European Journal of Medicinal Chemistry 70 (2013) 456–468

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Original article

Novel water-soluble prodrugs of acyclovir cleavable by the dipeptidyl-peptidase IV (DPP IV/CD26) enzyme

Alberto Diez-Torrubia [a], Silvia Cabrera [a,1], Sonia de Castro [a], Carlos García-Aparicio [a], Gwenn Mulder [a], Ingrid De Meester [b], María-José Camarasa [a], Jan Balzarini [c], Sonsoles Velázquez [a,*]

[a] Instituto de Química Médica (IQM-CSIC), c/ Juan de la Cierva 3, E-28006 Madrid, Spain
[b] Laboratory of Medical Biochemistry, University of Antwerp, Universiteitsplein 1, B-2610 Antwerp, Belgium
[c] Rega Institute for Medical Research, KU Leuven, Minderbroedersstraat 10, B-3000 Leuven, Belgium

ARTICLE INFO

Article history:
Received 2 September 2013
Received in revised form
27 September 2013
Accepted 1 October 2013
Available online 9 October 2013

Keywords:
Acyclovir
Antiviral
Prodrug
Peptide
Dipeptidyl peptidase IV
Solid-phase synthesis

ABSTRACT

We herein report for the first time the successful use of the dipeptidyl peptidase IV (DPPIV/CD26) prodrug approach to guanine derivatives such as the antiviral acyclovir (ACV). The solution- and solid-phase synthesis of the tetrapeptide amide prodrug **3** and the tripeptide ester conjugate **4** of acyclovir are reported. The synthesis of the demanding tetrapeptide amide prodrug of ACV **3** was first established in solution and successfully transferred onto solid support by using Ellman's dihydropyran (DHP) resin. In contrast with the valyl ester prodrug (valacyclovir, VACV), the tetrapeptide amide prodrug **3** and the tripeptide ester conjugate **4** of ACV proved fully stable in PBS. Both prodrugs converted to VACV (for **4**) or ACV (for **3**) upon exposure to purified DPPIV/CD26 or human or bovine serum. Vildagliptin, a potent inhibitor of DPPIV/CD26 efficiently inhibited the DPPIV/CD26-catalysed hydrolysis reaction. Both amide and ester prodrugs of ACV showed pronounced anti-herpetic activity in cell culture and significantly improved the water solubility in comparison with the parent drug.

1. Introduction

Dipeptidyl peptidase IV (DPPIV, also termed CD26) is a member of the prolyl oligopeptidase family, a group of atypical serine proteases able to hydrolyse the prolyl bond, that mediates the activities of a number of regulatory peptides [1–4]. It is endowed with an interesting (dipeptidyl) peptidase catalytic activity and specifically removes dipeptides from the *N*-terminus of peptide substrates that have a proline or, to a lesser extent an alanine at the penultimate amino acid position. It exists as an extracellular membrane-bound peptidase, highly expressed on endothelial cells, epithelial cells and lymphocytes, and as a soluble form that is found in plasma [5,6].

In 2005, it was first demonstrated that a synthetic small molecule [GPG-NH_2 (glycylprolylglycinamide)] can be converted to an antiviral drug (glycinamide) through the specific action of DPPIV/CD26 [7]. Based on these results, we previously reported [8] a novel type of DPPIV/CD26-directed prodrug technology that provides conjugates [(Xaa-Pro)$_n$]–[drug] of therapeutic drugs with a di- (or tetra) peptide moiety as a carrier (Fig. 1). In these prodrugs the compounds/drugs and the peptide moiety are directly coupled via an amide bond which is specifically cleaved by DPPIV/CD26 (Fig. 1). The presence of a proline at the penultimate *N*-position protects the amino acid sequence against non-specific proteolytic degradation since many exopeptidases do not recognize such amino acid sequence [9]. This approach was successfully applied to a broad variety of amine-containing drugs having a free amino group on alkyl chains (the N-3 aminopropyl derivative of the anti-HIV TSAO compounds) [10,11] and on heteroaromatic (6-aminoquinoline), carbohydrate (doxorubicine), heterocyclic pyrimidine (ara-C and TSAO-m^{5}C) and purine (ara-A) rings [12]. Our data revealed that purified DPPIV/CD26 could recognize these prodrugs as substrates and efficiently released the parent compound. The DPPIV/CD26-specific inhibitor Vildagliptin could efficiently block the enzymatic conversion in human, bovine and murine serum [12]. Interestingly, it was possible to modulate the enzymatic and serum

Abbreviations: DPP IV, dipeptidyl peptidase IV; ACV, acyclovir; VACV, valacyclovir; DKP, diketopiperazine; DHP, dihydropyran.

* Corresponding author. Tel.: +34 91 258 74 58; fax: +34 91 5644853.
E-mail addresses: iqmsv29@iqm.csic.es, sonsoles@iqm.csic.es (S. Velázquez).
[1] Present address: Departamento de Química Inorgánica, Facultad de Ciencias, Universidad Autónoma de Madrid, 28049 Madrid, Spain.

0223-5234/$ – see front matter © 2013 Elsevier Masson SAS. All rights reserved.
http://dx.doi.org/10.1016/j.ejmech.2013.10.001

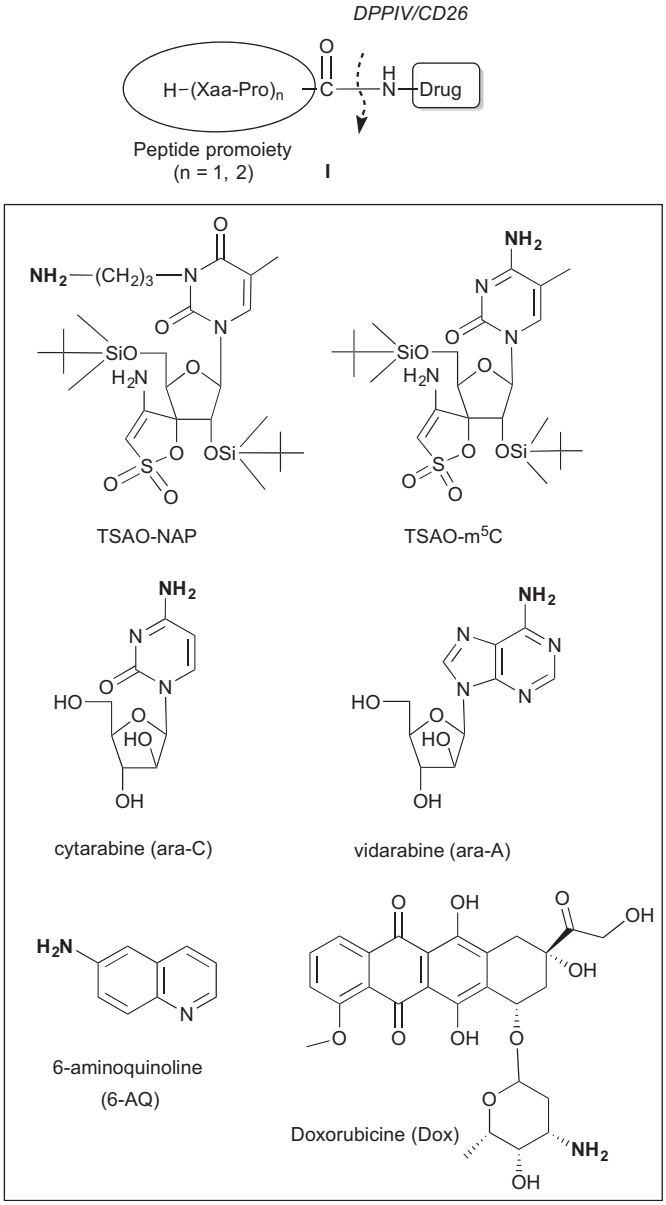


Fig. 1. Application of the DPP IV/CD26-based prodrug approach to amine-containing drugs.

hydrolysis rate (half-life) of the prodrug conjugates by changing the nature and the length of the peptide promoiety [13].

