Published in final edited form as:

Mol Pharm. 2013 February 4; 10(2): 445–458. doi:10.1021/mp300663j.

Evolution of an Amino Acid-based Prodrug Approach: Stay Tuned

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Abstract

Certain acyclic nucleoside phosphonates (ANPs) such as (*S*)-HPMPC (cidofovir, Vistide[®]) and (*S*)-HPMPA have been shown to be active against a broad spectrum of DNA and retroviruses. However, their poor absorption as well as their toxicity limit the utilization of these therapeutics in the clinic. Nucleoside phosphonates are poorly absorbed primarily due to the presence of the phosphonic acid group, which ionizes at physiological pH. When dosed intravenously they display dose-limiting nephrotoxicity due to their accumulation in the kidney. To overcome these limitations, nucleoside phosphonate prodrug strategies have taken center stage in the development pathway and a number of different approaches are at various stages of development. Our efforts have focused on the development of ANP prodrugs in which a benign amino acid promoiety masks a phosphonate P-OH via a hydroxyl side chain. The design of these prodrugs incorporates multiple chemical groups (the P-X-C linkage, the amino acid stereochemistry, the C-terminal and N-terminal functional groups) that can be tuned to modify absorption, pharmacokinetic and efficacy properties with the goal of improving overall prodrug performance.

Keywords

Acyclic nucleoside phosphonates; antiviral; amino acid; (*S*)-HPMPC; (*S*)-HPMPA; cidofovir; prodrugs

Introduction

ANPs constitute an important class of antimetabolites that are active against various DNA viruses and retroviruses.^{1,2} The prototype member, (*S*)-9-[3-hydroxy-2-(phosphonomethoxy)propyl]adenine ((*S*)-HPMPA)³ introduced by Hóly and De Clercq in 1986 was followed by several generations of newer ANPs that to two structural categories are exemplified by: (i) “HPMP” derivatives that retain a 2'-hydroxyl group in the modified sugar moiety and are represented by (*S*)-HPMPA and its cytosine analogue, (*S*)-1-[3-hydroxy-2-(phosphonylmethoxy)propyl]cytosine⁴ ((*S*)-HPMPC, cidofovir); and (ii) “PME” and “PMP” derivatives, represented by PMEA (adefovir) and (*R*)-PMPA, respectively, that lack the hydroxymethylene function in the modified sugar moiety (Figure 1). Certain ANPs have been marketed as antiviral drugs.⁵ In particular, (*S*)-HPMPC has been approved by the Food and Drug Administration (FDA) for intravenous treatment of cytomegalovirus (CMV) retinitis in AIDS patients.⁶

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ANPs mimic metabolism of natural nucleoside monophosphates, but contain a nonhydrolyzable phosphonate group and also a modified sugar moiety, in which the 3'-hydroxyl group is replaced or absent. Thus, upon intracellular phosphorylation, the resulting triphosphates have long intracellular half-lives and selectively target the viral DNA polymerase.⁷ However, other enzymes may be inhibited by the ANP compounds, such as HSV-1 encoded ribonucleotide reductase, and certain ANP compounds also target reverse transcriptase and act as chain terminators.⁸

Incorporation of a $\text{CH}_2\text{P}(\text{O})(\text{OH})_2$ group into the acyclic nucleoside analogue to generate ANP conveniently circumvents the rate-limiting step (first phosphorylation) in the nucleoside phosphorylation pathway and restores the activity of the nucleoside analogues against thymidine kinase mutant viruses.^{9, 10} However, addition of the $\text{CH}_2\text{P}(\text{O})(\text{OH})_2$ group also comes with limitations. Firstly, the phosphonate reduces cell membrane permeability due to the anionic status of the phosphonic acid at physiological pH.¹¹ Secondly, the ANPs concentrate in the kidney proximal tubule thus resulting in nephrotoxicity.¹² Nevertheless, recognition of the therapeutic potential of the nucleotide analogues has encouraged the design and development of various prodrug approaches that modify the phosphonic acid group to mask its charge and to improve oral bioavailability and *in vivo* intracellular delivery of these agents.^{13–18} Thus, acyloxyalkyl¹⁹ (pivaloyloxymethyl, POM), alkyloxyalkyl,²⁰ S-acylthioethyl²¹ (SATE), aryl,²² acyloxybenzyl²³ and cycloaligenyl²⁴ (*cycloSal*) phosphonate esters, and phosphoramidate derivatives^{25, 26} of ANPs have been examined. Several ANP prodrugs have been marketed for antiviral therapy, including the bis(pivaloyloxymethyl) ester of PME₃²⁷ (bis-POM-PME₃; Hepsera[®]) for the treatment of HBV infections and the bis(isopropyl-oxycarbonyloxymethyl) ester of (*R*)-PMPA²⁸ (bis-POC-(*R*)-PMPA fumarate; Viread[®]) for the treatment of HIV and chronic HBV infections. Despite a significant amount of research and development in this area,^{13–18} currently no prodrugs for (*S*)-HPMPC and (*S*)-HPMPA have been approved by the FDA for use in the clinic, although several are in clinical trials. In this article, we focus on an amino acid phosphonate ester prodrug approach developed in our laboratory, in which an amino acid containing a hydroxy side chain is used as an esterifying promoiety to mask the negative charge of the phosphonate group in these ANPs. We narrate the evolution of the approach to date and assess its current status and future prospects.

The concept of the amino acid phosphonate ester prodrug approach

As shown in Figure 2, (*S*)-HPMPC (**1**), and (*S*)-HPMPA (**2**), incorporate a hydroxymethylene function in their structure, which is able to esterify the ANP phosphonic acid group intramolecularly, producing cyclic forms of the ANPs, cHPMPC **3** and cHPMPA **4**. Thus, two classes of (*S*)-HPMPA and (*S*)-HPMPC prodrugs have been investigated: 1) cyclic phosphonate esters and 2) acyclic phosphonate monoesters. In the cyclic phosphonate ester prodrugs, one phosphonic acid OH group of the ANPs is masked via intramolecular cyclization with the hydroxyl group to afford the cyclic form of the drug (Figure 2; Pathway 1).²⁹ The remaining P-OH group is esterified with the side chain group of a natural amino acid (e.g. Ser, Thr, Tyr or Cys), serving as a single amino acid promoiety, or incorporated into a dipeptide promoiety. The second class of studied prodrugs, acyclic phosphonate monoester prodrugs, incorporate a single amino acid promoiety to mask one phosphonic acid P-OH, whereas the second P-OH group is unmasked (Figure 2; Pathway 2). The present discussion will chiefly focus on the cyclic phosphonate ester prodrugs.

