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.

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. These can act as competitive inhibitors of viral and cellular DNA or RNA polymerases or alternatively can be incorporated into growing DNA or RNA strands, causing chain termination [1]. Unfortunately, many nucleoside analogues are not phosphorylated effectively in vivo, in particular, the first phosphorylation step is often an 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 phosphate chemically and enzymatically much more stable than the corresponding phosphate. Different to the O-P linkage, the CH₂-P-bond, due to its chemical nature, is not susceptible to phosphodiesterase and phosphatase hydrolysis. Enzymatically and chemically stable phosphate 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 nucleoside phosphonates (ANPs) and cyclic nucleoside phosphonates (CNPs) [2].

ANPs, originally developed by the Holý group in the 1980s, 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 transcriptase for retroviruses). The diphosphorylated ANPs mimic nucleoside triphosphates and may therefore act as chain terminators of the viral DNA, inhibiting viral replication. After the description of the first member of ANPs,
(S)-9-(3-hydroxy-2-phosphonyl-methoxypropyl)adenine (1; (S)-HPMPA) 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, Figure 1), 2-(phosphonomethoxyethyl) (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 AIDS), 2,6-diaminopurine (5, (S)-HPMP-DAP), 2,4-diamino-3-hydroxy pyrimidine (6, (R)-HPMPO-DAPy) and 5-azacytosine...
(7, (S)-HPMP-5-azaC) were found to be especially potent [3,7]. 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 (5) is equivalent to (S)-HPMPA (1), that is, with regard to its activity against poxviruses such as vaccinia virus (VV) [9] and orf virus [10]. Similarly to (S)-HPMPA (1) and (S)-HPMPC (3), (R)-HPMPO-DAPy (6) 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)-HPMP-DAPy (6), similarly to (S)-HPMPC (3) 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-azaC (7) was also shown to be comparable to that of the reference drug (S)-HPMPC (3) against herpes simplex viruses 1 and 2 (HSV-1, HSV-2) and VV, or two- to sevenfold 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)-HPMPC-5-azaC (7) showed a 2–16-fold higher selectivity index (ratio of 50 % cytostatic concentration to 50% effective concentration) against DNA viruses in cell culture than (S)-HPMPC (3) [12]. In an effort to increase the cell permeability of HPMP-based nucleosides, investigators have also generated a cyclic version of this series, forming an internal phosphonate ester bond with the hydroxymethyl chain (cyclic HPMP; Figure 1). As a representative example, cyclic (S)-HPMPC (4) has been shown to have advantages when compared to (S)-HPMPC (3), such as reduced nephrotoxicity due to diminished uptake in the renal proximal tubular cells [13]. Researchers have identified a cellular cCMP phosphodiesterase that can convert (S)-cHPMPC (4) to (S)-HPMPC (3) in vivo [14].

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, adefovir) and (R)-PMPA (9, tenofovir) structures (Figure 2). The PME structures, unlike the HPMP and PMP series, do not contain a chiral carbon centre 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 antiretroviral 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 antiretroviral potency than (R)-PMPA (9) and has proved active against (both wild-type and lamivudine resistant) HBV with a potency comparable to that of (R)-PMPA (9) [16,17]. PMEG (10) was shown to be a potent inhibitor of DNA polymerase [18]. In animal models PMEG (10) has an antiproliferative effect. However its use for the treatment of human papilloma virus infections, which cause proliferative lesions, is limited by its toxicity [19]. ‘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 herpes viruses (HSV-1, HSV-2, VZV) as well as retroviruses such as HIV-1 and HIV-2 [20]. 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 [21].