We recently reported [14,15] the application of this prodrug approach to hydroxy-containing drugs of different nature (primary, secondary, tertiary or aromatic hydroxyl groups) (Fig. 2). In this case, the peptide promoiety cannot be linked directly to the hydroxyl group through an ester bond because the DPPIV/CD26 enzyme solely recognizes amide bonds. Thus, tripartite conjugates [Xaa-Pro]–[connector]–[drug] bearing heterobifunctional connectors to link the peptide moiety to the hydroxyl group of the drug were designed. Tripartite prodrugs bearing an amino acid (i.e. valine) as a heterobifunctional connector to link the dipeptide to the hydroxyl group of the drug through an ester bond (released by chemical or enzymatic hydrolysis) were first studied (conjugates of general formulae **II**, Fig. 2). The hydroxy-containing prodrugs showed different susceptibilities to hydrolysis by DPPIV/CD26 and serum depending on the nature of the compound. Several prodrugs showed a remarkable increase in water solubility and a markedly

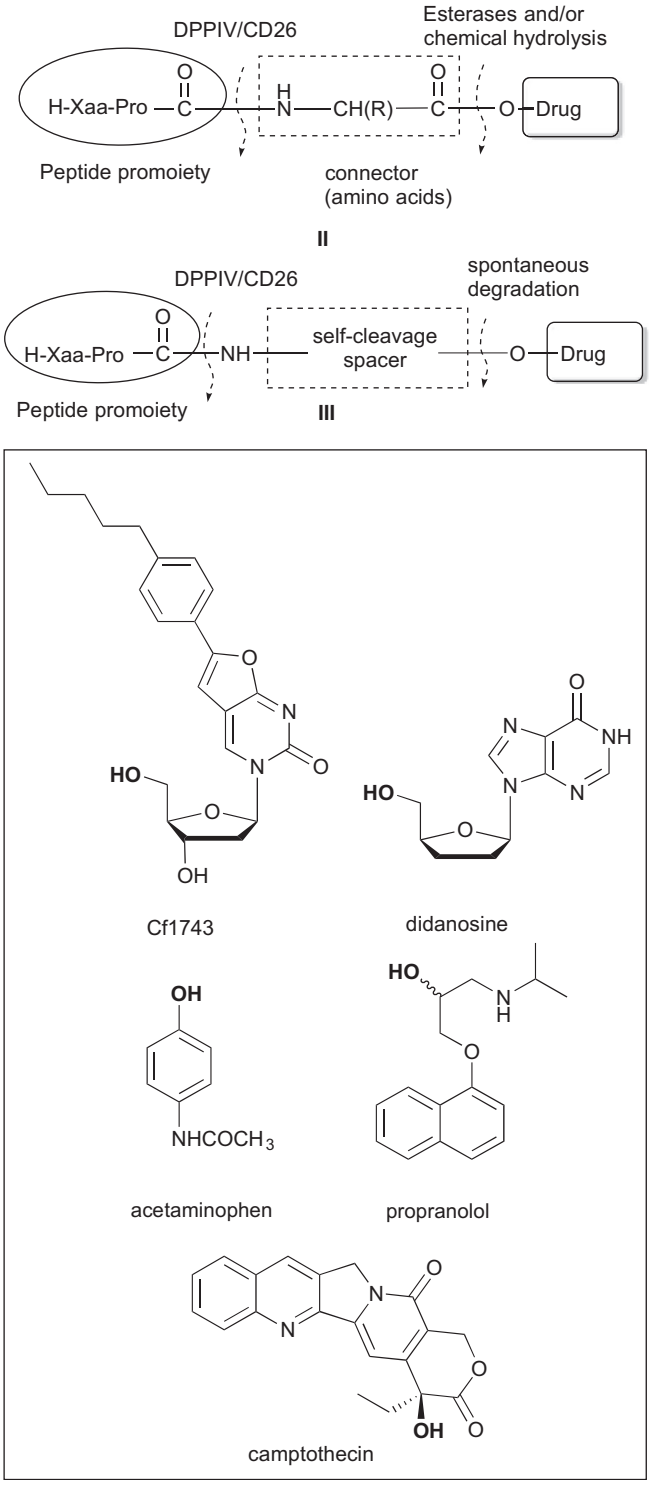


Fig. 2. Two-step activation of DPP IV/CD26-based prodrugs of hydroxy-containing drugs.

enhanced oral bioavailability in mice compared to the free parent compound [14]. We also investigated self-cleavage connectors in the design of novel two-step activation prodrugs (conjugates of general formulae **III**, Fig. 2) [16]. Both cyclization and electronic cascade self-cleavage spacers were explored. Prodrugs based on dipeptide cyclization spacers efficiently released the parent nucleoside upon hydrolysis by DPPIV/CD26 followed by spontaneous cyclization of the dipeptide conjugate intermediate via

