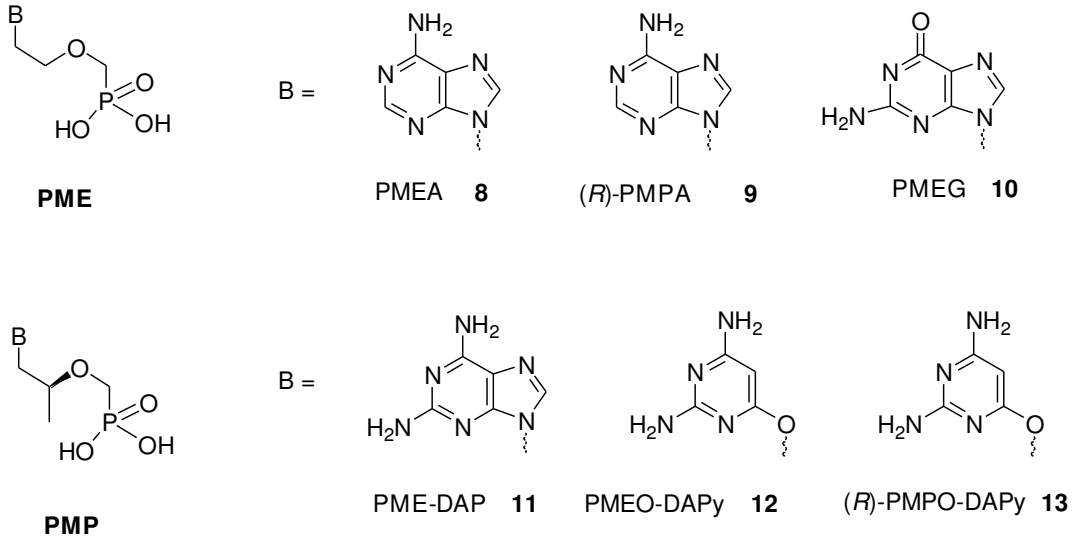


Figure 2. Structures of acyclic nucleoside phosphonate **PME** and **PMP** series.

CNPs are real nucleoside analogues as they contain a nucleobase and a sugar moiety. Compared to the large number of the ANPs described in the literature, only few examples of CNPs endowed with antiviral activity have been reported (Figure 3) [2]. The lack of antiviral activity of CNPs is generally explained by their poor substrate properties for cellular and viral kinases. On the other hand, the potent antiviral activity of ANPs is ascribed to their intracellular phosphorylation to their diphosphates and to a refractory incorporation of the modified nucleosides in nucleic acids. To try to overcome the drawback of CNPs, compounds where the phosphonoalkoxy group of the nucleoside phosphonates is bound at the 3'-position were designed. Interesting examples of this class of drugs are the (*L*)-2-deoxythreosyl phosphonate nucleosides PMDTT (**14**) and PMDTA (**15**), reported as selective anti-HIV agents [20]. The absence of a hydroxymethyl substituent in the 4'-position of the nucleoside can probably avoid OH hindrance during enzymatic phosphorylation allowing the formation of the diphosphate species.

In an effort to identify new nucleoside inhibitors of reverse trascriptase, phosphonometoxy analogs of cyclic pyrimidine nucleoside were synthesized and compared for their antiviral activity [21]. Among them, only d4TP (**16**) showed an antiviral activity below 200 μM. This approach have been recently extended to purine analogs leading to the discovery of the phosphonomethoxy-2'-fluoro-2',3'-dideoxy-2',3'-didehydroadenosine (**17**, GS-9148), a ribose-modified HIV-1 nucleotide reverse transcriptase inhibitor (NRTI) selected from a series of nucleoside phosphonate analogues for its low mitocondrial toxicity, minimal cytotoxicity in renal proximal tubule cells and other cell types, for its synergy in combination with other antiretroviral drugs and its unique resistance profile against multiple resistant HIV-1 strains [22].

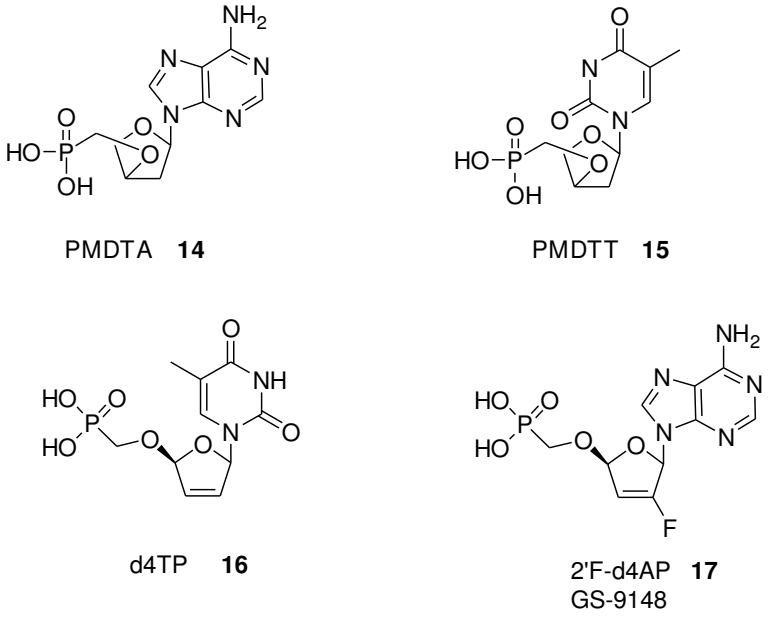


Figure 3. Structures of cyclic nucleoside phosphonates PMDTA (**14**), PMDTT (**15**), d4TP (**16**) and 2'F-d4AP (GS-9148, **17**).

Natural nucleosides are as well hydrophilic molecules and do not rapidly penetrate cell membranes by non-facilitated diffusion. Instead they permeate the cell by carrier-mediated endocytosis, which is an active or facilitated transport mechanism that requires energy and a

specific receptor or protein on the cell surface. Acyclic and cyclic nucleoside phosphonate analogues contain a phosphonic acid group completely ionized at physiological pH, resulting in a molecule largely impermeable to mucosal and cellular membranes. However carrier-mediated transport often requires very close structural resemblance to natural products. Because their low oral bioavailability ANP and CNP analogues are of limited value in the treatment of chronic diseases, where the oral therapies are highly desired.

Prodrugs are used in general to bypass physicochemical, pharmaceutical, pharmacokinetic and pharmacodynamic barriers to drug formulation and delivery, such as poor aqueous solubility, chemical instability, insufficient oral absorption, rapid presystemic metabolism and inadequate tissue penetration. In an attempt to overcome, the limited oral bioavailability of ANPs and CPs ionizable phosphonate can be masked by derivatization, thus generating a prodrug with increased liphophilicity, capable to alter cell and tissue distribution/elimination patterns of the parent drug [23].

Passive transcellular absorption is the most general route for absorption of lipophilic (pro)drugs through the intestinal mucosal membranes. After the drug molecule is dissolved in the aqueous media, absorption occurs by diffusion through the lipid bilayer and because it is driven by a concentration gradient, energy is not required. Absorption is thus dependent on the lipophilicity, intrinsic aqueous solubility and molecular weight of the drug molecule. Once the drug has reached the blood circulation it will pass through the endothelium of the capillary and distribute into tissues. Thus also the distribution is dependent on several structural and physicochemical properties including the molecular size, logP, hydrogen-bonding, charge state and finally on its ability to be a substrate for influx and efflux transporters and to bind plasma proteins. The liver is the major organ for drug metabolism, but metabolism occurs also in the GI-

tract and to some extent in lungs, skin and kidneys. First-pass metabolism consisting of phase I oxidation reactions catalyzed by cytochrome P450-enzymes and phase II conjugation reactions catalyzed by several enzymes occur in the liver. Excretion, elimination or clearance of a drug molecule from the body starts immediately after the drug has entered the circulation and two organs are mainly responsible for it: the liver and the kidney. Renal clearance occurs via filtration of the blood in the glomerulus of the kidneys and the further reabsorption of the compounds from the kidney tubule back to the systemic circulation with the molecular weight and liphophilicity of the drug molecule playing a key role in these events.

In order to achieve oral bioavailability and intracellular delivery an ideal oral prodrug must survive the gastrointestinal (GI) tract, be absorbed across intestinal mucosa and delivered into the systemic circulation following its active/passive transport, and then be distributed into cells where it has to be converted to the parent drug releasing a non-toxic promoiety, which in turn has to be rapidly excreted from the body. Thus, stability is one of the major factors influencing the design of prodrugs. The bioactivation mechanism for most prodrug structures is enzymatic or at least requires enzymes to initialize the bioactivation process, which can then further continue chemically.

Several prodrug approaches have been utilized to overcome the limitation of phosphonate-containing drugs. General reviews about prodrug strategies elaborated for phosphate-, phosphonate- and phosphinates-containing compounds [24-27] and more specific reviews directed toward delivery of nucleoside and nucleotide have been published [23, 28-30]. The aim of this review is to survey specifically the status and the recent progress in the design and development of acyclic and cyclic nucleoside phosphonate prodrugs, focusing on the most promising strategies currently under development in the antiviral area.

Bis-(POM) and bis-(POC) esters prodrugs

The development of acyloxy ester alkoxycarbonyl ester prodrugs of ANPs has been successful. Among these ester prodrugs two compounds are currently marketed for antiviral therapy: the bis (pivaloyloxymethyl) ester of adefovir (**18**, bis-(POM)-PMEA) (Hepsera®) approved in 2002 for the treatment of HBV infections and the disopropyloxycarbonyloxymethyl ester of tenofovir fumarate (**19**, bis-(POC)-(*R*)-PMPA fumarate) (Viread®) licensed in 2001 for the treatment of HIV infections (Figure 4).