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 a 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; whereas, 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 phosphonooxy 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 PMDTA (14) and PMDTT (15), reported as selective anti-HIV agents [22]. 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 transcriptase, phosphonooxy analogues of cyclic pyrimidine nucleosides were synthesized and compared for their antiviral activity [23]. Among them, only d4TP (16; Figure 3) showed an antiviral activity below 200 µM. This approach has been recently extended to purine analogues leading to the discovery of the [5-(6-aminopurin-9-yl)-4-fluoro-2,5-dihydrofuran-2-oxymethyl] phosphonic acid (17, GS-9148; Figure 3), a ribose-modified HIV-1 nucleotide reverse transcriptase inhibitor (NRTI) selected from a series of nucleoside phosphonate analogues for its low mitochondrial toxicity, minimal cytotoxicity in renal proximal tubule cells and other cell types, for its synergy in combination with other antiretroviral drugs and for
its unique resistance profile against multiple resistant HIV-1 strains [24].

Natural nucleosides are 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. Due to their low oral bioavailability, ANP and CNP analogues are of limited value in the treatment of chronic diseases, for which 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 CNPs ionizable phosphonate can be masked by derivatization, thus generating a prodrug with increased lipophilicity, capable of altering cell and tissue distribution/elimination patterns of the parent drug [25].

Passive transcellular absorption is the most general route for absorption of lipophilic (pro)drugs through the intestinal mucosal membranes. After the (pro)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 (pro)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 chemical and enzymatic stabilities, 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, which also occurs in the gastrointestinal (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 occurs in the liver. Excretion, elimination or clearance of a drug molecule from the body, starts immediately after the drug has entered the circulation and one organ is mainly responsible for it, 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 lipophilicity 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 GI tract, be absorbed across intestinal mucosa and be 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 phosphate-containing compounds [26–29] and more specific reviews directed toward delivery of nucleosides and nucleotides have been published [25,30–32]. 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) ester prodrugs**

The development of acyloxy ester and alkoxy carbonyl 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 bisopropylxycarbonyloxymethyl ester of tenofovir fumarate (19, bis-(POC)-(R)-PMPA fumarate; Viread®), licensed in 2001 for the treatment of HIV infections and approved to treat chronic HBV infections since 2008 (Figure 4).

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 to 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 due to its ability to reduce the viral load in plasma [33]. However, the toxicity due to its decomposition products (formaldehyde and pivalic acid) generated some concern (Figure 5) [34–36].

The pivalic acid was demonstrated to be responsible for altering carnitine homeostasis [37]. Additionally, it has been shown that bis-(POM)-phosphothriesters were chemically unstable and highly susceptible to serum-mediated hydrolysis, factors that limit their potential utility for intracellular drug delivery [38]. For these reasons, bis-(POM)-PMEA (18) was considered too toxic to permit long-term use at the dosage required in order to inhibit HIV and it was instead approved by the FDA only for the treatment of HBV. The pivaloyloxymethyl prodrugs of (R)-PMPA and (R)-PMP-DAP were also evaluated but due to toxicity concerns they were not selected for clinical trials.

The bisopropylxycarbonyloxymethyl ester (bis-(POC)) is a modification of the bis-(POM)-ester, offering the advantage of not generating 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 trials and subsequently formally approved as its fumarate salt.
(19) 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 humans [39,40]. Bis-(POM)-PMCDG is rapidly converted to the parent drug (PMCDG) in the liver and intestine, probably by esterases. The parent drug is further metabolized to a nucleotide analogue of guanosine monophosphate (II), by an oxidase such as aldehyde or xanthine oxidase (Figure 6). After phosphorylation to di- and triphosphate forms, the molecule inhibits viral replication following incorporation into viral DNA.

More recently, bis-POM esters of (S)-HPM-DAP and its cyclic analogue were reported by Krečmerová et al. [41]. Although these compounds proved to be less active than the corresponding alkoxyalkyl ester prodrugs (see Alkoxyalkyl ester prodrugs), they appeared to be less cytotoxic and cytostatic. Finally, bis-POM esters of novel ANPs have been described. Among them, bis-(POM)-(E)-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) [42].
The antiviral potency of CNPs can also be improved considerably by the application of bis-(POC) prodrug technology. As a representative example, bis-(POC)-d4TP (22) has been reported to improve 29-fold the HIV antiretroviral activity of the corresponding nucleoside (Figure 8) [23].