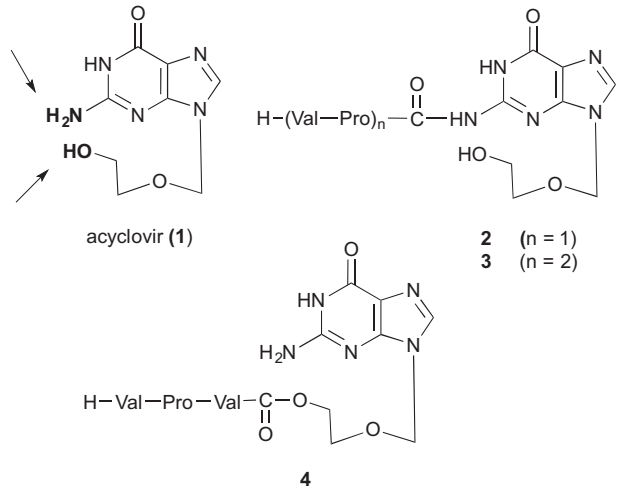


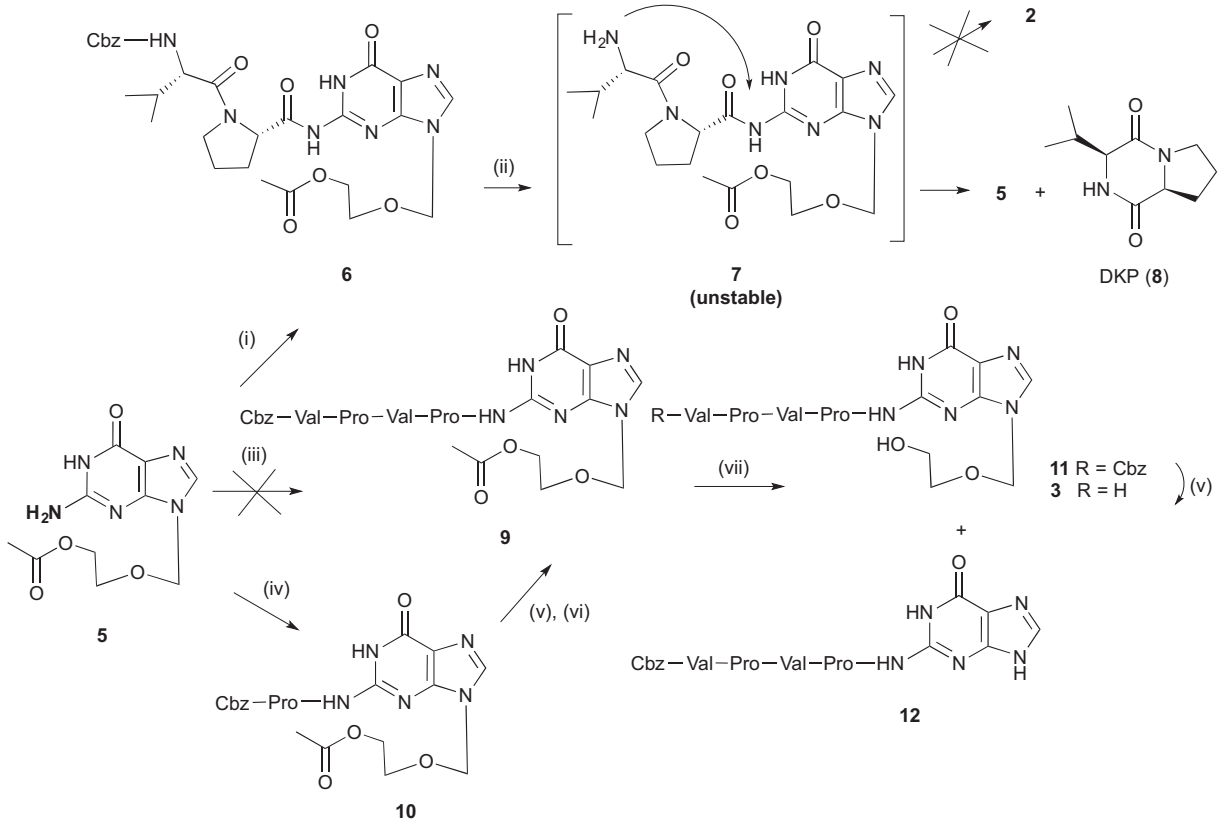
Fig. 3. Structure of acyclovir (**1**) and target amide and ester prodrugs (**2**–**4**).

diketopiperazine (DKP). A markedly increased transport through CaCo-2 cell monolayers and oral bioavailability was observed for several tetrapeptide prodrugs [16].

9-[2-(Hydroxyethoxy)methyl]guanine (acyclovir **1**, Fig. 3) is a widely used therapeutic agent for the treatment of herpes virus infections [17]. Acyclovir (ACV) is poorly water-soluble and has an oral bioavailability of 10–20% [18]. In order to overcome these limitations, a number of acyclovir prodrugs were previously reported [19–21]. Because the reactivity of the 4′-hydroxyl group is higher than that of the 2-amino group, most of these produgs were acylated at the primary hydroxyl group. Valacyclovir (VACV), the L-valine ester prodrug of ACV [22,23], was found to metabolise easily upon oral administration (by esterases and/or biphenyl hydrolase-like protein [24]) and exhibited four-folded higher bioavailability than ACV [25]. The oral absorption of this prodrug appears to be mediated by its substrate activity for the PepT1 peptide transporter abundantly present in the intestinal mucosa. The amino acid ester prodrugs of ACV have low stability at physiological pH and their delivery is still not optimal. Dipeptide [26,27] or peptidomimetic [28] prodrugs of ACV were also synthesized and evaluated for their chemical stability and pharmacokinetics.

The aim of this study was to assess for the first time the applicability of the DPPIV/CD26 prodrug strategy to guanidine drugs such as the antiviral acyclovir (**1**). In this compound both the amino and the hydroxyl group are amenable for prodrug derivatization. Depending on the site of attachment of the peptide promoiety, both peptidyl amide and ester prodrugs of acyclovir were envisioned (Fig. 3). As the peptide promoiety, we focused on the Val-Pro dipeptide (efficiently recognized by the DPPIV/CD26 enzyme) and the Val-Pro-Val-Pro tetrapeptide for the peptidyl amide prodrugs **2** and **3** and on the Val-Pro dipeptide and on the valine as connector for the peptidyl ester prodrug **4** based on our previous results (Fig. 3) [8,12,14,15].

The preparation of target peptidyl amide prodrugs **2** and **3** acylated at the exocyclic amino group of the guanine base represents a synthetic challenge due to the intrinsically low nucleophilicity of this amino function and the low solubility of this compound. We investigated several procedures for the acylation of the 2-amino group of acyclovir with peptides using both solution-phase and solid-phase strategies. It should be noted that although the amino group of guanine nucleosides (such as ACV) has been



Scheme 1. Synthetic strategies in solution for the synthesis of target acyclovir amide prodrugs **2** and **3**. Reagents and conditions: (i) Cbz-Val-Pro-OH, TFFH, collidine, DMF, 80 °C; (ii) H_2, Pd(C), MeOH; (iii) Cbz-Val-Pro-Val-Pro-OH, TFFH, collidine, DMF, 80 °C; (iv) Cbz-Pro-OH, TFFH, collidine, DMF, 80 °C; (v) H_2, Pd(C), CH_3OH; (vi) Cbz-Val-Pro-Val-OH, pyBOP, TEA, DMF, rt; (vii) NH_3/CH_3OH sat.

previously protected with simple acyl groups following different strategies [29,30], acylation with amino acids or peptides has never been reported.

We herein describe the synthesis, water solubility and chemical and enzymatic stability studies of target acyclovir prodrugs (**2**–**4**) in the presence and absence of a dipeptidyl peptidase inhibitor.

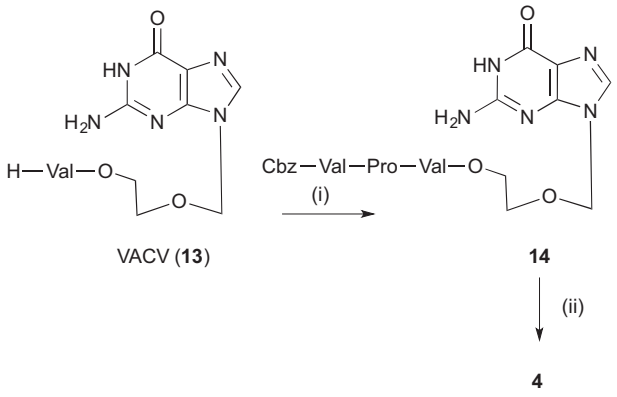
2. Results and discussion

2.1. Chemistry

2.1.1. Solution-phase synthesis of acyclovir amide and ester prodrugs

We first focused on the synthesis of the Val-Pro dipeptide amide prodrug of acyclovir **2** using a solution-phase approach. The hydroxyl function of acyclovir was first protected with an acetyl group (compound **5** [31]) before the coupling step under standard acetylation conditions (Ac_2O/pyridine) (Scheme 1). Initial attempts of coupling of **5** with the commercially available dipeptide Cbz-Val-Pro-OH in the presence of a variety of coupling reagents such as carbodiimides [1,3-diisopropylcarbodiimide (DIPCDI) with *N*-hydroxybenzotriazole (HOBt)] or more potent coupling reagents such as phosphonium-[(benzotriazol-1-yloxy)-tris[pyrrolidino] phosphonium hexafluorophosphate (PyBOP)] or uronium salts [hexafluorophosphate salt of the *O*-(7-azabenzotriazolyl)tetramethyl uronium (HATU)] in the presence of TEA or DIEA as base and dry DMF as solvent failed to give the desired dipeptide ACV conjugate **6** (Scheme 1). Increasing amounts of coupling reagents (3–5 equiv) and higher reaction temperatures (90–150 °C) in a sealed pressure tube did not afford the coupled product. Thus, the exocyclic amino group of the guanine moiety of acyclovir appears difficult to couple with dipeptides under similar conditions previously used in our group for the synthesis of dipeptide conjugates of other pyrimidine and purine (adenine) nucleosides [12]. Further attempts of coupling by in situ generation of protected dipeptide chlorides using thionyl chloride or triphosgene [bis(trichloromethyl)carbonate (BTC)] with protected compound **5** in the presence of pyridine and DMAP or 2,4,6-collidine as base in dry DMF also failed in all the experimental conditions investigated (room temperature or 90–150 °C in a sealed pressure tube). We next explored coupling reactions via acid fluorides. A remarkable progress in this field was the development of tetramethylfluoroformamidinium hexafluorophosphate (TFFH) by Carpino et al. [32] which act by in situ generating amino acid fluoride in difficult peptide coupling reactions. When protected acyclovir **5** was coupled with Cbz-Val-Pro-OH in the presence of 1.5 equiv. of TFFH as coupling reagent and 2,4,6-collidine as base in dry DMF at 150 °C the dipeptide conjugate **6** was isolated for the first time although in low yields (5%) after chromatographic purification. However, when the coupling reaction was performed at 80 °C in the presence of 5 equivalents of TFFH, the desired protected dipeptide conjugate **6** was obtained in higher yield (53%). Similarly to what has been observed with cytidine and adenosine dipeptide prodrugs [12], the *N*-deprotected dipeptide acyclovir conjugate **7** was chemically unstable. Thus, catalytic hydrogenation of protected conjugate **6** in the presence of 10% Pd/C in methanol showed that parent ACV derivative **5** was spontaneously released from the *N*-deprotected conjugate **7** with concomitant formation of the known DKP **8** [33] (Scheme 1).