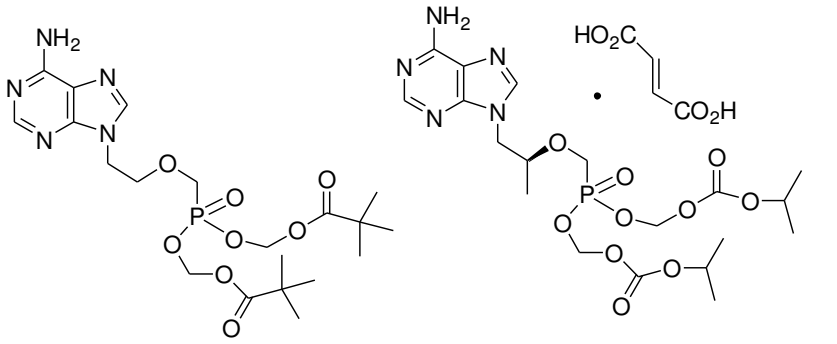


Figure 4. Structures of bis-(POM)PMEA (**18**), bis-(POC)-PMPA fumarate (**19**), bis-(POM)-PMCDG (**20**) and (*E*)-bis-(POM)TbutP (**21**).

In general, bis-POM derivatives are reported to exhibit a 9- to 13-fold greater *in vitro* antiretroviral activity than their corresponding unmodified compounds and show remarkable increased bioavailability *in vivo*. Bis-(POM)-PMEA (**18**), selected among a series of acyloxy methyl ester prodrugs, was first considered as a possible candidate for the treatment of HIV infection since it was able to reduce the viral load in plasma.[31] However, the toxicity due to its decomposition products (formaldehyde and pivalic acid) generated some concern (Figure 5) [32-34].

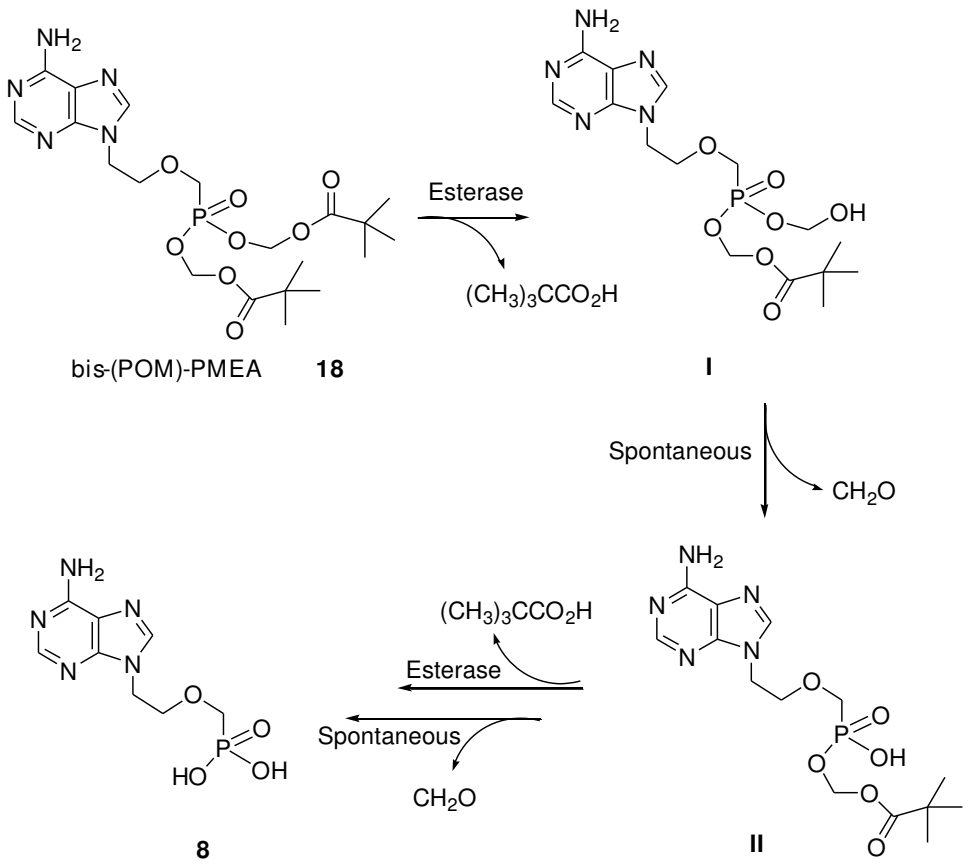


Figure 5. Metabolic pathway for bis-(POM)-PMEA (**18**).

In particular the pivalic acid was demonstrated to be responsible for altering carnitine homeostasis [35]. Additionally it has been shown that bis-(POM) phosphotriesters were chemically unstable and highly susceptible to serum-mediated hydrolysis, factors that limit their potential utility for intracellular drug delivery [36]. For these reason, bis-(POM)-PMEA (**18**) was considered too nephrotoxic to permit a long term use at the dosage required inhibition of HIV and it was instead approved from FDA only for the treatment HBV. The pivaloyloxymethyl prodrugs of (*R*)-PMPA and (*R*)-PMP-DAP were also evaluated but due to toxicity concern they were not selected for clinical trials.

The diisopropyloxycarbonyloxymethyl ester (bis-(POC) is a modification of the bis-(POM)-ester, offering the advantage to not generate pivalic acid during its bioconversion. The bis-(POC) motif was successfully applied to (*R*)-PMPA (**8**) leading to bis-(POC)-(*R*)-PMPA, which was selected for clinical trial and subsequently formally approved as its fumarate salt (**9**) for the treatment of HIV. Bis-(POC)-(*R*)-PMPA fumarate (**19**) is also currently commercially available in combination with emtricitabine (Truvada®) and emtricitabine and efavirenz (Atripla®).

Subsequently PMCDG dipivoxil (**20,** bis-(POM)-PMCDG, figure 6) emerged as a possible candidate as an oral prodrug for HBV treatment and it is currently in Phase II clinical trials for evaluation of efficacy in human [37, 38]. Bis-(POM)-PMCDG is rapidly converted to the parent drug in the liver and intestine probably by esterases. The parent drug is further metabolized to a nucleotide analogue of guanosine monophosphate, by oxidase such as aldehyde or xanthine oxidase. After phosphorylation to di- and tri-phosphate forms, the molecule inhibits viral replication following incorporation into viral DNA.

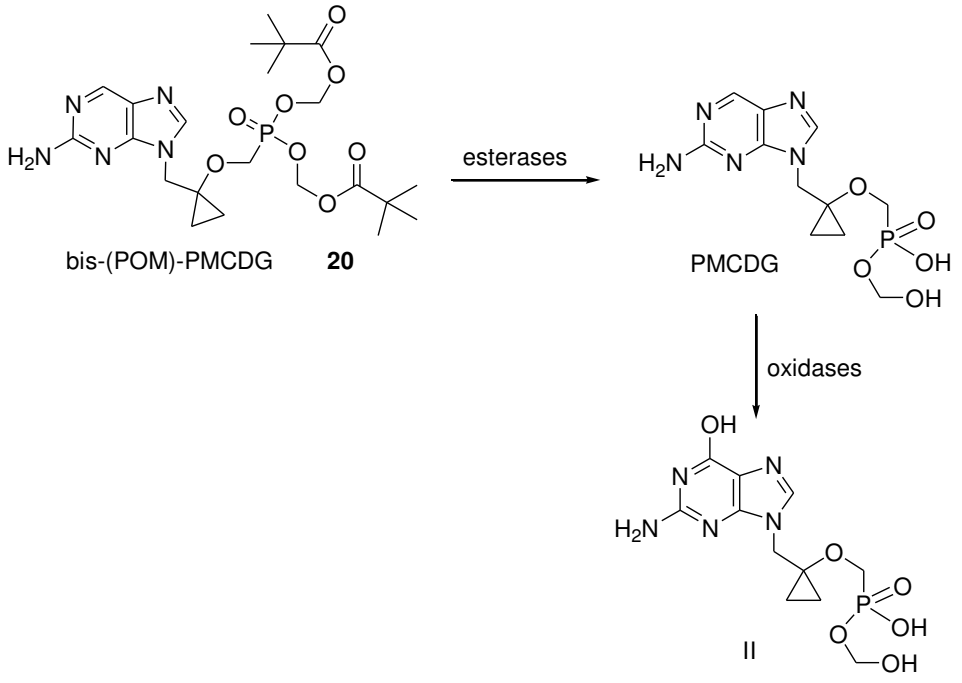
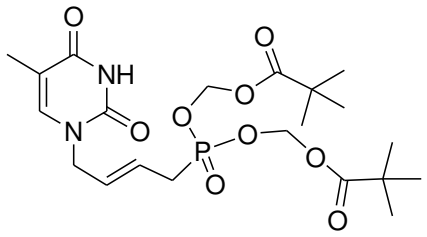


Figure 6. Metabolic pathway for bis-(POM)-PMCDG (**20**).

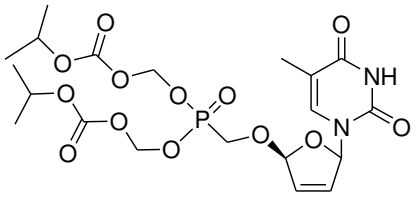
More recently bis-POM esters of (*S*)-HPM-DAP and its cyclic analog were reported by Krečmerová *et al. [39]* Although these compounds proved to be less active than the corresponding alkoxyalkyl ester prodrugs (see next section), they appeared to be less cytotoxic and cytostatic. Finally, bis-POM esters of novel ANPs have been described. Among them, (*E*)-bis-(POM)TbutP (**21**) emerged as a potent antiviral agent against several herpes viruses, (HSV-1, HSV-2, VZV), representing a new potential antiviral lead for further optimization (Figure 7) [40].



bis-(POM)-(*E*)-TbutP **21**

Figure 7. Structure for bis-(POM)-(*E*)-TbutP (**21**).