**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),
PMEA (8), (R)-PMPA (9) [43]. Two alkoxyalkyl esters of (S)-HPMPC, hexadecylxypropyl-(S)-HPMPC (23), HDP-(S)-HPMPC and octadecyloxyethyl-(S)-HPMPC (24, ODE-(S)-HPMPC) have been shown to be more active than (S)-HPMPC (3) 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 9) [44]. 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) [45,46]. 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. The authors state that such an increase in antiviral activity of 23 and 24 when compared to (S)-HPMPC (3) is due to the increased cellular uptake and conversion to (S)-HPMPC diphosphosphate.

In vivo, these compounds proved as effective as parental (S)-HPMPC (3) in the treatment of HCMV infection in a variety of murine models [47]. They also proved to be effective when tested in animal models of orthopox diseases such as cowpox, vaccinia and ectromelia virus infections [48,49]. When given orally to mice infected with ectromelia virus by small particle aerosol, HDP-(S)-HPMPC (23) and ODE-(S)-HPMPC (24) were almost fully protective by oral doses of 5 mg/kg and 10 mg/kg, administered 4 h after infection and sustained daily for five days. A dose of 10 and 12 mg/kg of HDP-(S)-HPMPC (23) and of ODE-(S)-HPMPC (24), respectively, given orally for five days prior to or a few days after nasal infection with cowpox, were reported to be able to significantly reduce the mortality. HDP-(S)-HPMPC (23) with the name CMX001 is currently under development by Chimerix Inc. as an oral drug for the treatment of HCMV and smallpox 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 [50].

Besides (S)-HPMPC derivatives, alkoxyalkyl derivatives of another ANP, (S)-HMPA (1) have been described (Figure 9). Esterification to HDP-(S)-HMPA (25) or ODE-(S)-HMPA (26) resulted in large increases in antipoxvirus activity ranging from 160 to 270-fold versus (S)-HMPA (1) [51,52]. Similar results have
been observed with CMV infection [53]. Among these derivatives, ODE-(S)-HPMPA (26) was the most active compound in adenovirus-infected cells with EC₅₀ values of 0.04–0.16 μM compared with 0.19–1.1 μM of HDP-(S)-HPMPC (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 [3,8] and the increased antiviral activity observed after alkoxyalkyl esterification does not appear to be specific for a particular type of virus [43]. Although (S)-HPMPA (1) was reported to be inactive against HIV, HDP-(S)-HPMPA (25) and ODE-(S)-HPMPA (26) are described as highly active in vitro (with low nanomolar EC₅₀ values), indicating that the alkoxy alkylester strategy can eventually widen the range of antiviral activity. The same strategy has been applied to PMEA (8) and (R)-PMPA (9; Figure 10). HDP-PMEA (27) and HDP-(R)-PMPA (28) have been found to be highly 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 favourable safety, tolerability and drug distribution profile for CMX157.

Other ANPs have been subjected to esterification with alkoxyalkyl groups. In particular, the antiviral activity of the cyclic version of (S)-HPMPC-5azaC (7) was reported to be further enhanced by introduction of alkoxyalkyl groups, the most active being the hexaethyleneoxyethyl (HDE) ester derivative (29) with EC₁₀ values in the range of 0.003–0.008 μg for HSV (Figure 11) [54].

Recently, Krečmerová et al. [41] selected (S)-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 (5) and its cyclic analogue, 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.

Hexaethylene glycol unit, hydroxylated decyl- and decyloxyethyl- chains were recently used by Krečmerová et al. [55] to mask either PMEA (8) or (S)-HPMPC (3). However, the antiviral activities of these prodrugs were, in general, lower or similar to the corresponding parent drugs. 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 approach because of the potentially poor solubility in aqueous solutions, arising from a lipid moiety [49,56].