Although the mechanism of the parent drug release from the amino acid prodrugs may be affected by the structure of the promoiety, it is known that simple alkyl/aryl esters of cyclic phosphonates can undergo both enzymatic or pH-dependent chemical hydrolysis, releasing cyclic drug or acyclic phosphonate monoester prodrugs (Figure 3).²² These intermediates

would then liberate the parent ANP drugs by enzymatic degradation given that chemical hydrolysis of the phosphonate monoesters is particularly slow at physiological conditions. Mendel et al. have shown that intracellular enzymatic conversion of cyclic (*S*)-HPMPC, **3** to the parent (*S*)-HPMPC is catalyzed by a cyclic CMP phosphodiesterase.³⁰

The rationale: why amino acids?

The initial rationale behind the utilization of an amino acid as the promoiety was based on: 1) the biologically benign properties of natural amino acids and their metabolites; 2) facile conjugation to the drug via a naturally occurring P–X–C linkage (X = O, S), which is reasonably stable at gastric acid pH (< 3.5) and can be activated at physiological pH (> 6.5), preferably by an endogenous phosphatase or similar enzyme activity; 3) the presence of various functional groups, modification of which would allow “fine-tuning” of the Absorption, Distribution, Metabolism and Excretion (ADME) profile of the resulting prodrugs; and 4) the possibility to target human oligopeptide transporter 1 (hPEPT1). hPEPT1 is a proton-coupled di- and tripeptide transporter located in the brush border membrane of enterocytes of the small intestine.³¹ Broad substrate specificity of hPEPT1 allows it to transport a wide array of chemically diverse compounds, such as β -lactam antibiotics,^{32–34} angiotensin-converting enzyme inhibitors^{35–37} and non-peptidic drugs,³⁸ thus making this transporter an attractive drug delivery target.³⁹ Prodrugs successfully targeting the hPEPT1 transporter include valacyclovir (Valtrex®, Figure 4),⁴⁰ which is a valine-containing conjugate of acyclovir (Zovirax®, Figure 4).

Acyclovir does not contain a phosphonate group, but a terminal hydroxyl group that can esterify (L)-valine to form valacyclovir. Valacyclovir has significantly higher oral bioavailability⁴¹ than the parent drug (54% vs. 15–30% for acyclovir⁴²) associated with transport of the prodrug by hPEPT1^{43–46} according to a study using radiolabeled valacyclovir in *Xenopus laevis* oocytes expressing the transporter.⁴⁷ Other phosphonate drugs and prodrugs, Fosinopril, and the dipeptidyl bisphosphonate prodrugs, Pro-Phe-pamidronate and Pro-Phe-alendronate, have been suggested to be transported by hPEPT1.^{48, 49} Efforts to define the structure-activity relationship (SAR) and structure-transport relationship (STR) properties of hPEPT1 have been made,^{50–53} however, reliable predictive rules for affinity and transport have been elusive.⁵⁴ In a search for a promoiety that, when conjugated to the phosphonate drug, can be recognized and translocated by hPEPT1, and also efficiently cleaved from the prodrug at the target site *in vivo*, various ethylene glycol (EG)-linked amino acid prodrugs and dipeptide prodrugs of cyclic (*S*)-HPMPC (Figure 5; **5–12**) have been previously reported as discussed in the following section.

Ethylene glycol-linked amino acid (*S*)-HPMPC prodrugs

The ethylene glycol-linked amino acid conjugates⁵⁵ of cyclic (*S*)-HPMPC closely resemble valacyclovir structurally (Figure 5; **5, 6**), but did not show an increased oral uptake and did not efficiently release the parent drug at the target site *in vivo*, owing to the inadequate stability of the promoiety under physiological conditions. For example, the cHPMPC prodrug **6**, incorporating EG-linked (L)-valine, exhibited half-lives in liver and intestinal homogenates ranging from 4 to 16 min. This compound showed submicromolar *in vitro* antiviral activity against human cytomegalovirus (HCMV), but failed to demonstrate improved absorption in a rat model compared to the parent drug.⁵⁵

Dipeptide (*S*)-HPMPC prodrugs

To increase the prodrug stability and also attempt to target the hPEPT1 transporter, dipeptides composed of a hydrophobic amino acid, such as Ala, Val, Phe or Leu, and a

hydroxyl amino acid, such as Ser were evaluated as promoieties (Figure 5; **7–12**).^{56–59} The dipeptide promoiety was conjugated to the drug via an ester linkage between the serine side chain OH and a P(O)OH group of ANP. Advantageously, this design allowed for the “fine-tuning” of the prodrugs’ pharmacokinetic properties by modification of the multiple functional groups introduced with construction of the promoiety. For example, utilization of *N*-terminal amino acids with a (D)-configuration to construct the dipeptide promoieties increased the enzymatic stability of the prodrug, and thereby increased its fraction transported into the plasma in the intact form. Depending on the promoiety structure, half-lives of these dipeptide prodrugs (**7–12**) ranged from 140 to 595 min in phosphate buffer (pH 6.5 at 37 °C) and from 43 to 216 min in intestinal homogenate (pH 6.5 at 37 °C).⁵⁷ Furthermore, the (*S*)-HPMPC prodrugs incorporating dipeptide promoieties demonstrated an 8–15-fold increase in the oral bioavailability of total cidofovir species compared to the parent drug in a rodent *in situ* perfusion model.^{58, 60}

In order to test the hypothesis that the observed enhancement of oral bioavailability was due to active transport by hPEPT1, (L)-Val-(L)-Ser(OMe) cHPMPC, **10**, was evaluated in a competitive binding assay in DC5 cells over-expressing hPEPT1. This dipeptide prodrug showed a significant affinity for the hPEPT1 transporter.⁵⁹ However, a further investigation of the dipeptide cHPMPC prodrugs **10** and **12** using radiotracer uptake and electrophysiological assays of *Xenopus laevis* oocytes overexpressing the transporter did not detect hPEPT1-mediated translocation of these compounds, although their ability to bind to the transporter with significant affinity was corroborated.⁵⁶