The antiviral potency of CNPs can also be improved considerably by the application of bis-(POC) prodrug technology. As representative example bis-(POC)-d4TP (**22**) has been reported to improve 29-fold the HIV antiretroviral activity of the corresponding nucleoside (Figure 8) [21].

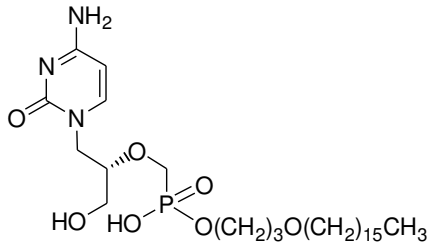


bis-(POC)-d4TP **22**

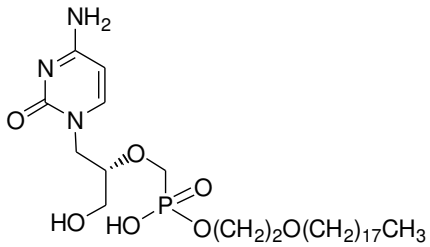
Figure 8. Structure of bis-(POC)-d4TP (**22**).

Alkoxyalkyl ester prodrugs

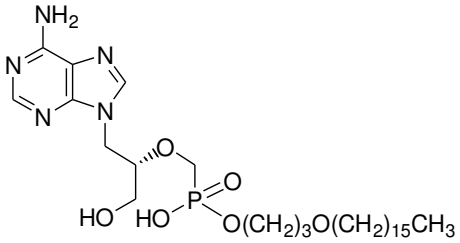
In order to enhance cellular drug uptake of ANPs, the Hostetler group and collaborators have developed a series of very promising ether lipid conjugates of ANPs, including (*S*)-HPMPC (**3**), (*S*)-HPMPA (**1**), PME (**8**), (*R*)-PMPA (**9**).[41] Two alkoxyalkyl esters of (*S*)-HPMPC, hexadecyloxypropyl-(*S*)-HPMPC (**23**, HDP-(*S*)-HPMPC) and octadecyloxyethyl-(*S*)-HPMPC (**24**, ODE-(*S*)-HPMPC) have been shown to be more active than (*S*)-HPMPC against HCMV and other herpes viruses, exhibiting 2.5- to 4-fold increases in antiviral activity depending on the *in vitro* antiviral assay used (Figure 7).[42] These alkoxyesters were also found to be 25- to 910-times more active than (*S*)-HPMPC (**3**) against variola virus (smallpox) with EC_{50} values ranging from 0.04 to 0.1 μM for HPD-(*S*)-HPMPC (**23**) and from 0.01 to 0.03 μM for ODE-(*S*)-HPMPC (**24**) [43, 44]. Similar results were also reported with monkeypox and cowpox viruses *in vitro*. When tested *in vitro* against VV, HPD-(*S*)-HPMPC (**23**) showed an increase in antiviral activity of 58-fold when compared to the parent drug, whereas ODE-(*S*)-HPMPC (**24**) was even more active with a 231-fold increase (with respect to (*S*)-HPMPC (**3**)) and with an EC_{50} value of 0.2 μM. An explanation of such increase in antiviral activity of **23** and **24** when compared to (*S*)-HPMPC (**3**) is found from the author in the increased cellular uptake and conversion to (*S*)-HPMPC diphosphosphate.



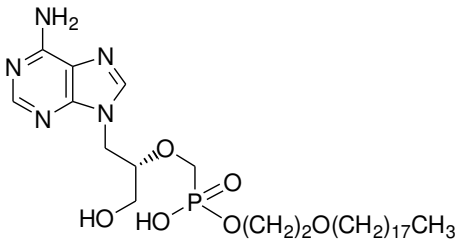
HDP-(*S*)-HPMPC **23**



ODE-(*S*)-HPMPC **24**



HDP-(*S*)-HPMPA **25**



ODE-(*S*)-HPMPA **26**

Figure 9. Structures of HDP-(*S*)-HPMPC (**23**), ODE-(*S*)-HPMPC (**24**), HDP-(*S*)-HMPA (**25**) and ODE-(*S*)-HMPA (**26**).

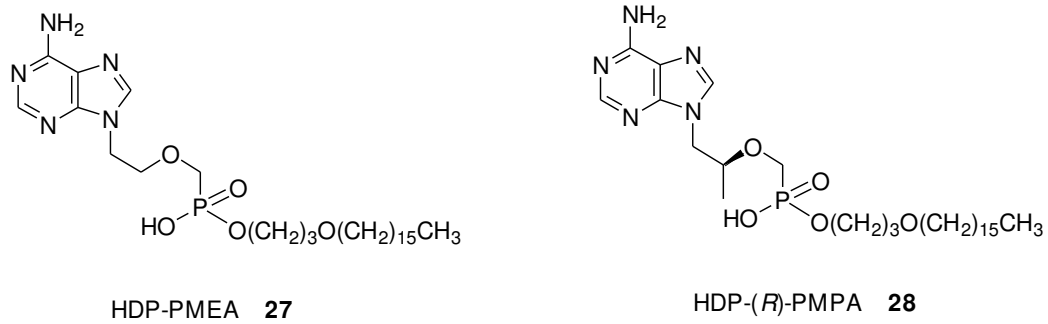
In vivo these compounds proved as effective as parental (*S*)-HPMPC (**3**) in the treatment of HCMV infection in a variety of murine models [45]. They also proved to be effective when tested in animal model of orthopox diseases such as cowpox, vaccinia and ectromelia virus infections [46, 47]. When given orally to mice infected with ectromelia virus by small particle aerosol, HDV(*S*)-HPMPC (**23**) and ODE(*S*)-HPMPC (**24**) were almost full protective by oral doses of 5 mg/kg and 10 mg/kg, administered four hours after infection and sustained daily for five days. A dose of 10 to 12 mg /kg of respectively HDP-(*S*)-HPMPC (**23**) and of ODE-(*S*)-HPMPC (**24**), given orally for five days prior to or few days after nasal infection with cowpox, were reported to be able to significantly reduce the mortality. HDP-(*S*)-HPMPC (**23**) with the name of CMX001 is currently under development by Chimerix, Inc. as an oral drug for treatment

of HCMV and small pox infection. Recently CMX001 has completed Phase I clinical trials in healthy volunteers and Phase II trials are now in progress in HCMV stem cell transplant patients and BK virus infection in kidney transplant patients. However, CMX001 recently failed in an *in vivo* efficacy trial (monkeypox model) likely due to metabolic differences between rodents and monkeys [48].

Besides (*S*)-HPMPC derivatives, alkoxyalkyl derivatives of another ANP, (*S*)-HPMPA (**1**) have been described (Figure 10). Esterification to HDP-(*S*)-HPMPA (**25**) or ODE-(*S*)-HPMPA (**25**) resulted in large increases in antipoxvirus activity raging from 160 to 270-fold versus (*S*)-HPMPA (**1**) [49, 50]. Similar results have observed with CMV infection [51]. Among these derivatives, ODE-(S)-HPMPA (**26**) was the most active compounds in adenovirus infected cells with EC_{50} values of 0.04-0.16 μM compared with 0.19-1.1 of HDP-(*S*)-HMPA (**25**). When compared to the alkoxyalkyl of (*S*)-HPMPC (**3**), the corresponding alkoxyalkyl ester of (*S*)-HPMPA (**1**) displayed similar activities against human and murine CMV.

Both (*S*)-HPMPC (**3**) and (*S*)-HPMPA (**1**) have been reported to inhibit replication of all double-stranded DNA viruses and the increased antiviral activity observed after alkoxyalkyl esterification does not appear to be specific for a particular type of virus. Although (*S*)-HPMPA (**1**) was reported to be inactive against HIV, HDP-(*S*)-HPMPA (**25**) and ODE-(*S*)-HPMPA (**26**) are described highly active *in vitro* (with low nanomolar EC_{50} values), indicating that the alkoxy alkylester strategy can eventually wide the range of antiviral activity. The same strategy has been applied to PME (**8**) and (*R*)-PMPA (**9**) (Figure 10). HDP-PMEA (**27**) and HDP-(*R*)-PMPA (**28**) have been found to be high active against HIV-1. HDP-(*R*)-PMPA (**28**) under the name of CMX157, is currently in clinical development by Chimerix, Inc. as an oral drug for treatment of

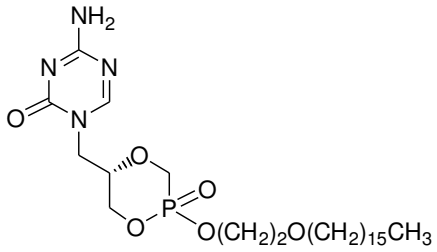
HIV infection. Recently it was announced that the first results in human Phase I clinical trials demonstrate a favorable safety, tolerability and drug distribution profile for CMX157.



HDP-PMEA **27** HDP-(*R*)-PMPA **28**

Figure 10. Structures of HDP-(*S*)-PMEA (**27**) and HDP-(*S*)-PMPA (**28**)

Other ANPs have been subjected to esterification with alkoxyalkyl groups. In particular the antiviral activity of (*S*)-HPMPC-5aza was reported to be further enhanced by introduction of alkoxyalkyl groups, the most active being the hexadecyloxyethyl (HDE) ester derivative (**29**) with EC_{50} values in the range of 0.003-0.008 μg/mL for HSV (Figure 11) [52].



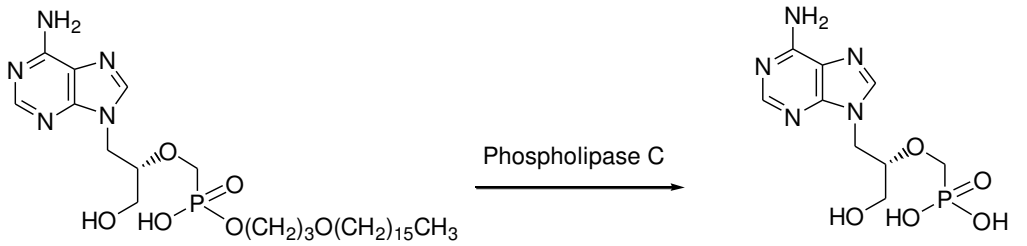
HDE-cHPMP-5-azaC **29**

Figure 11. Structure of HDE-(*S*)-cHPMP-azaC (**29**).