We next focused on the synthesis of tetrapeptide amide prodrug **3** in order to obtain chemically stable prodrugs. Coupling of **5** with Cbz-Val-Pro-Val-Pro-OH [12] under the optimized coupling conditions described above for the dipeptide acyclovir conjugate (TFFH and 2,4,6-collidine at 80 °C) did not afford the desired tetrapeptide conjugate **9**. An alternative strategy to the difficult direct tetrapeptide coupling, which consisted of coupling of **5** with an amino acid followed by coupling with the corresponding tripeptide, was next performed. The required tripeptide Cbz-Val-Pro-Val-OH was prepared by solid-phase synthesis using a Fmoc strategy on a 2-chlorotrityl polystyrene resin as previously described [12]. Thus, coupling of compound **5** with Cbz-Pro-OH in the presence of TFFH and 2,4,6-collidine in dry DMF at 80 °C (under similar conditions described above for the dipeptide conjugate **6**) gave the Cbz protected prolyl amide derivative **10** in 65% yield after chromatographic purification (Scheme 1). Catalytic hydrogenation of **10** in the presence of 10% Pd/C in methanol followed by coupling of the *N*-deprotected prolyl intermediate derivative with Cbz-Val-Pro-Val-OH in the presence of 3 equivalents of PyBOP as coupling reagent and TEA as base in dry DMF at room temperature afforded the desired tetrapeptide conjugate **9** in low overall yield (24% from **10**). Use of other coupling reagents usually employed in amide formation (HATU/HOAt/DIEA or coupling via acyl halides) and higher reaction temperatures did not lead to improved yields. Treatment of compound **9** with methanol saturated with ammonia at room temperature (removal of the *O*-acetyl group) gave the *O*-deprotected acyclovir conjugate **11** in a 32% yield together with the tetrapeptide guanine derivative **12** in a 26% yield due to C–N bond cleavage. Finally, catalytic hydrogenation of **11** in the presence of 10% Pd/C in methanol afforded the *N*-deprotected target amide prodrug **3** in 83% yield (Scheme 1).



Scheme 2. Synthesis of target acyclovir ester prodrug **4**. Reagents and conditions: (i) Cbz-Val-Pro-OH, PyBOP, TEA, DMF, rt; (ii) H_2, Pd(C), MeOH.

The synthesis of the tripeptide ester prodrug of acyclovir **4** was next carried out by standard peptide coupling of Cbz-Val-Pro-OH and commercially available L-valacyclovir **13** (Scheme 2). Thus, treatment of the hydrochloride salt of free amine of VACV **13** with Cbz-Val-Pro-OH in the presence of PyBOP as coupling reagent and TEA as base in dry DMF at room temperature overnight afforded the *N*-protected tripeptide conjugate **14** in good yields (81%) after chromatographic purification. Finally, removal of the Cbz group of compound **14** by catalytic hydrogenation in the presence of 10% Pd/C in methanol gave the target tripeptide ester conjugate of ACV **4** in 67% yield.

The target prodrugs **3** and **4** were characterised by ^{1}H NMR, mass spectrometry and microanalysis, all data confirming the structure and purity of the novel compounds.

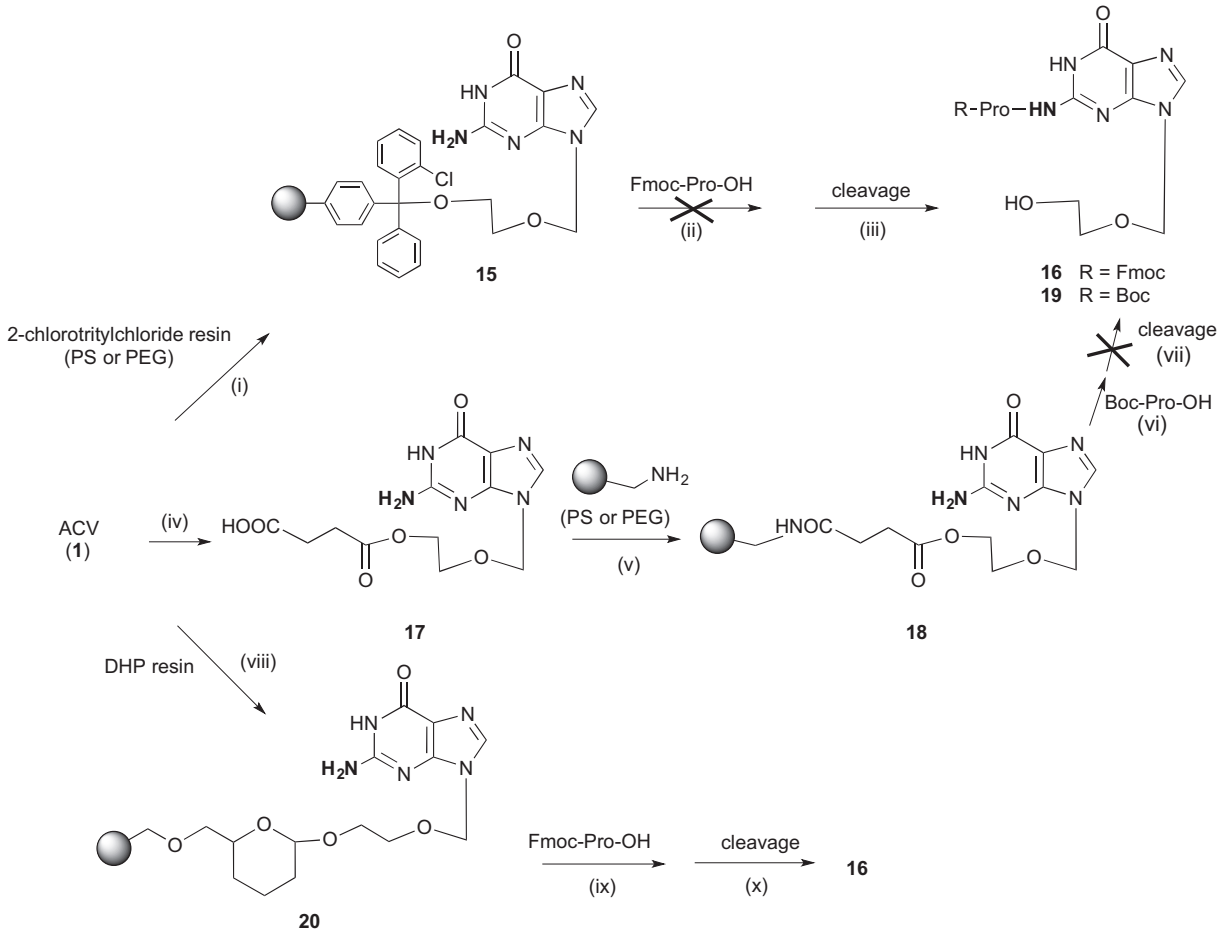
2.1.2. Solid-phase synthesis of acyclovir amide prodrug *3*