Similarly to the parent drug **1**, dipeptide prodrugs **7–12** were found to be up to 10-fold more potent *in vitro* against HCMV ($IC_{50} = 0.2–0.7 \mu M$) compared to ganciclovir, the positive control ($IC_{50} = 3 \mu M$),^{55,58,60} but in contrast, they did not show any activity against orthopox viruses (vaccinia and cowpox viruses, $IC_{50} > 100 \mu M$), despite the prodrugs’ improved metabolic stability and increased cell membrane permeability. The difference in potency against various viruses was attributed to the prodrugs’ inadequate activation in the viral assays caused by either lack of specific viral-encoded enzymes that activate the prodrugs or different assay incubation times (HCMV, 10 d; cowpox and vaccinia virus, 3 d).^{58, 60} To test the hypothesis that the shorter incubation time in the poxvirus assays did not allow sufficient time for the prodrug to exert a therapeutic effect via conversion to the parent drug, activation mechanism was evaluated in phosphate buffer (pH 6.5 and 7.4 at 37 °C) and in intestinal and liver homogenates as well as plasma.⁵⁷ These studies not only revealed the existence of multiple prodrug activation mechanisms, incursion simple phosphonate ester hydrolysis and β -elimination of the serine side-chain linked dipeptides (Figure 6; Pathway a), but also the presence of several prodrug degradation pathways, including de-esterification and cyclization of the promoiety to the corresponding diketopiperazine (DKP), which reduces the amount of the parent drug released and significantly decreases the efficiency of the dipeptide prodrug activation (Figure 6; Pathways b and c).⁵⁷

In summary, the dipeptide prodrugs of cyclic (*S*)-HPMPC, **7–12**, exhibited sufficient chemical and enzymatic stabilities and 8–15-fold enhanced oral bioavailability in a rodent *in situ* perfusion model. However, these compounds lacked efficient activation, releasing only a fraction of the parent drug along with various metabolically stable but biologically inactive metabolites.⁵⁷ Using the *Xenopus laevis* oocytes hPEPT1 system, it was found that the dipeptide conjugates were tightly bound to hPEPT1, but were not translocated. Finally, the dipeptide prodrugs were active against HCMV ($IC_{50} = 0.2–0.7 \mu M$), but revealed minimal antiviral activity against orthopox viruses (cowpox and vaccinia).

Single amino acid prodrugs

Given the lack of success in obtaining evidence for hPEPT1-mediated transport with the dipeptide prodrugs and with the goal of mitigating inefficient dipeptide prodrug activation, the effects of the removal of the non-linking, second amino acid from the dipeptide moiety were then examined^{61–63} using modifications of free CO₂H and NH₂ groups of the hydroxyl bearing amino single linking amino acid in (*S*)-HPMPA and (*S*)-HPMPC prodrugs. These prodrugs retained multiple sites to tune their biological and physicochemical properties: N-terminal, C-terminal, and stereocenter groups (Figure 7).

Initially, several serine-based prodrugs of cyclic (*S*)-HPMPA and (*S*)-HPMPC (Figure 8; **13–17**) were synthesized and evaluated for hPEPT1-mediated transport in the electrophysiological *Xenopus laevis* oocytes assay.⁵⁶

In these experiments the serine prodrugs **13–17** showed inhibition of the Gly-Sar uptake, but did not evoke an inward current. Variation of the nucleobase (adenine *vs.* cytosine), the serine configuration ((*L*) *vs.* (*D*)) and alkyl ester groups (Me *vs.* *i*-Pr) had no effect on the observed results.⁵⁶ Thus, it was concluded that, similar to the dipeptide prodrugs, the single amino acid conjugates of cyclic (*S*)-HPMPC and (*S*)-HPMPA are not transported by hPEPT1. An analogous observation has been recently reported for Fosinopril, which showed no hPEPT1-mediated transport based on an electrogenic transport experiment.⁶⁴

As active hPEPT1-mediated transport could not be detected, yet some of the compounds exhibited significant bioavailability in model studies, an alternative transport mechanism, such as passive diffusion, may operate for the single amino acid and dipeptide (*S*)-HPMPC and (*S*)-HPMPA prodrugs.⁵⁶ According to Fick's first law of diffusion, the driving force for diffusion of a compound from an aqueous phase (intestinal lumen) through the gastrointestinal (GI) cell membrane is the concentration gradient of the drug across the membrane.⁶⁵ It is essential for a molecule to have favorable characteristics, *e.g.* low molecular weight and relatively high lipophilicity, in order to pass through the intestinal membrane by this mechanism. The lipophilicity of a drug, measured as the partition coefficient (for non-ionized molecules) between water and *n*-octanol (log *P*) or as the distribution coefficient (for ionized molecules) between buffer with fixed pH and *n*-octanol (log *D*), is commonly used physico-chemical property to predict permeability across biological membranes.⁶⁶

Thus, in a further search for an adequately transported single amino acid promoiety, calculated log *D* (Clog *D*) values at pH 6.5 and 7.4 were used to preliminarily estimate cell membrane permeability to identify new prodrugs exhibiting a suitable balance between stability and efficient conversion to the active parent drug *in vivo*. On this basis, a library of mono amino acid prodrugs was synthesized and subjected to SAR studies.

Initial SAR studies of the single amino acid prodrugs

The first SAR studies aimed to identify the optimal single amino acid side chain, which upon conjugation with the phosphonate drug, forms a pH-responsive P–X–C ester linkage (X = O, S) sufficiently stable at gastric acid pH (< 3.5), but at the same time, efficiently hydrolyzable at pH 6.5–7.4. Desirable half-life of the prodrugs under physiological conditions should be similar to or longer than the transit time of the prodrug through the intestine, which is approximately equal to 3 h. The amino acids serine, threonine, tyrosine and cysteine incorporate hydroxyl or thiol-containing side chains that once conjugated to the cyclic nucleoside phosphonates form aliphatic (Ser, Thr), aromatic (Tyr) or thio-(Cys) phosphonate esters, respectively. The susceptibility of such phosphonate esters to hydrolysis under neutral conditions was initially rationalized based on literature data. For example,

hydrolysis of the simple aliphatic phosphonate esters to the corresponding phosphonic acid typically requires pH extremes and vigorous conditions (*e.g.* refluxing in concentrated hydrobromic acid for 24 h).⁶⁷ However, with the intramolecular assistance of a neighboring group, the hydrolysis of the aliphatic phosphonate ester can be accomplished under mild physiological conditions. Several examples of intramolecularly catalyzed hydrolyses of various phosphonate and phosphate esters have been previously reported.^{19, 21, 23–26, 67–71} The phosphoryl-ethanolamine triesters, the close analogues to the serine- and threonine-based phosphonate prodrugs, represent a case in point; they can be rapidly hydrolyzed at pH 6–8 via the amino-group assisted C–O bond cleavage releasing the corresponding phosphodiester and aziridine.⁶⁹