In the case of (*S*)-HPMP-DAP (**5**), the ODE derivative has been shown to be a potent inhibitor of herpes virus and orthopoxvirus replication. Recently Krečmerová *et al.* selected HPMP-DAP (**5**) and its cyclic form for further evaluation, synthesizing a series of different

prodrugs and then assessing their *in vitro* antiviral activity. From these studies the HDP and the POM mono ester of (*S*)-HPMP-DAP and its cyclic analog, emerged as the most active prodrugs against VV, HSV, VZV and HCMV.

The esterification of ANPs with HDP and ODE chains was designed to increase oral bioavailability, based on the resemblance to lysophosphatidylcholine. As a representative example for this class of prodrugs, the metabolic pathway of HDP-(*S*)-HPMPC (**23**) is reported in figure 12. Phospholipase C, which is reported to be the only enzyme responsible for the metabolic cleavage, is common in mammalian tissues. Phospholipase C is not present in plasma or pancreatic secretions, providing stability for **23** and other compounds of this type during oral absorption and transport in plasma to tissues.



HDP-(*S*)-HPMPC **23** (*S*)-HPMPC **3**

Figure 12. Metabolic pathway for HDP-(*S*)-HPMPC (**23**).

Hexaethyleneglycol unit or hydroxylateddecyl- or decyloxyethyl- chains were recently used by Krecmerova *et al.* to mask either PME or (*S*)-HPMPC [53]. However the antiviral activity of these prodrugs was found in general lower or similar to the corresponding parent drug. Considering these results the authors conclude that these prodrugs are taken up less efficiently or are not suitable substrates for the phospholipase C.

Despite the notable success of the alkoxyalkyl prodrugs developed by Hostetler there are still challenges around this this approach because of the potential poor solubility in aqueous solutions, arising from a lipid moiety [47, 54].

Aryl and phenyl ester prodrugs

Rappresentative examples of these prodrugs are the phenyl (**30**) and the salicylate ester prodrugs (**31** and **32**) of (*S*)-cHPMPC (**4**) reported by Oliyai *et al*. (Figure 13) [55, 56].

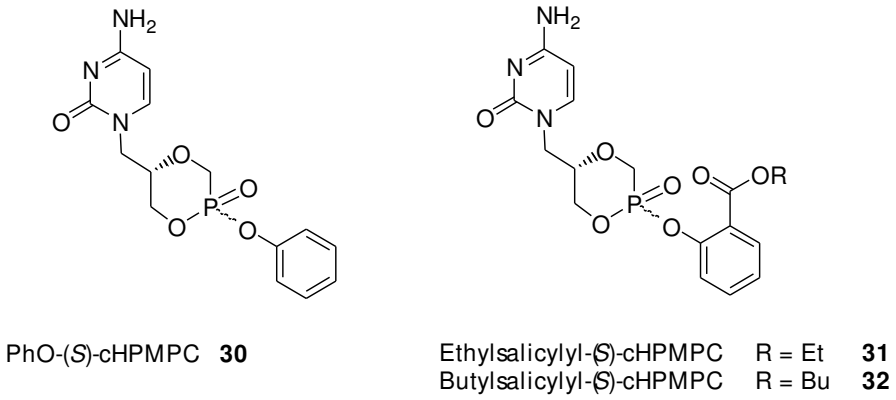


Figure 13. Structures of PhO-(*S*)-cHPMPC (**30**), ethylsalicylyl- (**31**) and butylsalicylyl-(*S*)-cHPMPC (**32**).

Esterification of (*S*)-cHPMPC (**4**) *via* a phosphoester bond, either with a phenol or a salicylic ester produces a new chiral center at the phosphorus, leading to two different diastereoisomers of the resulting prodrug (SR_p and SS_p). The carboxylate function on the salicylate prodrugs provides an additional site for chemical modification to tune the lipophilicity, solubility and biological reactivity of the prodrug. The compounds were synthesized in a stereospecific manner and investigated for their physicochemical and pharmacokinetics properties. The authors demonstrated that the chemical stability was dependent on the phosphorus stereochemistry (with

the axial isomer more stable than the equatorial) and not greatly with nature of the salicylate ester, which on the contrary plays a key role for the enzymatic stability. The axial isomer of salicylate prodrugs (**I**, Figure 14), selected for *in vivo* oral bioavailability evaluation produces (*S*)-cHPMPC (**4**) as the major metabolites together with (*S*)-HPMPC (**3**) and the mono ester of (*S*)-HPMPC (**II**) whereas the corresponding equatorial isomer generates only (*S*)-cHPMPC (Figure 12). The results of these studies proved that salicylate ester prodrugs can successfully deliver (*S*)-cHPMPC (**4**) to the systemic circulation with an oral bioavailability of **4** ranging from 18.5 (for **31**) to 46.3% (for **32**). This prodrug approach presents the advantage to allow oral delivery of (*S*)-cHPMPC (**4**), while minimizing (*S*)-HPMPC (**3**) related toxicity.

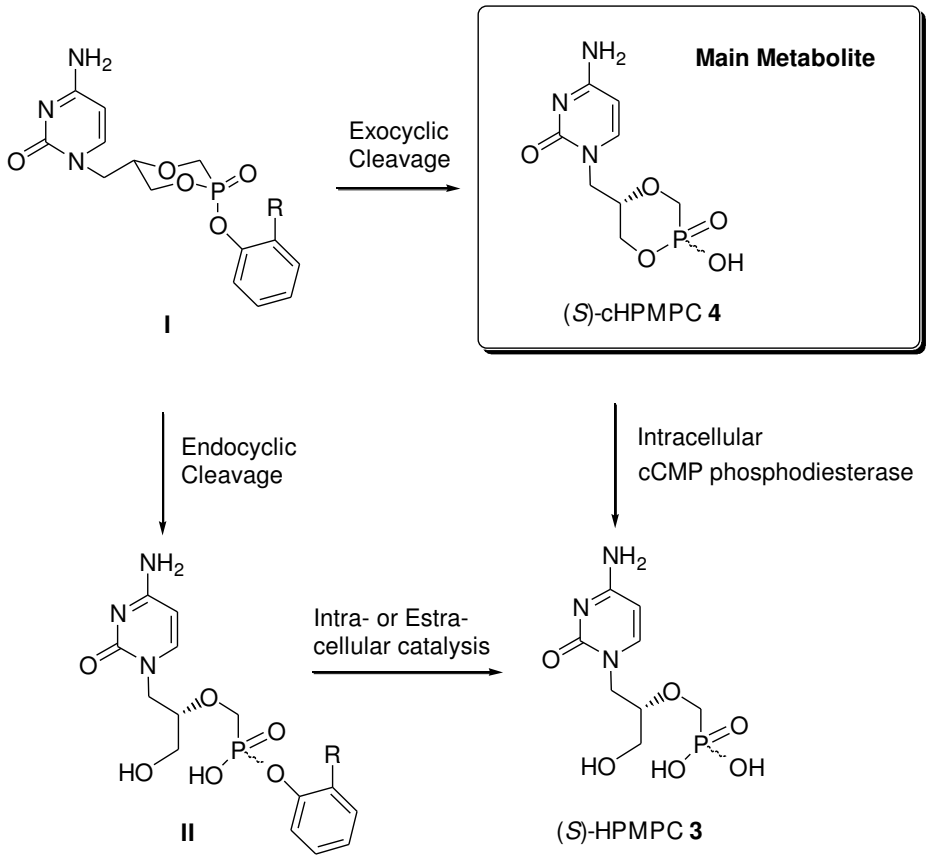
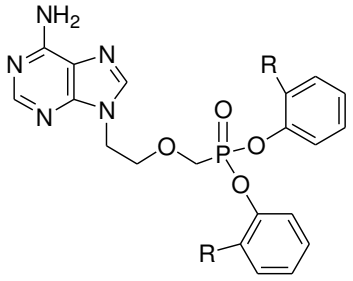


Figure 14. Metabolic pathway for axial isomers of aryl (*S*)-cHPMPC prodrugs.

A number of esters of PMEA have been synthesized and evaluated for their oral bioavailability [57]. Among them, the diphenyl ester was identified as the preferred prodrug because it is well absorbed and efficiently converted to the parent compound with an oral biovailability of 50% (Figure 15). However, further studies revealed that the diphenyl ester even if moderately absorbed is poorly converted to PMEA due to oxidation of the ethyl side chain by P450 enzymes to generate the inactive metabolite 2-adenylacetic acid. The same metabolite was observed from the activation of bis-(*o*-ethoxy)phenyl making these two aryl esters unsuitable for further evaluation as prodrugs.



bis-(PhO)-PMEA R = H **33**
bis-[(*o*-EtO)PhO]-PMEA R = OEt **34**

Figure 15. Structures of bis-(PhO)-PMEA(**33**) and bis-[(*o*-EtO)PhO]- PMEA (**34**).