We next investigated for the first time a solid-phase method for acylation of the exocyclic amino group of acyclovir by attaching the hydroxyl group of ACV to a solid-support. In this approach, the use of a large excess of reagents, which can be easily eliminated through simple resin washes, may facilitates the completion of the notoriously difficult coupling reaction of peptides with the guanine

moiety observed above in the solution-phase strategy. Our solid-phase strategies for the synthesis of the tetrapeptide amide prodrug of acyclovir **3** involve anchoring acyclovir through the hydroxyl group to an appropriate solid-support, followed by a stepwise coupling of *N*-protected amino acids (method A), direct tetrapeptide coupling (method B) or a [1 + 3] fragment coupling (method C) and a final *N*-deprotection/cleavage step.

We first focused on the selection of a convenient solid-support to anchor acyclovir via the hydroxyl group followed by an exploratory model coupling reaction with a conveniently *N*-protected amino acid (Scheme 3). We initially investigated the widely used acid labile 2-chlorotrityl chloride resin in solid phase peptide synthesis using Fmoc strategy. The steric bulk of the 2-chlorotrityl resin prevents diketopiperazine formation during the attachment of the first two amino acids. This would allow us to explore the stepwise coupling of amino acids (method A) that could not be used in the solution-phase approach. Both, the classical polystyrene (PS)-based solid support and the more hydrophilic ChemMatrix®, a totally poly(ethylene glycol)(PEG)-based resin developed by Albericio et al. [34] were used. This resin swells better than PS-based resins in polar solvents such as acetonitrile, DMF or DMSO that were required to dissolve the highly polar acyclovir compound. A variety of bases (DIEA or py), solvents (DMF or DMSO) and reaction conditions (rt or 60 °C) were explored for the loading of the primary alcohol of acyclovir onto the 2-chlorotrityl resin (PS or PEG). The best loading conditions (DIEA, DMSO, rt) were determined by cleavage of a small amount of resin in the presence of 1% TFA in DCM and analytical HPLC (254 nm) determination. No significant differences in loadings were observed between PS- and PEG-based 2-chlorotrityl resins. Supported acyclovir compound **15** (Scheme 3) was then reacted with Fmoc-Pro-OH in dry DMF in the presence of a variety of coupling reagents (TFFH, HATU, PyAOP, DIPCDI, HCTU), additives (HOAt) and bases (2,4,6-collidine, DIEA or DMAP). The coupling reactions were heated in a pressure tube or using microwave irradiation at 80 °C. In all cases, only traces of the desired prolyl acyclovir derivative **16** were detected by HPLC-MS after cleavage (1% TFA) of a small amount of resin being the major compound acyclovir.

In order to reduce steric hindrance of the resin, that may explain the failure of the amino acid coupling (even in the PEG resin which minimizes the steric effects caused by the support), in a second attempt, we chose base-labile aminomethyl-resins (both PS and ChemMatrix® PEG-resin) using a BOC strategy (Scheme 3). Acyclovir was first esterified with succinic anhydride according to the method of Colla et al. [22a,35], followed by coupling of compound **17** through its carboxylic group to the aminomethyl-resins. The efficiency of the coupling reaction was easily monitored by the Kaiser ninhydrin test. Coupling of **17** to the aminomethyl-PS resin in the presence of DIPCDI/HOAt in DMSO at room temperature for 2 h did not result in a negative Kaiser test even after three coupling cycles (incomplete coupling). However, reaction of **17** to the more soluble ChemMatrix® PEG-support under the same coupling conditions provided a negative Kaiser test after two coupling cycles. The resulting supported-bound acyclovir **18**



Scheme 3. Resin selection for the solid-phase synthesis of acyclovir prodrugs. Reagents and conditions: (i) DIEA, DMSO, rt; (ii) Fmoc-Pro-OH, HATU, HOAt, or PyAOP, HOAt, or DIPCDI, DMAP, TFFH, DIEA or HCTU, DIPEA, DMF, rt or 90 °C; (iii) TFA, 1% CH_2Cl_2; (iv) succinic anhydride, pyridine, DMF, rt; (v) DIPCDI, HOAt, DMSO, rt; (vi) Boc-Pro-OH, HATU, HOAt, or PyAOP, HOAt, DMF, rt; (vii) DBU or 2 N NH_3, EtOH; (viii) PPTS, DMF, 80 °C; (ix) Fmoc-Pro-OH, HCTU, DIPEA, DMF, MW, 80 °C; (x) TFA:H_2O, 95:5, rt.

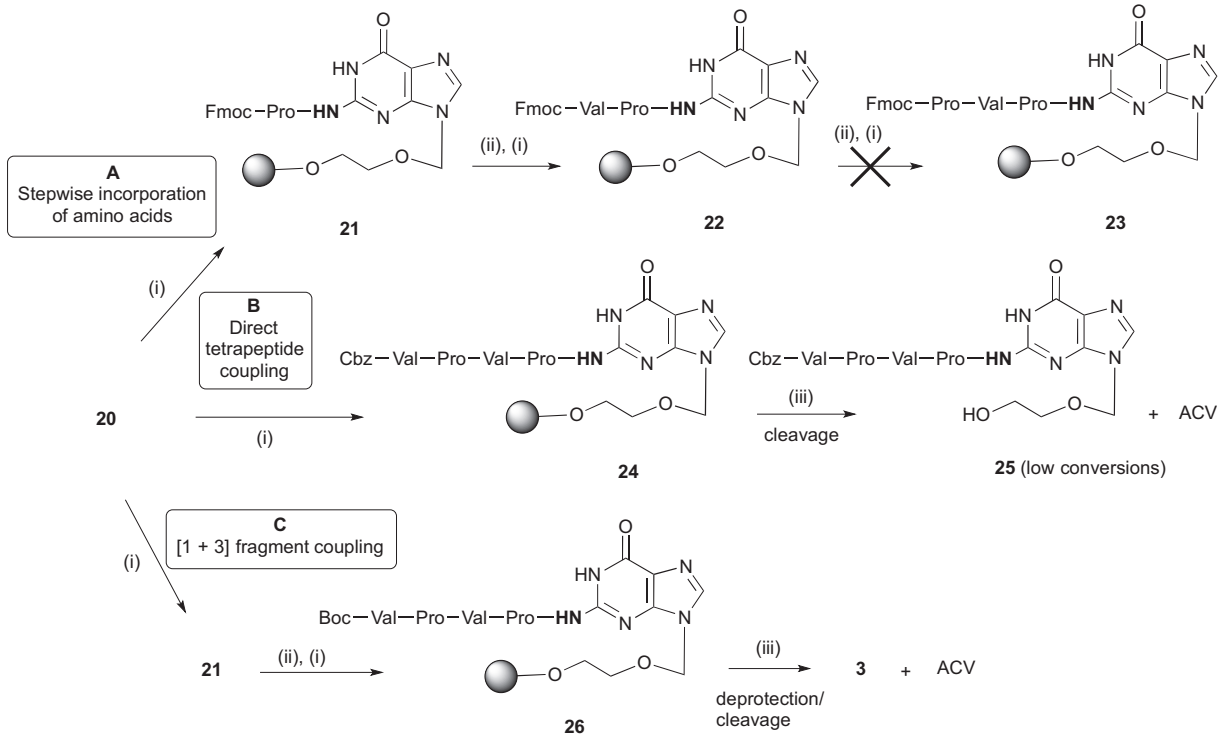
(Scheme 3) was then coupled to Boc-Pro-OH in the presence of HATU/HOAt and PyAOP/HOAt as coupling reagents in dry DMF. Monitoring of this reaction was carried out by cleaving a small amount of resin from the reaction and then analysing the crude by HPLC-MS. Unfortunately, the standard basic cleavage conditions (DBU or 2 N NH_3 in ethanol) were found to be completely ineffective and no acyclovir or Boc-prolyl acyclovir **19** were detected by HPLC-MS. When stronger cleavage conditions were used (ammonia gas overnight) C–N bond cleavage occurred and a signal corresponding to the guanine moiety was detected in the HPLC-MS chromatogram as the major product.