**Aryl and phenyl ester prodrugs**

Representative 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. [57,58] (Figure 13). Esterification of ([S]-cHPMPC ([4]) via a phosphoester bond, either with a phenol or a salicylic ester produces a new chiral centre at the phosphorus, leading to two different diastereoisomers of the resulting prodrug ([SR] and [SS]). 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. These compounds were synthesized in a stereospecific manner and investigated for their physico-chemical and pharmacokinetic 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 upon the nature of the salicylate ester, which, on the contrary, plays a key role for the enzymatic stability. The axial isomer of salicylate prodrugs ([I]), selected for *in vivo* oral bioavailability evaluation, produces ([S]-cHPMPC ([4]) as the major metabolite, together with ([S]-HPMPC ([3]) and the mono ester of ([S]-HPMPC ([II]), whereas, the corresponding equatorial isomer generates only ([S]-cHPMPC ([4]). 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.
A number of esters of PMEA (8) have been synthesized and evaluated for their oral bioavailability [59]. Among them, the diphenyl ester (33) was identified as the preferred prodrug because it is well absorbed and efficiently converted to the parent compound with an oral bioavailability of 50% (Figure 15). However, further studies revealed that the diphenyl ester (33), even if moderately absorbed, is poorly converted to PMEA (8) due to the 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 ester (34) making these two aryl esters unsuitable for further evaluation as prodrugs [60].

Cycosaligenyl ester prodrugs (CycloSal)

The cycosaligenyl prodrug strategy was elaborated by Meier et al. [61], first to deliver monophosphates

Figure 14. Metabolic pathway for the axial isomer of aryl (S)-cHPMPC prodrugs

![Metabolic pathway for the axial isomer of aryl (S)-cHPMPC prodrugs](image14)

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

![Structures of bis-(PhO)- (33) and bis-[(o-EtO)PhO]-PMEA (34)](image15)
and only later was it applied to ANPs. In particular, the same group reported the synthesis, the stability and the biological evaluation of cycloSal-PMEA prodrugs. 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 (8). 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 (Figure 16). 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 (8) as the only product of hydrolysis (Figure 16).

Although considerably chemically and enzymatically more stable than cycloSal-PMEA prodrugs, cycloAmb-PMEA derivatives were, unexpectedly, reported to exhibit antiviral activity two- to threefold lower compared to the PMEA (8) and 10-fold less active than the cycloSal-PMEA prodrugs. The reduced antiviral activity was attributed by the authors [61] to the slow release of PMEA (8) from II, 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 [62]. 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.

S-Acylthioethyl ester prodrugs (SATE)

On the basis of the favourable 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) [63]. Among them, the bis-(t-bu-SATE)-PMEA (35) 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 (Figure 17). The metabolic pathway for bis-(SATE) phosphonate prodrug 35, assumed to be identical to the one reported for the phosphate prodrug, is depicted in Figure 17 [64]. The prodrug, once inside the cell, preferentially forms an unstable 2-thioethyl intermediate (I) by the action of a carboxyesterase 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 of the parent drug PMEA (8) from II. Other reports 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 [65]. The only concern for this class of prodrugs is a potential toxicity, which may limit their further development. In contrast, 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 [65]. Compared to the parent drugs, characterized by weak anti-HIV activity (EC_{50}=100 \mu M), the anti-HIV activity of the prodrugs (EC_{50}=40 \mu M) is moderately improved, but its cytotoxicity is increased from >100 \mu M to 50 \mu M.

**Aryl phosphonamidates and phosphonodiamidate prodrugs**

The ProTide (pronucleotide) approach, developed by McGuigan et al. [66] was successfully applied to nucleoside phosphates and then investigated in application to nucleoside phosphonates. The ProTide of a nucleoside phosphate 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 phosphate inside the cell releasing the two 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 18). The putative mechanism for the activation of the phosphonamidates then 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 an enzyme endowed with a phosphonamidase activity or may result from simple hydrolysis in more acidic subcellular compartment, to eventually release the parent drug V.

The ProTide technology was successfully applied by Ballatore et al. [66] to PMEA (8) and (S)-PMPA (9). 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 (36) versus PMEA (8) and 50–100-fold...
increase for phenyloxy methyl-(L)-alaninyl phosphonamidate (R)-PMPA prodrug (37) versus (R)-PMPA (9; Figure 19). Mirroring this work, Gilead Sciences reported an extensive study on the application of the ProTide approach to (R)-PMPA (9) investigating the mechanism of hydrolysis and the metabolism of this class of prodrugs [67]. As results of these studies, the phenyloxy isopropyl-(L)-alaninyl phosphonamidate prodrug of (R)-PMPA (38, GS-7340) emerged as a lead compound with improved biological properties (Figure 19). In addition, cathepsin A was found to be the primary enzyme that activates 38 in human lymphatic tissues [68].