Aryl phosphonamidates and phosphonodiamidate prodrugs

The ProTide (pronucleotide) approach, developed by McGuigan *et al.* was succesfully applied to nucleoside phosphates and then investigated in application to nucleoside phosphonates. The ProTide of a nucleoside phosphonate is a phosphonamidate prodrug consisting of an amino acid ester promoiety linked via a P-N bond to a nucleoside aryl phosphonate. Such a prodrug should be able to facilitate passive diffusion through the cell membrane and when it is cleaved, it should deliver the nucleoside phosphonate inside the cell releasing non-toxic masking groups. The metabolic activation of the phosphonamidates is

generally assumed to follow the same two enzymatic steps involved in the activation of the phosphoramidates (Figure 16). The putative mechanism for the activation of the phosphonamidates involves an initial carboxylic esterase or carboxypeptidase mediated hydrolysis of the carboxylic ester of the amino acid leading to intermediate **II**. The ester cleavage is followed by an internal nucleophilic attack of the acid residue on the phosphorus centre, displacing the aryloxy group and giving the transient formation of the putative five-membered cyclic intermediate **III**. This cyclic mixed anhydride is rapidly hydrolyzed to the corresponding aminoacyl phosphonamidate ester **IV**. The ester is then believed to undergo P-N cleavage, mediated by a phosphonamidase or may result from simple hydrolysis in more acidic subcellular compartment, to eventually release the parent drug **V**.

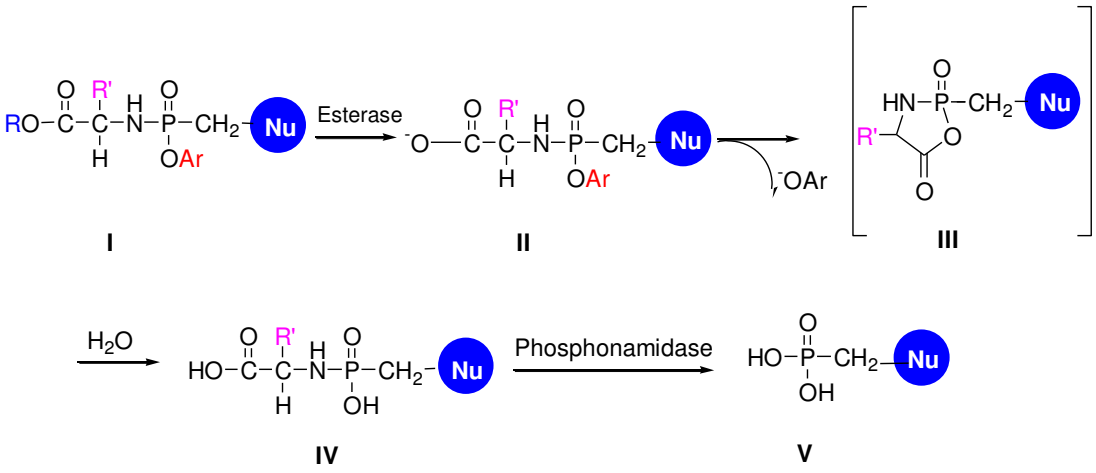


Figure 16. Metabolic pathway for aryl phosphonamidate prodrugs.

The ProTide technology was successfully applied by McGuigan and coauthors to PMEA (**8**) and (*S*)-PMPA (**9**) [58]. In these studies similar SARs were found for the phosphonamidates of PMEA and (*S*)-PMPA as earlier noted for nucleoside phosphoramidate analogues, with the (*L*)-alanine derivatives showing greatly enhanced antiviral potency against HIV compared with the parent nucleotide analogue (50 fold increase for phenyloxy methyl-(*L*)- alaninyl

phosphonamidate PMEA prodrug (**35**) *vs* PMEA (**8**) and 50-100 fold increase for phenyloxy methyl-(*L)*- alaninyl phosphonamidate PMPA prodrug (**36)** *vs* (*S*)-PMPA (**9**) (Figure 17). In analogy to this work, Gilead science reported an extensive study on the application of the ProTide approach to (*S*)-PMPA (**9**) investigating the mechanism of hydrolysis and the metabolism of these class of prodrugs [59]. As results of these studies, the phenyloxy isopropyl-(*L*)-alaninyl phosphonamidate prodrug of PMPA (**37**, GS-7340, Figure 17) emerged as a lead compound with improved biological properties. In addition, cathepsin A was found to be the primary enzyme that activates **37** in human lymphatic tissues [60].

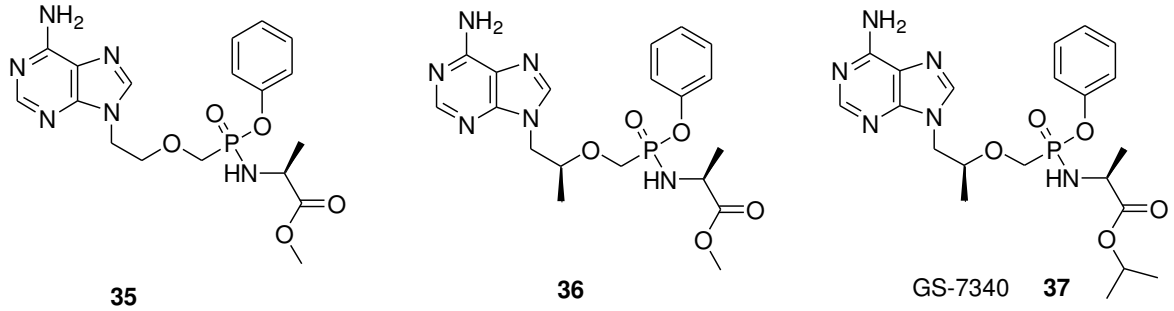
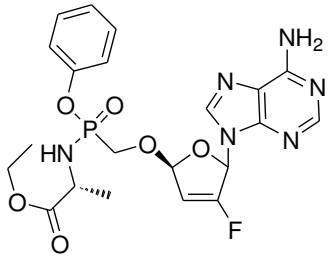


Figure 17. Structures of PMEA phosphonoamidate derivatives **35** and **36** and GS-7340 (**37**).

A series of aryl phosphonamidate prodrugs of GS-9148 (**16**), were also designed by Gilead to effectively deliver **16** and its active diphosphorylated metabolite into target cells [61]. The phenyloxy ethyl (*L*)-alaninyl phosphonamidate prodrug (**38**, GS-9131, Figure 18), improved the *in vitro* antiviral activity of **16** by approximately 50-fold against HIV, demonstrating the most favorable esterase (cathepsin) substrate properties in addition to a good *in vitro* intestinal and hepatic stabilities [22, 62]. Following oral dosing (3mg/kg) of **38** in beagle dogs, high levels of GS9148 diphosphate were observed inside the cells with a mean oral bioavailability of 26%.[19] All these favorable properties lead to the selection of GS-9131 (**38**) as a clinical candidate.



GS-9131 **38**

Figure 18. Structure of phosphonoamidate derivative GS-9131 (**38**).

Phosphonodiamidate analogues having two identical amino acids as masking groups of the phosphonate moiety trough a P-N bond were designed. They offer two distinct advantages compared to phosphonamidate analogues: 1) due to their symmetric structure no phosphorus chirality arises; 2) exclusively non toxic promoieties are released. The activation mechanism is presumed to be similar to the one previously shown for the cognate phosphonoamidates. As first steps the hydrolysis of one amino acid ester lead trough spontaneous cyclization to intermediate **III**, structurally identical to the one derived from the activation of an aryl phosphonamidate (see intermediate **III** in figure 16). Then exactly the same pathway follows (Figure 19)

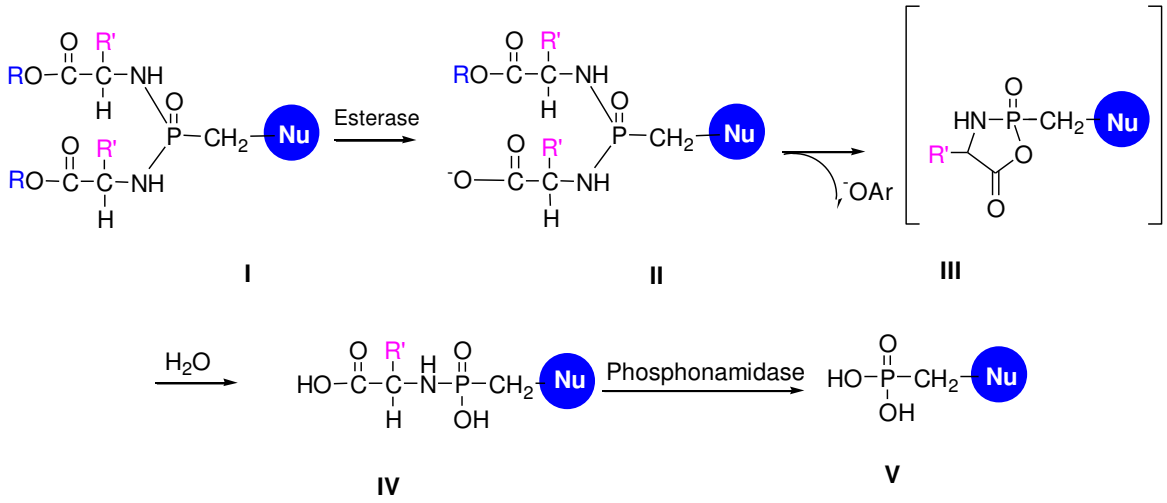


Figure 19. Metabolic pathway for phosphonodiamidate prodrugs.

Several phosphonodiamidate of ANPs containing alkyl (*L*)-alanine were reported to exhibit potent antiviral activities, being bis(butyl-(*L*)-alaninyl)PME-N6-(cyclopropyl)DAP (**39**, Figure 20) the most active of the series against poxviruses [63]. After prodrug cleavage, the cPrPMEDAP is intracellularly deaminated by N6-methyl-AMP amino hydrolase to yield the potent antiproliferative and antiviral agent PMEG (**10**).