In contrast to aliphatic esters, aromatic phosphonate esters are more labile in alkaline solution due to resonance stabilization of the leaving group, phenoxide anion. The hydrolysis rate of these esters can also be increased by participation of a neighboring group, for example at pH = 7.8, *p*-nitrophenyl phenacyl methylphosphonate was reported to be hydrolyzed *ca.* 9000 times faster ($t_{1/2} = 41.0$ s) compared to ethyl *p*-nitrophenyl methylphosphonate ($t_{1/2} = 3.63 \times 10^5$ s) due to assistance of the neighboring carbonyl group.⁷²

Finally, when oxygen is substituted for sulfur in the P–O–C linkage in the case of thiophosphonate esters, the rate of hydrolysis greatly increases because the sulfur atom cannot conjugate effectively with phosphorus in order to reduce its electron demand. In addition, this primary effect is greatly enhanced because the mercaptide anion is a better leaving group than the alkoxide anion.⁷³ Therefore, hydrolysis of phosphonothioates can be accomplished under mild physiological conditions without the need for catalysis from a neighboring group.

Thus, in the first round of SAR studies, four (*S*)-HPMPA prodrugs incorporating (L)-serine, (L)-threonine, (L)-tyrosine and (L)-cysteine methyl esters were synthesized (Figure 9, compounds **13**, **18–20**) and their chemical and enzymatic stabilities were evaluated.

All compounds were chemically robust under acidic conditions (pH 1–4). Chemical stability was determined by evaluating the hydrolysis rates of the prodrugs in 200 mM phosphate buffer solution (PBS) at physiologically relevant pH (6.5 and 7.4) at 37 °C. To assess enzymatic stability the rate of disappearance of each prodrug was determined in rat intestinal homogenate (pH 6.5) at 37 °C. The chemical and enzymatic hydrolyses of each prodrug followed pseudo-first-order kinetics over several half-lives ($t_{1/2}$). Half-lives of compounds **13**, **18–20** in PBS are reported in Table 1.⁶³

The prodrugs **13** and **18–20** exhibited half-lives ranging from 19 to 147 min (note: mixtures of two diastereomers due to the chiral P-atom in the phosphonate ring). The tyrosine-based prodrug **19** was found to be 2–6-fold chemically more stable ($t_{1/2} = 147$ min) than the serine, threonine and cysteine-based compounds **13**, **18** and **20**. Upon hydrolysis, the serine and threonine (*S*)-HPMPA prodrugs **13** and **18** released the cyclic form of (*S*)-HPMPA (cHPMPA, **4**) with a yield *ca.* 90% (Figure 10). In contrast, the tyrosine and cysteine cHPMPA prodrugs **19** and **20**, when studied in the same conditions, produced two products, cHPMPA **4** and the corresponding acyclic prodrug **21** or **22** (Figure 10, yields 40–50%).⁶³

The difference in product distribution for the serine/threonine and tyrosine/cysteine prodrugs can be explained by the distinct mechanisms of hydrolysis. The serine and threonine phosphonate ester prodrugs **13** and **18** contain the amino group in the same position with respect to the P–O–C linkage as in the previously studied phosphorylethanolamine triesters.⁶⁹ Therefore, it is hypothesized that hydrolysis of the prodrugs at physiological pH

most likely proceeds through the same mechanism as in the case of phosphorylethanolamine triesters, via C–O bond cleavage intramolecularly assisted by the NH₂-group (Figure 11, left).

In contrast, no intramolecular assistance by a neighboring amino group is possible for the tyrosine/cysteine-containing prodrugs **19** and **20**, and hydrolysis happens via hydroxide attack at the phosphorus atom followed by P–X ester bond cleavage (X = O, S). The OH[–] attack is nonselective and can occur at either position opposite the exocyclic P–X bond (Figure 11; Pathway a), resulting in placement of the exocyclic ester in an axial position in the trigonal bipyramid intermediate leading to cyclic (*S*)-HPMPA **4**, or opposite to the endocyclic P–O bond (Figure 11; Pathway b), yielding the analogous placement for the endocyclic ester. Since pseudorotation of the latter intermediate is energetically prohibited,²² it is then transformed to the acyclic monoesters **21** and **22**.

When evaluated in intestinal homogenate, the single amino acid phosphonate ester prodrugs **13**, **18**, and **19** exhibited significantly shorter half-lives ($t_{1/2} < 30$ min) than in buffer solution, indicating enzymatic degradation of the prodrugs. All prodrugs tested in the first round of SAR studies had a methyl carboxylate ester group, which underwent rapid enzymatic deesterification (presumably by esterases) in intestinal homogenate and released the corresponding free carboxylic acid group (Figure 12; **23–25**).

In vitro antiviral activities of the single amino acid prodrugs **13**, **18**, **19** and of the parent (*S*)-HPMPA **2** were evaluated against HCMV, cowpox and vaccinia viruses and are summarized in Table 2. These prodrugs were also tested for cytotoxicity in human nasopharyngeal carcinoma cells (KB cells). In these experiments, the prodrugs **13**, **18**, **19** had antiviral potencies (IC₅₀ values) similar to the activity of the parent (*S*)-HPMPA against all the viruses tested (Table 2). None of the compounds showed any significant cytotoxicity towards growing KB cells up to a concentration of at least 100 μM.

In conclusion, the first round of SAR studies revealed that the tyrosine (*S*)-HPMPA prodrug **19** is 2–6-fold more chemically stable ($t_{1/2} = 147$ min) than the serine/threonine/cysteine-based conjugates. It was found that upon hydrolysis in PBS (pH 6.5, 37 °C), this prodrug released only two products: cHPMPA **4**, and acyclic tyrosine prodrug **21** (both compounds demonstrate similar *in vitro* antiviral activity against HCMV, cowpox and vaccinia). Thus, the tyrosine-based prodrug **19** exhibited significantly more efficient activation compared to previously reported dipeptide prodrugs.⁵⁷ In enzymatic media, the prodrugs **13**, **18** and **19** underwent rapid enzymatic deesterification of the amino acid ester group ($t_{1/2} < 30$ min in intestinal homogenate, pH 6.5, 37 °C) and released corresponding carboxylic acids, which are ionized at physiological pH and, therefore, prevent the prodrug from efficient cell membrane penetration. When evaluated *in vitro* against HCMV, cowpox and vaccinia viruses, the prodrugs **13**, **18** and **19** showed activity similar to or better than the parent (*S*)-HPMPA. Due to the high chemical stability and efficient activation in PBS of the tyrosine (*S*)-HPMPA prodrug **19**, tyrosine was then identified as the most favorable amino acid promoiety for further prodrug development.