At this point, we examined Ellman's dihydropyran (DHP) PS-resin as an alternative solid phase support for the inmobilization of primary or secondary alcohols [36]. The DHP linker was chosen because it is less sterically hindered than the trityl linkers and the bound alcohols can be cleaved from the resin under acidic conditions. Acyclovir (**1**) was coupled to the DHP resin (0.90 mmol/g of resin) by employing pyridinium *p*-toluenesulfonate (PPTS) in DMF at 80 °C [36] to achieve maximum dissolution. The reaction was carried out using microwave irradiation on a Biotage Initiator reactor for 8 h maintaining the vessel temperature at 80 °C. The loading level of the resin (0.843 mmol/g) was based on the mass balance recovered acyclovir, which was obtained by subjecting a portion of the resin to cleavage by 95:5 trifluoroacetic acid (TFA)/water for 20 min. The resulting acyclovir on DHP-resin (**20**, Scheme 4) was coupled with Fmoc-Pro-OH in the presence of 4 equivalents of HCTU as coupling reagent, DIEA as base in dry DMF at room temperature overnight. After cleavage of a small portion of resin (95:5 TFA/water), the desired Fmoc-Pro-acyclovir compound **16** was detected by HPLC-MS analysis (254 nm) albeit with recovering of acyclovir in a 1:5 proportion (20% conversion). The use of other coupling reagents such as TFFH/2,4,6-collidine or HATU/HOAt did not improve conversion. These results showed for the first time that the coupling of Fmoc-Pro-OH with the exocyclic amino of acyclovir was viable in a solid-phase approach using a DHP-resin and HCTU as coupling reagent. A variety of coupling reaction conditions were next examined. The best results were obtained by repeating coupling cycles under microwave heating at 80 °C (5 cycles × 10 min) using 1.2 equivalents of HCTU and DIEA in dry DMF. Under these conditions, an improved conversion (50%) to the prolyl-acyclovir compound **16** was detected by HPLC-MS after cleavage of a small portion of resin.

Next, we focused on the synthesis of target tetrapeptide amide prodrugs of acyclovir **3** from acyclovir attached to the DHP resin **20** (Scheme 4). We first investigated the stepwise introduction of amino acids (method A). Thus, coupling of Fmoc-Val-OH onto the prolyl-acyclovir resin **21** under optimized conditions (HCTU/DIEA/80 °C/MW) gave the expected dipeptide derivative **22**. However, Fmoc deprotection of resin **22** under standard conditions (piperidine 20% in DMF) and coupling of the third amino acid failed to give the tripeptide-acyclovir derivative **23** and only acyclovir was detected by HPLC-MS after cleavage of a small portion of the resin. This could be explained by DKP formation before the coupling of the third amino acid similarly to what had occurred in the above solution-phase approach.

We next examined the direct tetrapeptide coupling (method B) on the acyclovir DHP-resin (**20**) under the optimized coupling conditions (HCTU/DIEA/80 °C/MW). Unfortunately, although the desired tetrapeptide prodrug **25** was detected by HPLC-MS after cleaving a small portion of the resin, the acyclovir starting material (**1**) was recovered as major compound.

These results prompted us to investigate the amino acid and tripeptide coupling strategy (method C) as an alternative for the synthesis of the target tetrapeptide prodrug using a Boc protecting strategy. Thus, supported prolyl derivative **21** was reacted with tripeptide Boc-Val-Pro-Val-OH in the presence of HCTU as coupling reagent under the optimized coupling conditions to give the desired supported tetrapeptide-acyclovir derivative **26** (Scheme 4).



Scheme 4. Synthetic strategies in solid phase for the synthesis of target tetrapeptide acyclovir prodrug **3** from acyclovir attached to the DHP resin (**20**). Reagents and conditions: (i) R-XX_a-OH or peptide, HCTU, DIPEA, DMF, MW, 80 °C, 5 × 10 min; (ii) 20% piperidine, DMF, rt; (iii)TFA/H_2O 95:5, rt.

The final product was cleaved from the resin using the standard acidic cleavage protocol (95:5, TFA/water) which result in the simultaneous deprotection of the Boc group. Purification of the cleavage crude by reverse-phase chromatography on a *Biotage Isolera* provided the target prodrug **3** in a 20% overall yield together with unreacted acyclovir. The overall yield for the four-step process (loading, amino acid and tripeptide couplings and cleavage) was moderate but the target prodrug could be isolated at high purity after chromatographic purification without isolation of intermediates.

2.2. Water solubility of prodrug conjugates

The water-solubility of the acyclovir target prodrugs **3** and **4** were determined and compared to that of the poorly soluble parent compound. The amide prodrug **3** and the ester prodrug **4** improved the water-solubility (22.87 mg/mL and 12.19 mg/mL) more than 17-fold and 9-fold, respectively, in comparison with the parent compound **1** (1.29 mg/mL). This result suggests that the CD26 prodrug approach could be useful for increasing the water-solubility of polar drugs.

2.3. Biological studies

2.3.1. Susceptibility of the prodrugs to the action of purified CD26 and CD26 present in human and bovine serum

The stability of valyl ester prodrug **13** (Val-ACV) in PBS and sera was investigated. It was found that during the first two hours, only a few percent of the compound was converted to the parental ACV derivative (Fig. 4) in both PBS and human/bovine serum (HS and BS) (Fig. 4). However, after 24 h, almost 50% of the prodrug proved to be converted to ACV in PBS and HS and this conversion had even proceeded to ~60% in BS (Fig. 4). CD26 had no additional influence on this chemical conversion process as expected.

Next, the tripeptide ester prodrug of ACV **4** was investigated. In contrast with Val-ACV (**13**), the tripeptide ester conjugate was fully stable in PBS. Even after 24 h, no traces of free ACV were observed (Fig. 5). Exposure to CD26 resulted in rapid and efficient conversion to Val-ACV. The appearance of ACV after 24 h to the amount of ~40% is apparently due to spontaneous (chemical) conversion as earlier demonstrated in Fig. 4. Administration of HS and BS to [ValPro-Val]-ACV also efficiently converted the prodrug to Val-ACV. Within 15 min, 80% (HS) and 40% (BS) of the ACV tripeptide derivative had been converted to Val-ACV. Thus, in contrast with Val-ACV, [ValPro-Val]-ACV proved chemically fully stable, but was efficiently converted by CD26 and human/bovine sera to Val-ACV.

Finally, the conversion of the tetrapeptide amide prodrug **3** was investigated. This ACV prodrug was virtually completely stable in PBS. Only a few percent of the compound converted to ACV after 24 h (Fig. 6). CD26 efficiently hydrolysed the tetrapeptide to ACV. Interestingly, no traces of dipeptide-ACV that were expected to appear during the 2-step conversion of the tetrapeptide-ACV to ACV could be demonstrated. This observation strongly suggests that the ACV dipeptide derivative is highly unstable and spontaneously converts to ACV as soon as formed in the CD26-catalysed hydrolysis reaction. Also, in HS and BS, solely ACV could be found in the efficient hydrolysis reaction during the eventual conversion of the ACV-tetrapeptide to ACV (Fig. 6).