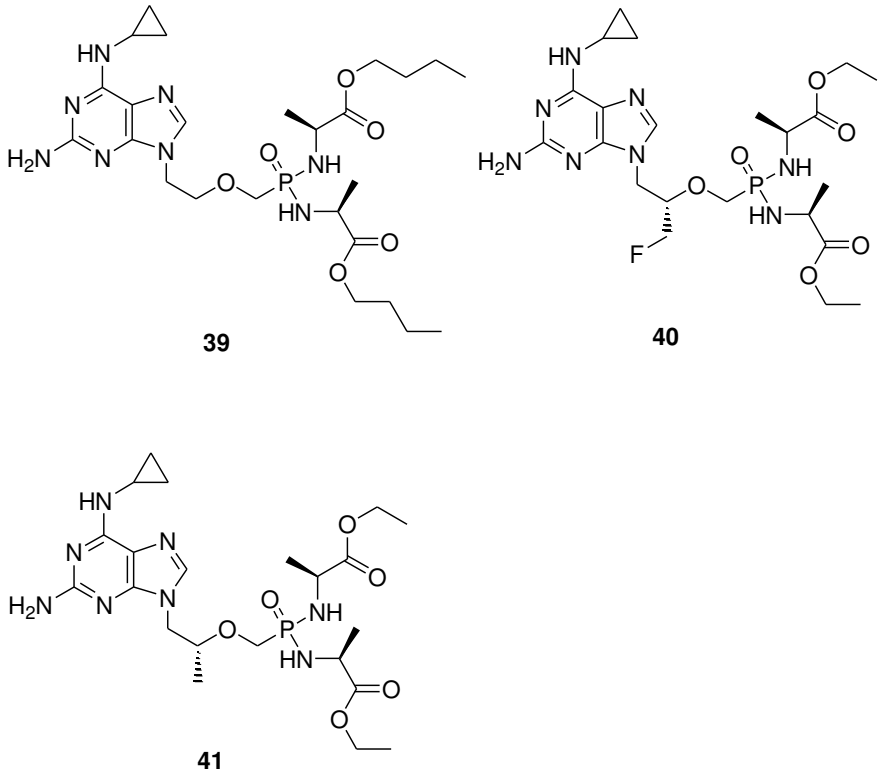


Figure 20. Structures of phosphonodiamidate derivatives of ANPs.

Recently, the development of a novel and efficient one-pot synthesis of phosphonodiamidates directly from the phosphonic acid diesters, significantly improved the preparation of this class of prodrugs [64]. In this report the methodology has been applied to the synthesis of **40** and **41**,

which exhibit anti-HIV activity with submicromolar EC_{50} values and no detectable cytotoxicity up to 100 μM, the highest concentration tested.

Diamidate prodrugs of GS-9148 (**16**) have been reported. Despite their potent *in vitro* anti HIV activity these diamidates were quite ineffective in delivering **16** into the cells, producing after intravenous administration to dogs only approximately 3-fold higher intracellular levels of GS-9148 compared to that from the same dose of GS-9148 by itself [61].

Cyclosaligenyl ester prodrugs (CycloSal)

The cyclosaligenyl prodrug strategy was elaborated by Meier and coworkers first to deliver monophospates and only later was applied to ANPs. In particular the same group reported the synthesis, the stability and the biological evaluation of cycloSal-PMEA prodrugs [65]. The structure of the cyclosal PMEA prodrugs present a salicyl alcohol as the only masking unit for both the two hydroxyl moieties of the phosphonic acid of PMEA. Due to an unexpected low hydrolytic stability of these cycloSal-PMEA prodrugs, the authors had to investigate the more stable cycloAmb-PMEA derivatives, in which the salicyl alcohol has been replaced with a 2-amino benzyl alcohol. From this study it was proven that the mechanism of activation of cycloSal phosphonate is identical to that of the cycloSal-phosphate with the PMEA as the only product of hydrolysis (Figure 21).

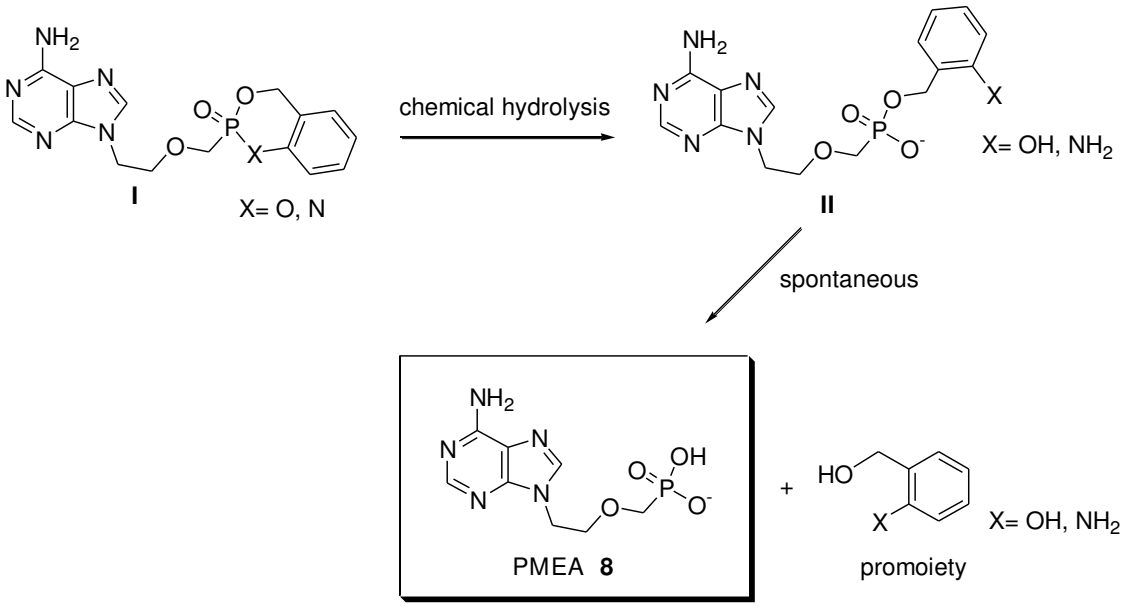


Figure 21. Hydrolysis mechanism for cycloSal- and cycloAmb-PMEA prodrugs.

Although considerably chemically and enzymatic more stable than cycloSal-PMEA prodrugs, cycloAmb-PMEA derivatives surprisingly were reported to exhibit antiviral activity somewhat (2- 3-fold) lower compared to the PMEA and10-fold lower than the cycloSal-PMEA prodrugs. The reduced antiviral activity was attributed by the authors to the slow release of PMEA from the cycloAmb-PMEA, the rate-limiting step being the cleavage of the intermediate benzyl phosphonate ester **II**. To achieve higher antiviral activity different cycloAmb-PMEA derivatives with new substitution patterns were reported. However these new prodrugs failed to accelerate the decay of the hydrolysis of the intermediate **II**, presenting only a slightly improved antiviral activity when compared to the previous cyclo-Amb prodrugs [66].

S-Acylthioethyl (SATE) esters prodrugs

On the basis of the favorable results obtained with the S-acylthioethyl (SATE) prodrug for delivering monophosphate drugs, the SATE approach was applied successfully to ANPs. In

particular a series of bis-(SATE)-PMEA derivatives were reported. These compounds proved enzymatically more stable than the bis-(POM)-PMEA (**18**) [67]. Among them the *t*-bu-SATE PMEA (**42**) emerged as the most promising compound, combining an antiviral potency against HIV similar to bis (POM) PMEA with a markedly greater chemical and enzymatic stability. The metabolic pathway for bis-(SATE) phosphonate prodrug **42**, assumed to be identical to the one reported for the phosphate prodrug,[68] is depicted in figure 22. The prodrug, once inside the cell, preferentially forms an unstable 2-thioethyl intermediate (**I**) by the action of a carboxyesterase or a reductase and then the 2-thioethyl moiety collapses to episulfide releasing the mono SATE nucleotide (**II**). Exactly the same sequence of events leads to the release from **II** of the parent drug PMEA (**8**). Other report of SATE prodrugs of novel ANPs appeared recently indicating that this class of prodrugs may serve to study the *in vitro* delivery of phosphonate drugs [69]. The only concern for this class of prodrugs remains the toxicity which may limit their further development On the contrary the SATE approach applied to CNPs did not give significant results. To eliminate the potential cell penetration issue associated with the anionic phosphonate moiety, several bis-SATE derivatives of adenosine phosphonate analogues were prepared [69]. Compared to the parent drugs, characterized by weak anti-HCV activity, the anti-HCV activity of the prodrugs is moderately improved, but on the other hand its cytotoxicity is drastically increased.

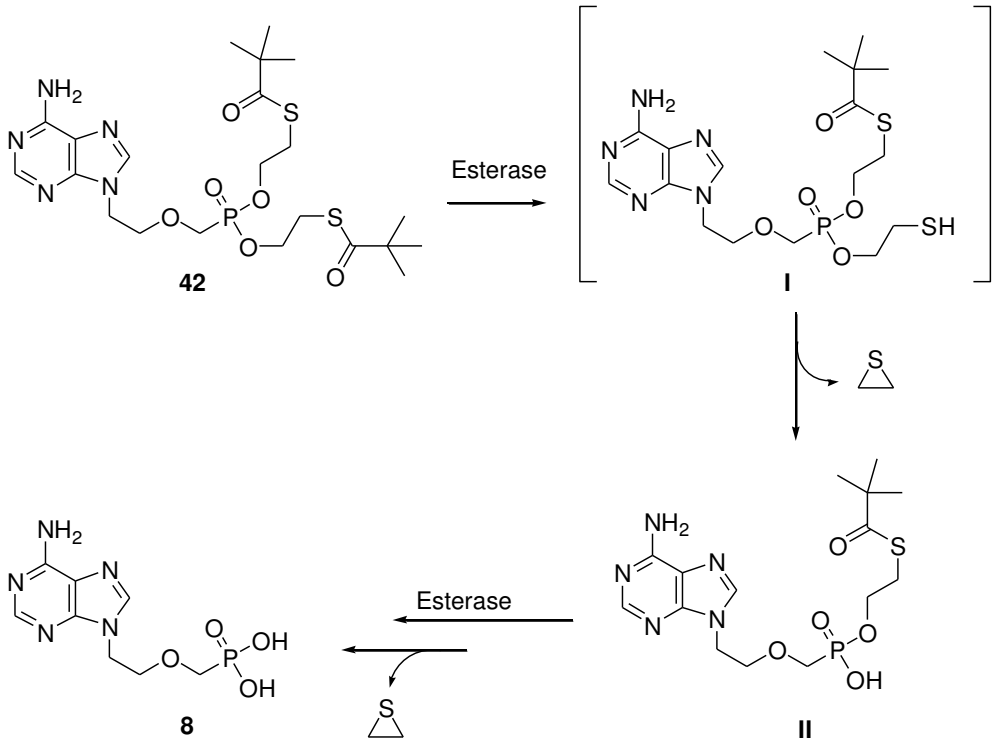


Figure 22. Metabolic pathway for *t*-bu-bis(SATE) PMEA prodrug (**42**)