Second round of SAR studies of the single amino acid prodrugs

In order to increase the enzymatic stability of the tyrosine methyl ester promoiety, a second round of SAR studies was performed involving alteration of the tyrosine (L)- vs. (D)-configuration and C-terminal group. Utilization of (D)-tyrosine was rationalized based on the fact that (D)-enantiomers of natural amino acids are not recognized by most natural enzymes and therefore usually exhibit increased enzymatic stability.^{74,75} Replacement of the methyl ester with a sterically hindered isopropyl ester group was patterned after the

diisopropyl carbonate ester prodrug of tenofovir; tenofovir disoproxil was reported to exhibit good chemical and enzymatic stability and is currently approved by the FDA for the chronic treatment of HIV and Hepatitis B infections.^{28, 76} Finally, the tyrosine carboxyl ester group was alternatively replaced by an enzymatically stable *N*-alkyl amido group (*N*-*iso*-butyl or *N*-*tert*-butyl) in order to completely avoid enzymatic deesterification.^{77–79} The alkyl moiety thus provided means to adjust lipophilicity of the prodrug independently of the essential scaffold. Thus, a library of six cyclic (*S*)-HPMPA and (*S*)-HPMPC prodrugs, incorporating (L)-TyrOMe, (D)-TyrOMe, (L)-TyrO*i*-Pr, (L)-TyrNH*i*-Bu and (L)-TyrNH*t*-Bu were synthesized and evaluated for stability in different media, and antiviral potency (Figure 13; **19**, **26–30**).

The hydrophobicities of the tyrosine prodrugs **19**, **26–30** were estimated by calculating the log *D* values at pH 6.5 and 7.4 using Marvin Sketch (version 5.2.0) software (Table 3). A comparison of these values predicted the lipophilicity for the tyrosine *iso*-butyl amide cHPMPA prodrug **29** (Clog $D_{(pH\ 6.5)} = 0.85$) and for the tyrosine isopropyl ester cHPMPC conjugate **28** (Clog $D_{(pH\ 6.5)} = 0.35$). Interestingly, according to these calculations, the Clog *D* value of the tyrosine *tert*-butyl amide conjugate of cHPMPC **30** (Clog $D_{(pH\ 6.5)} = -1.06$) is more than 1 order of magnitude lower than that of the corresponding *N*-alkyl amide (*S*)-HPMPA conjugate **29**.

The chemical and enzymatic stabilities of the tyrosine prodrugs were evaluated in PBS and intestinal homogenate using the same procedure as in the first round of SAR studies. Half-lives of the prodrugs **19**, **26–30** are summarized in Table 4. Since a significant difference in stability between the two diastereomers of the tyrosine prodrugs was observed (about 3- to 10-fold), the half-life for each diastereomer is reported separately.⁶¹ Similar differences in stabilities of diastereomers were reported previously for salicylate and aryl ester prodrugs of cHPMPC (5.4- and 9.4-fold) and were attributed to the greater ground state free energy of one diastereomer relative to the other.²²

As can be seen from Table 4, these structural modifications increased the chemical stability of the tyrosine prodrugs by 1–6-fold when compared to the (L)-tyrosine methyl ester conjugates. For example, incorporation of the *iso*-propyl ester, *iso*-butyl amide or *tert*-butyl amide functionality increases the chemical stability of the tyrosine prodrugs **28–30** in PBS 2–3-fold compared to the corresponding Me-ester derivatives **19** and **26**.

In intestinal homogenate, an enhanced enzymatic stability was observed when (D)-tyrosine was used instead of the natural (L)-amino acid component of the promoiety structure. However, the highest enzymatic stabilities among the compounds tested were found for the tyrosine *N*-alkyl amide prodrugs **29** and **30**. These compounds undergo the same activation pathway (release only two metabolites: the cyclic form of the parent drug and the acyclic form of the prodrug) and exhibit nearly identical half-lives in both enzymatic and non-enzymatic media at physiological conditions (Table 4). The activation pathway of **29** in PBS and in intestinal homogenate, along with HPLC analyses of the metabolites (cHPMPA **4** and (L)-TyrNH-*i*-Bu-HPMPA **31**) at the beginning of the reaction (*t* = 0 min) and after incubation of the reaction mixtures at 37 °C for 270 min and 300 min, are shown in Figure 14.

Additional enzymatic stability studies of **29** were performed in rat plasma (pH 7.4) and revealed the same metabolic pathway as was observed in intestinal homogenate (the same metabolites **4** and **31** were formed). The half-lives of the two diastereomers under these conditions differed nearly 10-fold: 23 min for (*S_p*)-diastereomer and 213 min for (*R_p*)-diastereomer.⁶¹

As in the case of the tyrosine methyl ester (*S*)-HPMPA prodrug **19**, the ratio of the hydrolysis products for the tyrosine *N*-alkyl amide prodrugs **26** and **27** can be explained by non-selective hydrolytic attack at the phosphorus opposite the exocyclic or endocyclic P–O bonds to form a trigonal bipyramid intermediate that is transformed subsequently to the respective products. The ratio of exocyclic and endocyclic P–O bond cleavage for compound **29** reached 1:1 to 1:2.3, depending on the pH and the diastereomeric content of the compound.⁶¹

The antiviral potential of the tyrosine-based prodrugs **19**, **26–30**, the metabolites **4**, **31** and the parent (*S*)-HPMPC **1** and (*S*)-HPMPA **2** was evaluated against HCMV, cowpox and vaccinia viruses. The results obtained are summarized in Table 5. All tyrosine conjugates were also tested for cytotoxicity in KB cells. The tyrosine conjugates **19**, **26–30** and the metabolites **4**, **31** exhibited antiviral potencies similar to or modestly improved over the activity of the parent (*S*)-HPMPA **2** against all the viruses tested (Table 5). Antiviral activities of these prodrugs were found to be independent of tyrosine stereochemistry and C-terminal functional group. None of the tyrosine-based prodrugs showed any significant cytotoxicity towards growing KB cells up to a concentration of at least 100 μ M.

In addition, the tyrosine *N*-isobutyl amide cyclic (*S*)-HPMPA prodrug **29** (the diastereomeric mixture enriched with the (*S_p*)-isomer, de $\approx$ 62%) was also evaluated for oral bioavailability in a murine model.⁶¹ It was found that the oral uptake of the prodrug was significantly enhanced over the parent drug, (*S*)-HPMPA. The total bioavailability derived from the oral administration of **29** was estimated to be 39% compared to < 5% for the parent (*S*)-HPMPA.