A series of aryl phosphonamidate prodrugs of GS-9148 (17), were also designed by Gilead to effectively deliver 17 and its active diphosphorylated metabolite into target cells [69]. The phenyloxy ethyl (L)-alaninyl phosphonamidate prodrug (39, GS-9131), improved the in vitro antiviral activity of 17 by approximately 50-fold against HIV, demonstrating the most favourable esterase (cathepsin) substrate properties in addition to good in vitro intestinal and hepatic stabilities [24,70] (Figure 20). Following oral dosing (3 mg/kg) of 39 in beagle dogs, high levels of GS-9148 diphosphate were observed inside the cells with a mean oral bioavailability of 26%. All these favourable properties lead to the selection of GS-9131 (39) as a clinical candidate.

Phosphonodiamidate analogues having two identical amino acids as masking groups of the phosphonate moiety through a P–N bond were designed. They offer two distinct advantages compared to phosphonamidate analogues: due to their symmetric structure no phosphorus chirality arises and exclusively non-toxic

---

**Figure 18. General structure of aryl phosphonamidate prodrugs (I) and their metabolic pathway**

**Figure 19. Structures of PMEA phosphonoamidate prodrug 36 and (R)-PMPA phosphonamidate prodrugs 37 and GS-7340 (38)**
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 through spontaneous cyclization to intermediate III, structurally identical to the one derived from the activation of an aryl phosphonamidate (see intermediate III in Figure 18). Then exactly the same pathway follows (Figure 21).

Several ANP phosphonodiamidates containing alkyl (L)-alanine were reported to exhibit potent antiviral activities, being bis(butyl-(L)-alaninyl)- (40) and bis(isobutyl-(L)-phenylalanyl)phosphonodiamidate-PME-N6-(cyclopropyl)DAP (43, GS-9191) the most active of the series respectively against pox and papilloma viruses (HPV; Figure 22) [71,72]. After prodrug cleavage, the cPrPMEDAP is intracellularly deaminated by N6-methyl-AMP amino hydrolase to yield the potent antiproliferative and antiviral agent PMEG (10). GS9191 is currently in Phase I clinical trials as a topical prodrug for the treatment of HPV lesion.

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 [73]. In this report, the methodology has been applied to the synthesis of 41 and 42, which exhibit anti-HIV activity with submicromolar EC₅₀ values and no detectable cytotoxicity up to 100 µM, the highest concentration tested (Figure 22).

Diamidate prodrugs of GS-9148 (17) have been reported. Despite their potent in vitro anti-HIV activity these diamidates were quite ineffective in delivering 17 into the cells, producing, after intravenous administration to dogs, only approximately threefold higher intracellular levels of GS-9148 compared to that from the same dose of GS-9148 by itself [69].

**Peptidomimetic prodrugs**

Very significantly enhanced oral bioavailability of valacyclovir resulting from esterification of the hydroxyl group of acyclovir with the carboxylic group of an (L)-valine, has encouraged the use of amino acids or dipeptides as promoieties. In this context, to increase the oral bioavailability of acyclic nucleoside phosphonate McKenna and coworkers [74] have proposed an approach in which a non-toxic promoiety such as a...
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 [74]. 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 an (L)-serine as well as single amino acid ((L))-valine or (L)-phenyalanine) prodrugs coupled by an ethylene glycol (EG) linkage were reported (Figure 23) [75–77]. All the dipeptide and the EG-amino acid conjugates 44 and 45 showed, in general, an in vitro antiviral activity (HCMV) similar to the parent drug. Whereas 44 and 45 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 (46) displayed an eightfold increase in oral bioavailability relative to (S)-cHPMPC (4) in in vivo murine model transport studies.