Peptidomimetic prodrugs

Very significantly enhanced oral bioavailability of valacyclovir resulting from esterification of the hydroxyl group of acyclovir with the carboxylic group of a (*L*)-valine, has encouraged the use of amino acids or dipeptides as promoieties. In this context to increase the oral biovailability of acyclic nucleoside phosphonate McKenna and coworkers have proposed an approach in which a non-toxic promoiety such as a dipeptide or an amino acid is conjugated to ANPs ((*S*)-HPMPC (**3**) or (*S*)-HPMPA (**1**)) by esterification of the phosphonic acid group with an alcoholic amino acid side chain [70]. The other phosphonic OH group was either left free or masked by intramolecular esterification affording (*S*)-cHPMPC or (*S*)-cHPMPA derivatives or by esterification with an ethyl group. In their initial investigation the synthesis and biological evaluation of several phosphono dipeptide ester prodrugs of (*S*)-cHPMPC with a dipeptide

attached via the hydroxyl group of a (*L*)-Serine as well as single amino acid ((*L*)-Valine, (*L*)-Phenyalanine) prodrugs coupled by an ethylene glycol (EG) linkage were reported (Figure 23) [71-73]. The dipetide and EG-amino acid conjugates **43** and **44** showed in general an *in vitro* antiviral activity (HCMV) similar to the parent drug. Whereas **43** and **44** did not exhibit increased bioavailability compared to the parent compound after direct injection into the gastrointestinal tract of rats, interestingly (*L*)-Val-(*L*)-Ser-OMe (*S*)-cHPMPC (**45**) displayed an 8-fold increase in oral bioavailability relative to (*S*)-cHPMPC (**4**) in an *in vivo* murine model transport studies.

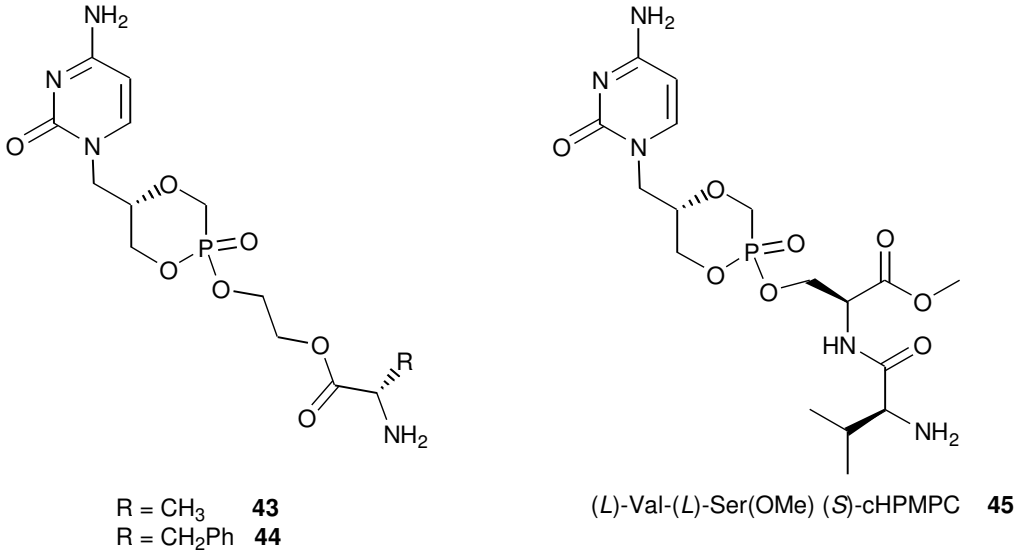


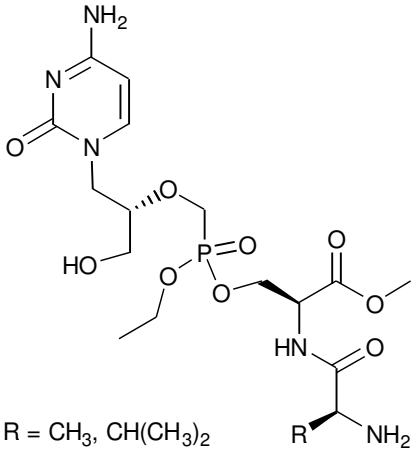
Figure 23. Structures of ethylenglycole aminoacid (**43** and **44**) and serine peptide (*S*)-cHPMPC prodrugs (**45**).

Since valacyclovir and valganciclovir are actively transported by the peptide-specific intestinal transporter PEPT1 which is highly expressed in the gastrointestinal tract, McKenna and coauthors investigated the possibility that hPEPT1 was involved in the ((*L*)-Val-(*L*)-Ser-OMe (*S*)-cHPMPC conjugate (**45**) observed transport [74]. Under the conditions studied, the authors showed that the Val-Ser-OMe dipeptide (*S*)-cHPMPC stereoisomer conjugates as well as

different serine mono amino acid (*S*)-cHPMPC and (*S*)-cHPMPA conjugates are recognized, but not transported by hPEPT1. According to the author this is probably due to the steric and/or polar structural specifics of the linked (*S*)-cHPMPC drug cargo, suggesting that an alternative transport mechanism operates for these prodrugs.

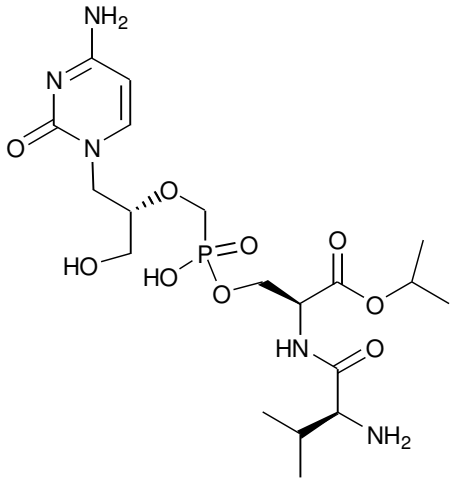
Despite an enhanced bioavailability, the dipetide (*S*)-cHPMPC conjugates having the second phosphonic OH masked with an ethyl group (**46**-**47**), proved to be not suitable as prodrugs due to the fact that the ethyl group was not cleaved during *in vivo* experiments and that the P-OEt monoester (*S*)-HPMPC metabolite did not exhibit significant antiviral activity in an *in vitro* vaccinia plaque reduction assay (Figure 24) [75].

Intriguingly, among the serine dipeptide (*S*)-HPMPC conjugates series, despite the presence of an ionizable P–OH group, (*L*)-Val-(*L*)-Ser-O*i*Pr (*S*)-HPMPC (**48,** Figure 22) displayed the greatest oral biovailability with a 15-fold increase in total cidofovir species in the plasma (related to **3** and **4**) after oral administration. This enhanced oral bioavailability was tentatively justified by the authors as the results of a higher chemical and enzymatic stability compared to its cyclic analogue [75].



(*L*)-Ala-(*L*)-Ser(Me) EtO-(*S*)-HPMPC **46**

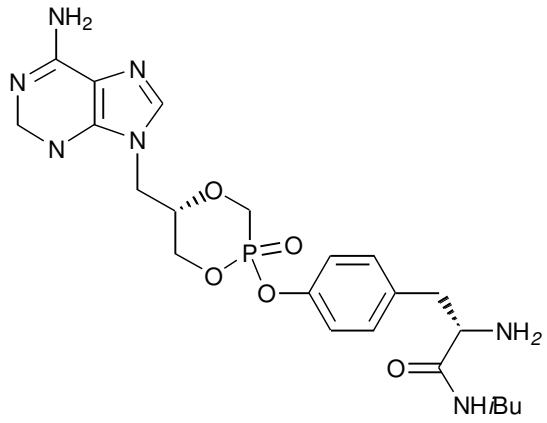
(*L*)-Val-(*L*)-Ser(OMe) EtO-(*S*)-HPMPC **47**



(*L*)-Val-(*L*)-Ser(O*i*Pr) (*S*)-HPMPC **48**

Figure 24. Structures of serine dipeptide (*S*)-HPMPC prodrugs (**46-48**).

More recently, the same authors have reported cyclic (*S*)-HPMPC and (*S*)-HPMPA amino acid or dipeptide prodrugs in which the phosphonic acid has been masked by esterification with a tyrosine hydroxyl group [76]. In this report the authors provided also a convenient method for the partial conversion of the prodrug into the more stable R_p diastereomer by a transesterification reaction from the corresponding S_p diastereomer. Along with this new series of peptidomimetic prodrugs, (*L*)-Tyr-NH-*i*Bu (*S*)-cHPMPA (**49**, Figure 25) was converted in rat or mouse plasma solely to two active metabolites and had significantly enhanced oral bioavailability *vs* parent drug in a mouse model (39% vs <5%). More recently, Krečmerová and coworkers successfully applied the peptidomimetic approach to cyclic (*S*)-HPMP-DAP (**5**)[39]. These results suggested that these peptidomimetic prodrugs are attractive candidates for further *in vivo* evaluation**.**



(*L*)-Tyr-NH*i*Bu (*S*)-cHPMPA **49**

Figure 25. Structure of (*L)*-Tyr-NH*i*Bu-(S)-cHPMPA (**49**).