In summary, among all structural modifications of the tyrosine amino acid promoiety performed in the second round of SAR studies, incorporation of an *N*-alkyl amide functionality instead of a carboxyl ester group resulted in the most significant increase in the enzymatic stability of the prodrugs. The tyrosine *N*-alkyl amide prodrugs **29** and **30** undergo the same activation pathway and demonstrate nearly identical half-lives in both enzymatic (rat intestinal homogenate) and non-enzymatic (PBS) media that mimic physiological conditions. Upon activation, these prodrugs released only two metabolites, the cyclic form of the parent drug plus an acyclic form of the respective prodrug. In addition, all the tyrosine-based prodrugs (**19**, **26–30**) and the metabolites (**4** and **31**) exhibited *in vitro* antiviral activities against HCMV, cowpox and vaccinia virus similar to or better than that of the parent ANPs, (*S*)-HPMPC and (*S*)-HPMPA. The oral bioavailability of prodrug **29** in mice was approximately 8 times higher than that of the parent drug, (*S*)-HPMPA (39% vs. < 5%).

Third round of SAR studies of the single amino acid prodrugs

After discovering that the tyrosine *N*-alkyl amide prodrugs **29** and **30** were sufficiently stable and efficiently activated in both enzymatic and non-enzymatic media, a third round of SAR studies aimed at exploring the correlation between the hydrophobicity and antiviral potency of the prodrugs was performed. Hydrophobic prodrugs exhibit improved cell membrane permeability (by passive diffusion, membrane flippase activity or phospholipid uptake), which might translate into enhanced potency. A possible strategy to increase a drug's hydrophobicity is to conjugate it to a long alkyl chain.⁸⁰ For example, Hostettler *et al.* reported that esterification of the phosphonic acid moiety of (*S*)-HPMPC and (*S*)-HPMPA with a hydrophobic alkyl group, particularly hexadecyloxypropyl (HDP) or octadecyloxyethyl (ODE), resulted in prodrugs exhibiting 2.5- to 4-log increases in antiviral activity depending on the antiviral assay used.⁸¹

It is by no means evident what specific alkyl group length (or other structural features) will result in an optimal balance of lipophilicity, solubility and other important prodrug properties including ultimate bioavailability, efficacy and toxicity. Thus, for the third round of SAR studies, a series of tyrosine *N*-alkyl amide cyclic (*S*)-HPMPA and cyclic (*S*)-HPMPC prodrugs, incorporating alkyl chains varying in length were synthesized and evaluated to “tune” the overall hydrophobicity of the prodrugs.⁸² Selected lipophilic tyrosine-based prodrugs exhibited antiviral potencies several logs higher than the parent ANPs. The obtained results will be reported in full detail in due course.

Prodrug synthesis

The target cyclic phosphonate diester prodrugs have been synthesized using a solution-phase method (Scheme 1) and a solid-phase approach (Scheme 2). The solution-phase procedure was originally developed for the synthesis of the EG-linked amino acid and dipeptide (*S*)-HPMPC prodrugs^{56, 59} and was later used to obtain mono amino acid prodrugs. This method includes three steps: the first two steps, intramolecular cyclization of the parent ANP drug and subsequent esterification of the remaining P–OH group with the side chain hydroxyl or thiol group of a *t*Boc-protected amino acid or dipeptide promoiety, were accomplished in one pot using a PyBOP-mediated coupling procedure (Scheme 1, steps i) and ii)). The last step was *t*Boc-deprotection of the promoiety conjugated to the drug using trifluoroacetic acid (TFA) in dichloromethane (DCM). The final prodrugs were obtained in overall moderate to good yields (40–65%).

In addition to the solution-phase synthesis, a solid-phase approach to the mono amino acid and dipeptide cyclic phosphonate prodrugs was developed and is outlined in Scheme 2.^{56, 83} In contrast to the solution phase method, this strategy involved the replacement of the *t*Boc-protecting group at the amino acid with a tritylchloride polystyrene (TCP) resin. The cyclization and conjugation of the (*S*)-HPMPC **1** or (*S*)-HPMPA **2** to the amino acid or dipeptides bound to the resin were accomplished using the same conditions as in the solution-phase approach (PyBOP, DIEA, DMF, 40 °C).

After completion of the coupling reaction, all the byproducts were washed, leaving the product on the resin. Finally, the products were cleaved from the resin using either TFA in DCM or HCl in dioxane, and were subsequently crystallized from a saturated methanolic solution by precipitation with diethyl ether. The solid-phase strategy produced the single amino acid prodrugs and the dipeptide conjugates in 20–35% overall yield from (*S*)-HPMPA or (*S*)-HPMPC, and provided the following advantages over the solution-phase prodrug synthesis: easy product purification (column chromatography is not required), scalability and potential for automation, if needed in the future.

HPMP-based ANPs, in particular (*S*)-HPMPA and (*S*)-HPMPC are chiral compounds, exerting their maximal antiviral effect as a single (*S*)-enantiomer. Their cyclization followed by conjugation of the remaining P–OH group with the promoiety leads to the formation of a new stereocenter at the phosphorus atom, resulting in generation of prodrugs as a mixture of (*S*_p)- and (*R*_p)-diastereomers with two distinct ³¹P NMR signals. As was pointed out above, (*S*_p)- and (*R*_p)-diastereomers of the tyrosine-based prodrugs exhibit 3- to 10-fold difference in chemical stability at physiologically relevant pH, which might translate into their different pharmacokinetic properties.

In order to correlate unequivocally the absolute phosphorus configuration with the prodrugs' chemical stabilities, the single diastereomers of model prodrugs, namely phenyl esters of cyclic (*S*)-HPMPA and (*S*)-HPMPC have been obtained and analyzed by X-ray crystallography and NMR.⁸⁴ Thus, the less stable diastereomer was identified to have (*S*_p)-

configuration at the phosphorus atom and corresponded to the downfield ^{31}P NMR signal (Figure 15).

Additionally, by analyzing ^1H NMR spectra of the diastereomeric model compounds in solvents of different polarity (CDCl_3 and ACN-d_3), it was shown that in solution (R_p)-diastereomers mainly exist in the same (*e*, *a*) chair conformation found in the crystal (Figure 16, left). In contrast, (S_p)-diastereomers of the model compounds exist largely in an equilibrium of (*a*, *a*)/(*e*, *e*) conformers (Figure 16, right).