Since valacyclovir and valganciclovir are actively transported by the human peptide-specific intestinal transporter (hPEPT1) which is highly expressed in the gastrointestinal tract, Peterson et al. [78] investigated the possibility that hPEPT1 was involved in the transport of (L)-Val-(L)-Ser-OMe (S)-cHPMPC conjugate (46) observed in in vivo murine model transport studies. 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 authors, 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 dipeptide (S)-cHPMPC conjugates having the second phosphonic OH masked with an ethyl group (47 and
Medicinal chemistry of phosphonate prodrugs

Antiviral Chemistry & Chemotherapy 22.5

197

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) [79].

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 (49; Figure 24) displayed the greatest oral bioavailability 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 [79].

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 [80]. In this report, the authors also provided a convenient method for the partial conversion of the prodrug into the more stable R<sub>p</sub> diastereomer by a transesterification reaction from the corresponding S<sub>p</sub> diastereomer.

Figure 23. Structures of ethylene glycol amino acid (44 and 45) and serine dipeptide (S)-cHPMPC prodrugs (46)

Figure 24. Structures of serine dipeptide (S)-HPMPC prodrugs (47–49)
Along with this new series of peptidomimetic prodrugs, (L)-Tyr-NH-i-Bu-(S)-cHPMPA (50, Figure 25) was converted in rat or mouse plasma solely to two active metabolites: (S)-cHPMPA 2 and the acyclic form of the respective prodrug (51) which was shown to possess in vitro antiviral activity as well (Figure 25). In addition, 50 had significantly enhanced oral bioavailability versus parent drug in a mouse model (39% versus <5%). Krečmerová et al. [41] also successfully applied the peptidomimetic approach to cyclic (S)-HPMP-DAP (5). These results suggested that these peptidomimetic prodrugs are attractive candidates for further in vivo evaluation.

Independently, a different group described a series of bis-(L)-amino acid ester prodrugs of PMEA (8) as potent anti-HBV agents with reduced toxicity [81]. Several of these compounds demonstrated more potent anti-HBV activity and higher selective index (SI) than adefovir dipivoxil (18), which was used in this study as a positive control (Figure 26).
Cyclic 1-aryl-1,3-propanyl ester prodrugs (HepDirect)

The cyclic 1-aryl-1,3-propanyl ester is a type of prodrug moiety recently reported to target phosphate and phosphonate-containing drug to the liver [82]. This prodrug class, called HepDirect prodrugs, is selectively activated by a 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 (8), were developed. The lead prodrug pradefovir (56) identified among several HepDirect PMEA prodrugs exhibited a high rate of activation in hepatocytes together with good oral bioavailability in rat and dog species, which was further increased by using the mesylate salt to boost water solubility [83] (Figure 27). Evaluation of its individual isomers led to the selection of single prodrug isomer (4S, Rp) as the lead compound. Phase II studies of pradefovir in hepatitis B patients were completed in USA by Ligand Pharmaceuticals. The trials, however, were put on hold based on increased tumour incidence in animal studies [84]. Since January 2011 pradefovir is under clinical evaluation in hepatitis B patients by Chiva Pharmaceuticals in China.

Discussion

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

However, further research is needed in this area. In fact, there is still some concern about adefovir dipivoxil (18) and tenofovir disoproxil (19) due to their potential toxicity during long-term treatment. The same concern can be extended to the clinical candidate PMCDG dipivoxil (20). In contrast to the PME and PMP series, there is not a commercially available prodrug so far available for HPMP-derivatives; cidofovir (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. Cidofovir 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 programme 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 from potential drawbacks 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 phosphonamidate prodrugs for which additional diastereoisomer issues need also to be taken in account.

In these scenarios, other prodrug strategies like the phosphono-diamidate (one already in clinical trial) and the peptidomimetic conjugate prodrugs are thus of extreme importance and may offer valuable alternatives to the other prodrugs. Hep-direct prodrugs are the obvious option when targeting the liver is required.

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

Disclosure statement
The authors declare no competing interests.

References