To demonstrate the involvement of CD26 in the conversion of [ValPro-ValPro] amide prodrug **3** and [ValPro-Val] ester prodrug **4** in HS, the enzyme reaction was investigated in the presence of increasing concentrations of Vildagliptin, a potent inhibitor of dipeptidyl peptidase activity (Fig. 7). The CD26 inhibitor dose-dependently prevented the release of the ValPro moiety of both tetrapeptide- and tripeptide-ACV prodrugs and proved particularly effective in case of the tetrapeptide-ACV derivative (Fig. 7) that was still intact by >80% after 24 h in the presence of 25 μM Vildagliptin. Without this inhibitor, the tetrapeptide-ACV was virtually completely converted to ACV within 1 h.

To investigate whether these prodrugs can survive the low pH environment of the stomach in case they would be administered

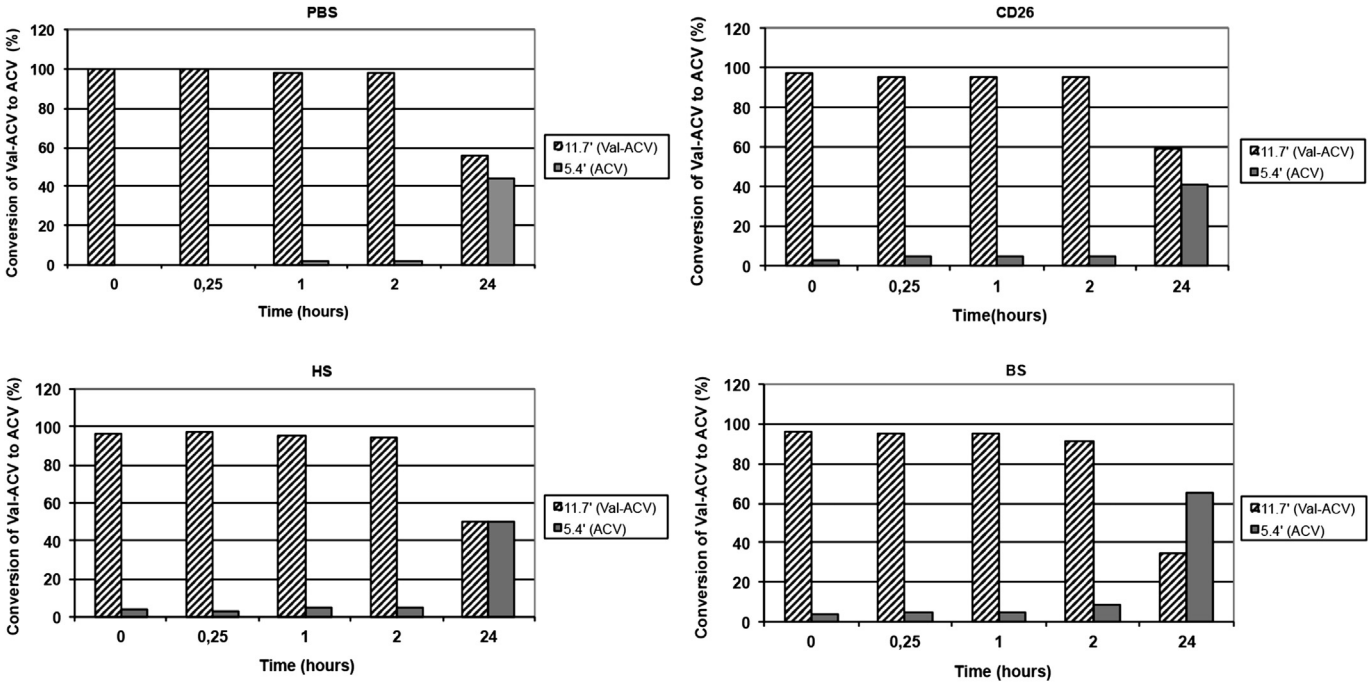


Fig. 4. Stability in PBS and conversion of valacyclovir **13** to the parent compound in the presence of DPPIV/CD26 and 10% human (HS) or bovine (BS) serum.

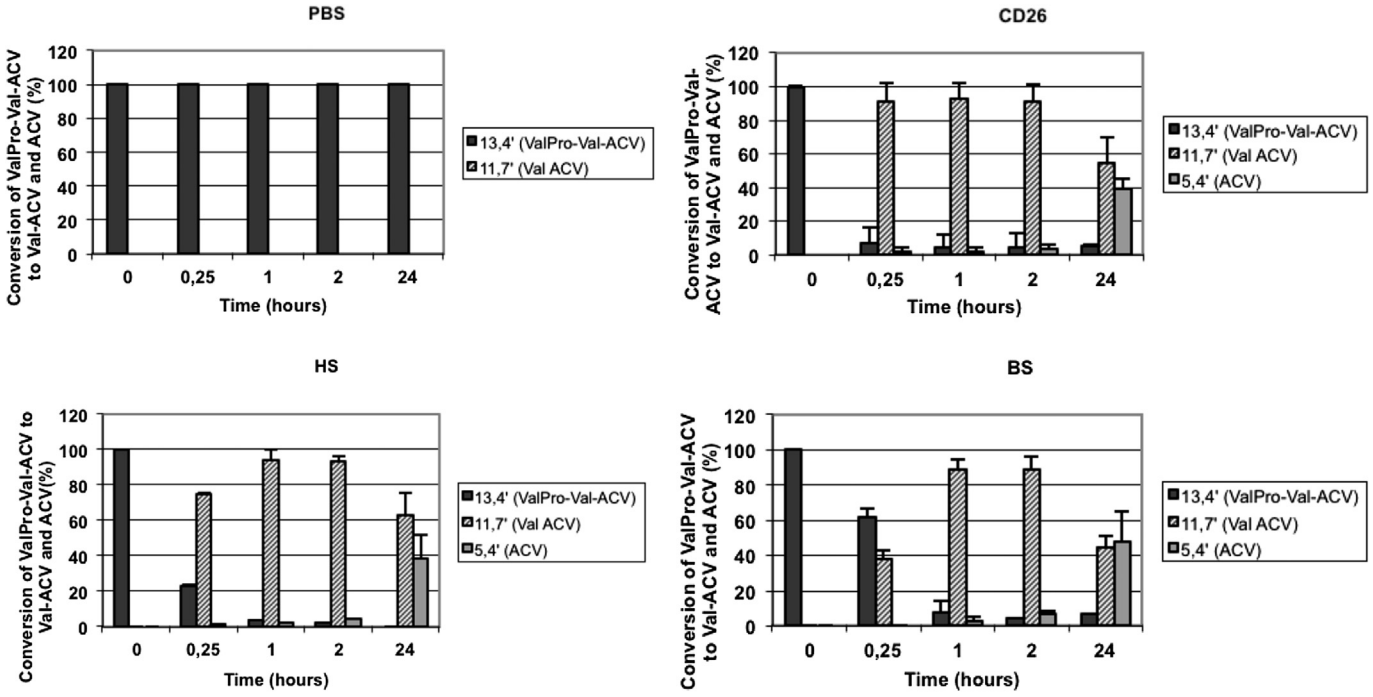


Fig. 5. Stability in PBS and conversion of [ValPro]-[Val] ester prodrug **4** to the parent compound in the presence of DPPIV/CD26 and 10% human (HS) or bovine serum (HS).

orally, their stability was examined in sodium phosphate buffer pH 3.2. It was found that both the [ValPro-ValPro] amide ACV prodrug **3** and the [ValPro-Val] ester ACV prodrug **4** were fully stable at such low pH for at least 6 h.