These results led to the conclusion that the *axial/equatorial* terminology frequently used in the literature to differentiate cyclic (*S*)-HPMPC and (*S*)-HPMPA diastereomeric diesters could be misleading since it is based on the assumption that the nucleobase is invariably equatorial and therefore broader (*R*)/(*S*) nomenclature should be used instead.⁸⁴

Conclusion

An evolutionary progression of EG-linked amino acid, dipeptide and single amino acid cyclic (*S*)-HPMPC and (*S*)-HPMPA prodrugs was designed and investigated with the aims: 1) to define structural effects on the interactions of peptidomimetic phosphonate prodrugs with hPEPT1, and 2) to tune the promoiety conjugated to the ANPs to obtain prodrugs able not only to withstand the rigors of metabolism, but also to release efficiently the active parent drug at the target site *in vivo*. Upon evaluation of the serine-based dipeptide and single amino acid (*S*)-HPMPC and (*S*)-HPMPA prodrugs **10**, **12–17** for hPEPT1-mediated transport in the electrophysiological *Xenopus laevis* oocytes assay, it was concluded that the mono amino acid and dipeptide cyclic (*S*)-HPMPC and (*S*)-HPMPA prodrugs were not transported by hPEPT1, most likely due to the presence of a phosphonate ester group, and it was decided to focus on the less complex mono amino scaffold. Three consecutive rounds of SAR studies involving synthesis and evaluation of the single amino acid ANP prodrugs were therefore performed. The studies were designed to probe the effects of various P–X–C linkages, the amino acid stereochemistry ((*L*) vs. (*D*)) and the C-terminal functional groups (ester vs. *N*-alkyl amide) on chemical and enzymatic stability, activation, antiviral potency and oral bioavailability of the corresponding prodrugs. The results demonstrate that the tyrosine is a superior mono amino acid promoiety, providing drug conjugates with good chemical stability and efficient activation to active drug species. Second, the stability of the tyrosine promoiety in liver and intestine homogenates or plasma was significantly increased by replacement of the carboxyl ester group with an *N*-alkyl amide moiety. Thus, the tyrosinamide prodrugs **29**, **30** undergo similar metabolism and stabilities in enzymatic and non-enzymatic media. Thirdly, the antiviral activities of the tyrosine-based *N*-alkyl amide cyclic (*S*)-HPMPA and (*S*)-HPMPC prodrugs against HCMV, cowpox and vaccinia virus can be greatly enhanced by incorporation of a lipophilic alkyl group into the tyrosinamide moiety. Tyrosine *N*-alkyl amide is a promising mono amino acid promoiety scaffold that is currently being further tuned to optimize its potential for creating more effective prodrugs of (*S*)-HPMPA, (*S*)-HPMPC and other antiviral ANPs.

Acknowledgments

This work was supported by grants AI061457 and AI056864 from the National Institutes of Health.

Abbreviations

ADME	absorption, distribution, metabolism, excretion
ANP	acyclic nucleoside phosphonate

(S)-HPMPA	((S)-9-[3-hydroxy-2-(phosphonomethoxy)-propyl]adenine)
FDA	food and drug administration
hPEPT1	human oligopeptide transporter 1
SAR	structure-activity relationship
STR	structure-transport relationship
HCMV	human cytomegalovirus
GI	gastrointestinal
PBS	phosphate buffer solution
HDP	hexadecyloxypropyl
ODE	octadecyloxyethyl
HFF	cells, normal human fibroblasts
KB	cells, human nasopharyngeal carcinoma cells
TFA	trifluoroacetic acid
DCM	dichloromethane
DMF	N,N-dimethylformamide
DIEA	diisopropylethylamine
PyBOP	benzotriazol-1-yloxytripyrrolidinophosphonium hexafluorophosphate
TCP	trityl chloride polystyrene
PMEA	9-(2-phosphonomethoxyethyl)adenine
PMPA	(R)-9-[2-(phosphonomethoxy)propyl]adenine
DKP	diketopiperazine

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Figure 1.
Structures of selected ANPs.

Figure 2.

The concept of the amino acid phosphonate ester prodrug approach: 1) cyclic phosphonate esters; 2) acyclic phosphonate monoesters.

Figure 3.

General representation of the activation pathway of amino acid phosphonate ester prodrugs:

a) enzymatic or pH-dependent chemical hydrolysis; b) enzymatic hydrolysis.

Acyclovir

Valacyclovir

Figure 4.
Structures of acyclovir and its prodrug valacyclovir.

Figure 5. Structures of EG-linked amino acid and dipeptide cyclic (*S*)-HPMPC prodrugs.^{55–59}

Figure 6.

Activation (a) and degradation (b, c) pathways observed for dipeptide cyclic (*S*)-HPMPC and (*S*)-HPMPA prodrugs at physiologically relevant pH (6.5 and 7.4).

Figure 7.
Tunable sites in the single amino acid promoiety.

Figure 8.

Structures of the serine-based cyclic (*S*)-HPMPC and (*S*)-HPMPA prodrugs evaluated for hPEPT1-mediated transport in the electrophysiological *Xenopus laevis* oocytes assay.

Figure 9.
Structures of single amino acid cyclic (*S*)-HPMPA prodrugs.

Figure 10.
Chemical activation of the mono amino acid (*S*)-HPMPA prodrugs **13**, **18–20** in PBS, pH 6.5 at 37 °C.

Figure 11.
Suggested mechanisms for the serine/threonine (left) and cysteine/tyrosine (right) cyclic (S)-HPMPA prodrug hydrolysis in PBS, pH 6–8.

Figure 12.

Enzymatic degradation of the mono amino acid cyclic (*S*)-HPMPA prodrugs **13, 18, 19** in intestinal homogenate, pH 6.5 at 37 °C.

Figure 13.

Tyrosine (*S*)-HPMPA and (*S*)-HPMPC prodrugs **19**, **26–30** synthesized and evaluated in the second round of SAR studies.

Figure 14.

A) Chemical and enzymatic activation of (L)-TyrNH*i*-Bu-cHPMPA **29** in PBS and intestinal homogenate at pH 6.5 and 37 °C. B) HPLC analysis of (L)-TyrNH*i*-Bu-cHPMPA **29** metabolism at pH 6.5 and 37 °C in PBS (left HPLC traces) and in intestinal homogenate (right HPLC traces). Top HPLC traces correspond to the reaction mixtures at $t = 0$ min; bottom HPLC traces correspond to the reaction mixtures after incubation for 270 min or 300 min; (X-axis is time, min; Y-axis is a relative absorbance).