Independently, a different group described a series of bis-(*L*)-amino acid ester prodrugs of PMEA as potent anti-HBV agents with reduced toxicity [77]. Several of these compounds

demonstrated more potent anti-HBV activity and higher selective index (SI) than adefovir dipivoxil, which was used in this study as a positive control (Figure 26).

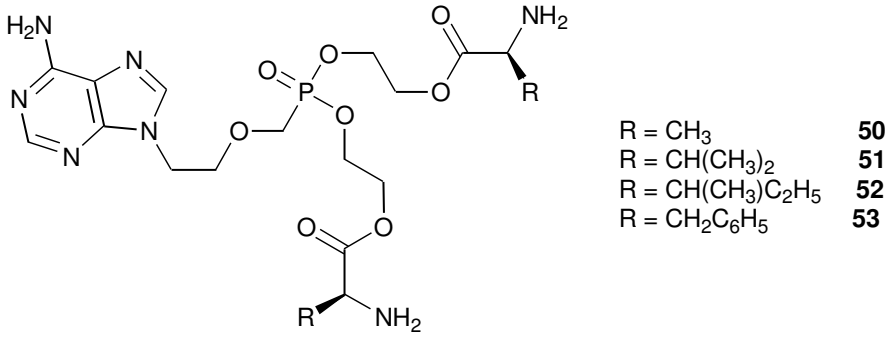


Figure 26. Structures of amino acid and peptide PMEA prodrug **50**-**53**.

Cyclic 1-aryl-1,3-propanyl ester

The cyclic 1-aryl-1,3-propanyl ester is a new type of prodrug moiety recently reported to target phosphate and phosphonate-containing drug to the liver, reducing as consequence their systemic exposure [78]. This prodrug class, called HepDirect prodrugs, is selectively activated in the liver by the liver specific cytochrome P450 isozyme CYP3A4 (Figure 27). In an effort to discover new drugs for treating diseases such as HBV, cyclic 1-aryl-1,3-propanyl ester prodrugs of PMEA, were developed. The lead prodrug pradefovir (**54**, Figure 27) identified among several Hept Direct PMEA prodrugs exhibit a high rate of activation in hepatocytes together with good oral biovailability in rat and dog species, which was further increased by using the mesylate salt to boost water solubility [79]. Evaluation of its individual isomers led to the selection of single prodrug isomer (4S, R_p) as the lead compound. Pradefovir is currently under clinical evaluation in hepatitis B patients.

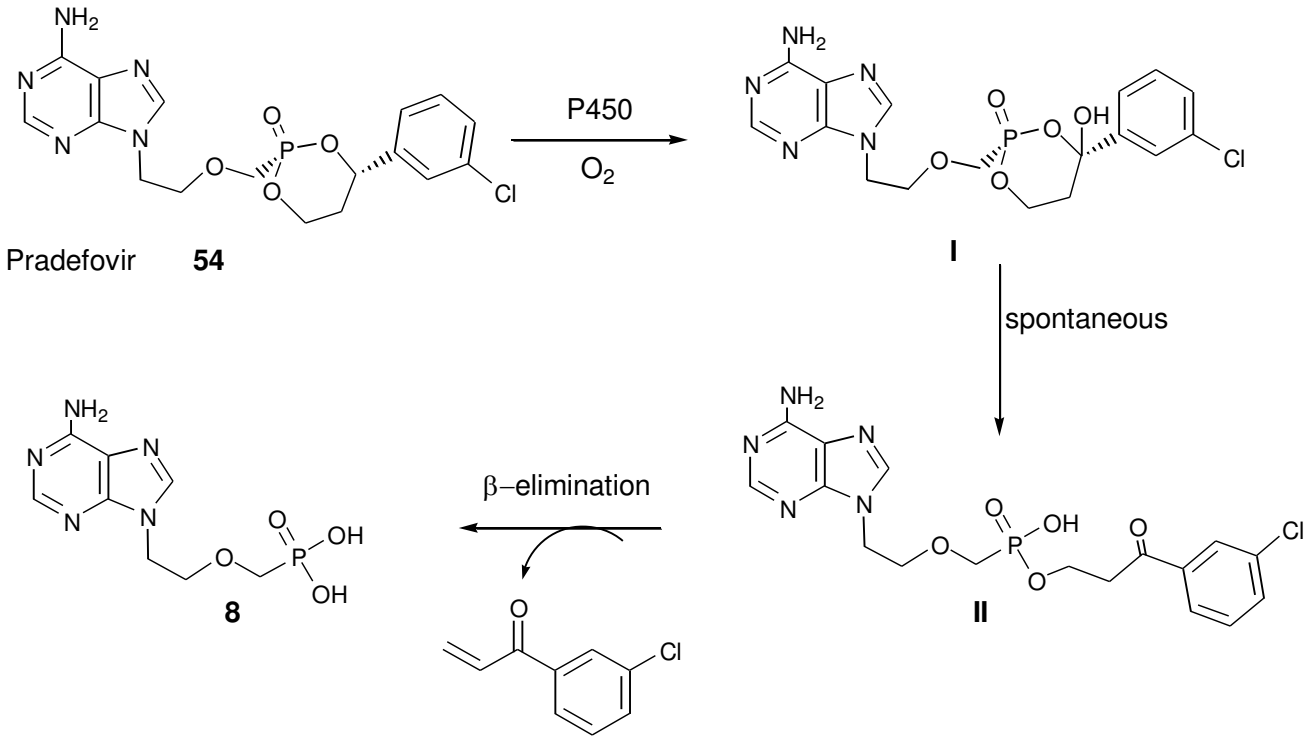


Figure 27. Structure of pradefovir (**54**) and his metabolic pathway in hepatocytes.

Discussion

As a result of extensive efforts in the development of ANPs and CNPs prodrugs, two prodrugs of adefovir and tenofovir (Hepsera® and Viread®) and their two formulations (Truvada® and Atripla®) in combination with other drugs are on the market (Table 1).

Table 1 ANPs and CNPs prodrugs on the market for the treatment of antiviral infection

Compounds	**On the Market**
Adefovir dipivoxil bis-(POM)-PMEA	Hepsera® licensed for the treatment of HBV infections**.**
Tenofovir disoproxyl fumarate bis-(POC)-(*S*)-PMPA fumarate	Viread® licensed for the treatment of HIV infections
Tenofovir disoproxyl fumarate in	Truvada® licensed for the treatment of

conbination with **emtricitabine.**	HIV-1 infections
Tenofovir disoproxyl fumarate in combination with emtricitabine and efavirenz	Atripla® licensed for the treatment of HIV infections

However further research is needed in this area. There is still some concern about adefovir dipivoxil and tenofovir disopropoxil due to their potential toxicity during long term treatment. The same concern can be extended to the clinical candidate PMCDG dipivoxil. Furthermore in contrast to PME and PMP series, there is no a commercially available prodrug so far available for HPMP-derivatives: cidofovir ((*S*)-HPMPC, **3**), approved with the brand name of Vistide® for the treatment of cytomegalovirus retinitis in AIDS patients is applied as an intravenous infusion of the free acid. Cidoforir is also accepted as an effective therapy for smallpox infection, caused by variola virus, a member of the Orthopoxvirus genus. Although this disease was eradicated after an intensive program of vaccination in 1980, there is increasing concern that variola virus might be used as a bioterrorist weapon because of its ease of dissemination, contagiousness, and high mortality rate. Lack of an oral form of cidofovir significantly limits its usefulness under the disruptive conditions of a large-scale biowarfare attack.

A considerable number of ANP and CNP prodrugs have been evaluated, but only a few of them passed the preclinical studies and are now in clinical trials (Table 2). This likely arises from the complexity in designing prodrugs appropriate for clinical use in terms of stability, metabolism, toxicology and side effects. Some of them appear promising, such as the long chain alkyl ester prodrugs developed by Hostetler and applied to different ANPs or the phosphonamidate of 2'-F-d4AP. However, they may suffer of potential drawn back such as lack of solubility for the long chain prodrugs or possible toxicity due to the releasing of the phenol in

the case of the phosphonoamidate prodrug for which additional diastereoisomer issue need also to be taken in account.

In these scenario other prodrugs strategies like the phosphono-diamidate and the peptimomimetic conjugates prodrugs are thus of extremely importance and may offer valuable alternative to those prodrugs already in clinical trials. Hep-direct prodrugs appear instead the obvious option when targeting the liver is required.

Table 2 ANPs and CNPs prodrugs in clinical trials for treatment of antiviral infections

Compounds	**In clinical trials**
Hexadecyloxypropyl-(*S*)-HPMPC HDP-(S)-HPMPC (**23**)	CMX001 is under clinical development by Chimerix, Inc. as an oral drug for the treatment of HCMV and small pox infections. Currently in Phase II trials
Hexadecyloxypropyl-(*R*)-PMPA HDP-(*R*)-PMPA (**28**)	CMX157 is under clinical development by Chimerix, Inc. as an oral drug for the treatment of HIV infections. Currently in Phase I trials.
PMCDG dipivoxil bis-(POM)-PMCDG (**20**)	LB80380 is under clinical development by LG Life Sciences as an oral drug for the treatment of chronic HBV infection and of lamivudine-resistant disease. Currently in Phase II clinical trials
Phenyloxy-ethyl-(*L*)-alaninyl phosphonamidate 2'F-d4AP GS-9131 (**38**)	Preclinical studies for the treatment of HIV infections.
Pradefovir (**59)**	MB06866 is under clinical development by Valeant Pharmaceuticals International, Inc. and Schering-Plough Corporation as an oral drug for chronic HBV infection.

In summary a variety of ANP and CNP prodrugs with diverse chemical structures are presented in this review. Although extensive efforts have been made in developing these prodrugs for the management of antiviral infection there are still exciting future prospects for either novel ANPs and CNPs and/or their prodrugs.

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