Figure 15.

X-ray crystal structures and ^{31}P NMR chemical shifts of (S_p) - and (R_p) -diastereomers of phenyl esters of cyclic (S) -HPMPA prodrugs. Reprinted with permission from Krylov, I. S.; Zakharova, V. M.; Serpi, M.; Haiges, R.; Kashemirov, B. A.; McKenna, C. E. *J. Org. Chem.* **2012**, *77*, 684–689. Copyright 2012 American Chemical Society.

Figure 16.

Possible conformer equilibria of (R_P) -diastereomers (**I-II**) (left) and (S_P) -diastereomers (**III-IV**) (right) in solution based on analysis of solvent effect on ^1H NMR coupling constant values. Reprinted with permission from Krylov, I. S.; Zakharova, V. M.; Serpi, M.; Haiges, R.; Kashemirov, B. A.; McKenna, C. E. *J. Org. Chem.* **2012**, 77, 684–689. Copyright 2012 American Chemical Society.

Scheme 1.

Conventional syntheses of the dipeptide and mono amino acid cyclic (*S*)-HPMPA and (*S*)-HPMPC prodrugs. *Reagents and conditions:* i) and ii) PyBOP, DIEA, DMF, 40 °C; iii) TFA, CH₂Cl₂, r.t.

Scheme 2.

Solid-phase synthesis of the single amino acid and dipeptide prodrugs of cyclic (*S*)-HPMPA and (*S*)-HPMPC. *Reagents and conditions:* i) PyBOP, DIEA, DMF, 40 °C; ii) TFA, CH₂Cl₂ or HCl, dioxane, 25 °C.

Table 1

Half-lives ($t_{1/2}$) of single amino acid cyclic (S)-HPMPA prodrugs **13**, **18–20** in PBS (pH 6.5) at 37 °C.

Prodrug (mixture of two diastereomers)	$t_{1/2}$, min
(L)-SerOMe-cHPMPA, 13	19
(L)-ThrOMe-cHPMPA, 18	64
(L)-TyrOMe-cHPMPA, 19	147
(L)-CysOMe-cHPMPA, 20	complete hydrolysis after 45 min

Table 2

In vitro antiviral activities against HCMV, cowpox and vaccinia and cytotoxicities of the single amino acid prodrugs of cyclic (*S*)-HPMPA, **13**, **18**, **19**.

Prodrug	Antiviral activity, IC ₅₀ (μM)			Toxicity, (μM)
	HCMV	Cowpox	Vaccinia	KB cells
(<i>S</i>)-HPMPA, 2	2.4	15	15	> 100
(L)-SerOMe-cHPMPA, 13	0.5	20	25	> 100
(L)-ThrOMe-cHPMPA, 18	0.62	20	30	> 100
(L)-TyrOMe-cHPMPA, 19	4.7	10	2	> 100

Table 3

Comparison of calculated log *D* (Clog *D*) values for the tyrosine-based (*S*)-cHPMPA and (*S*)-cHPMPC prodrugs with the values for parent (*S*)-HPMPC **1** and (*S*)-HPMPA **2** and their cyclic derivatives **3**, **4**.

Prodrug	Clog <i>D</i> (pH 6.5)	Clog <i>D</i> (pH 7.4)
(<i>S</i>)-HPMPA, 2	−4.51	−4.59
(<i>S</i>)-cHPMPA, 4	−3.43	−3.41
(L)-Tyr-OMe-cHPMPA, 19	−1.87	−1.62
(L)-Tyr-NH <i>i</i> -Bu-cHPMPA, 29	0.85	0.87
(<i>S</i>)-HPMPC, 1	−4.60	−4.70
(<i>S</i>)-cHPMPC, 3	−3.52	−3.52
(L)-Tyr-OMe-cHPMPC, 26	−0.41	0.04
(L)-Tyr-O <i>i</i> -Pr-cHPMPC, 28	0.35	0.80
(L)-Tyr-NH <i>i</i> -Bu-cHPMPC, 30	−1.06	−0.25

Table 4

Half-lives ($t_{1/2}$) of the tyrosine-based (*S*)-cHPMPA and (*S*)-cHPMPC prodrugs **19**, **26–30** in PBS and in rat intestinal homogenate at pH 6.5 and 37 °C.⁶¹

Prodrug	Isomer	$t_{1/2}$, min	
		PBS	Intestinal Homogenate
19	<i>R</i> _p	330	< 30
	<i>S</i> _p	55	< 30
26	<i>R</i> _p	256	< 30
	<i>S</i> _p	56	< 30
27	<i>R</i> _p	385	210
	<i>S</i> _p	91	72
28	<i>R</i> _p	770	< 30
	<i>S</i> _p	121	< 30
29	<i>R</i> _p	771	771
	<i>S</i> _p	122	114
30	<i>R</i> _p	1732	1732
	<i>S</i> _p	210	231

Table 5

In vitro antiviral activities against HCMV, cowpox and vaccinia and cytotoxicities of the tyrosine-based prodrugs of cyclic (*S*)-HPMPA and (*S*)-HPMPC, **19**, **26–31**.

Prodrug	Antiviral activity, IC ₅₀ (μM)			Toxicity, (μM) KB cells
	HCMV	Cowpox	Vaccinia	
(<i>S</i>)-HPMPA, 2	0.41	4	2	> 100
(<i>S</i>)-cHPMPA, 4	1.3	0.6	1	> 100
(<i>L</i>)-Tyr-OMe-cHPMPA, 19	0.45	7	3	> 100
(<i>L</i>)-Tyr-NH <i>i</i> -Bu-cHPMPA, 29	0.3	7	3	> 100
(<i>L</i>)-Tyr-NH <i>i</i> -Bu-HPMPA, 31	0.29	3	0.5	> 100
(<i>S</i>)-HPMPC, 1	0.28	30	20	> 100
(<i>S</i>)-cHPMPC, 3	0.25	30	40	> 100
(<i>L</i>)-Tyr-OMe-cHPMPC, 26	0.23	50	4	> 100
(<i>D</i>)-Tyr-OMe-cHPMPC, 27	0.2	35	25	> 100
(<i>L</i>)-Tyr-O <i>i</i> -Pr-cHPMPC, 28	0.12	40	30	> 100
(<i>L</i>)-Tyr-NH <i>i</i> -Bu-cHPMPC, 29	0.1	20	10	> 100