2.3.2. Antiviral evaluation

The antiviral activity of ACV and its peptide amide and ester prodrugs were also evaluated in confluent HEL cell cultures. Whereas ACV proved substantially active against herpes simplex virus type 1 (KOS) and type 2 (G) at an EC_{50} of 0.32 and 0.50 μM, respectively, the [ValPro-Val] and [ValPro-ValPro]-ACV prodrugs showed EC_{50}'s of 0.60 and 1.8 μM against HSV-1 and 1.6 and 4.9 μM against HSV-2. The antiviral activities of the [ValPro-Val] and [ValPro-ValPro]-ACV prodrugs against a thymidine kinase-deficient HSV-1 (KOS ACV^r) strain were 32 and 73 μM *versus* 18 μM for ACV. Thus, the peptide prodrugs displayed marked anti-herpetic activity in cell culture within a comparable order of magnitude as parent ACV.

3. Conclusions

In conclusion, various synthetic studies toward peptide amide and ester prodrugs of acyclovir cleavable by the DPPIV/CD26 enzyme are reported. Solid-phase synthesis for the challenging acylation of the exocyclic amino group of acyclovir with amino acids or peptides is a convenient procedure that does not require protection of the hydroxyl group of acyclovir. In our opinion the method could be extended to other nucleosides containing amino functions and/or other acylating reagents. Purified CD26 as well as

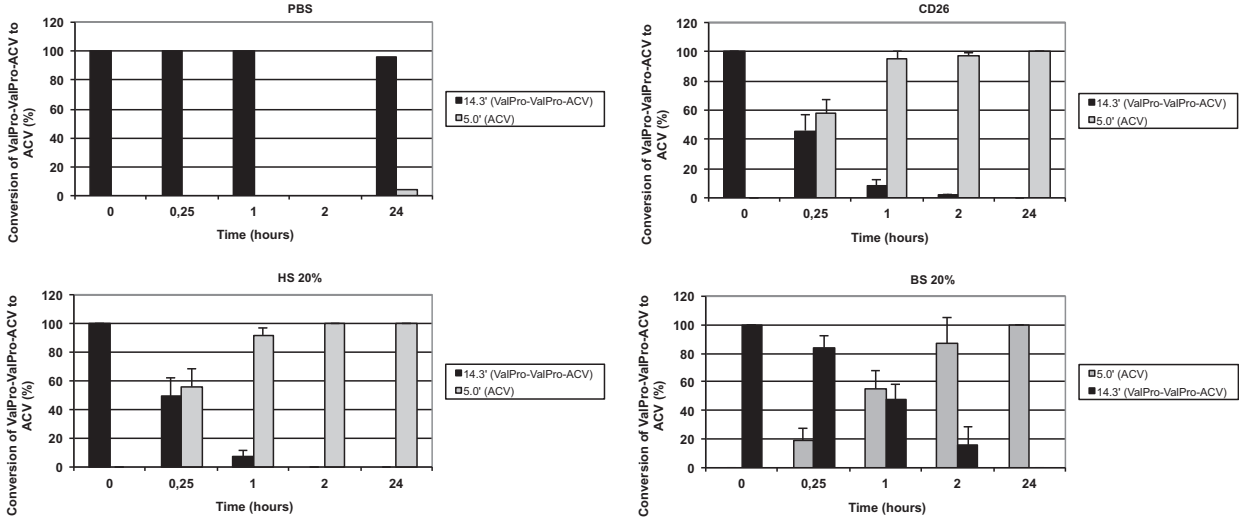


Fig. 6. Stability in PBS and conversion of [ValPro]-[ValPro] amide prodrug **3** to the parent compound in the presence of DPPIV/CD26 and 10% human (HS) or bovine serum (BS).

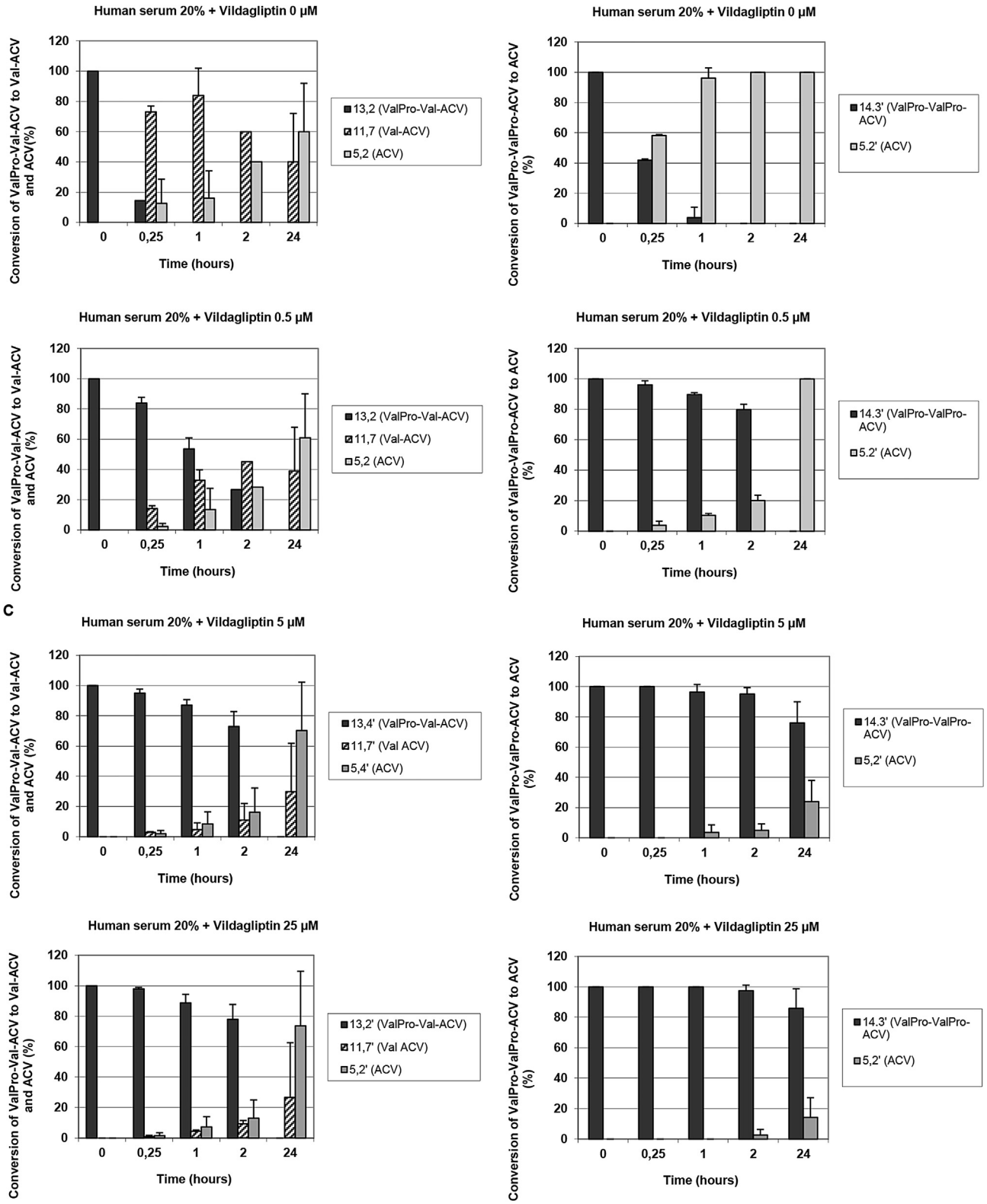


Fig. 7. Hydrolysis of [ValPro]-[ValPro] amide prodrug **3** and [ValPro]-[Val] ester prodrug **4** in the presence of a specific inhibitor of DPPIV/CD26.

(CD26-containing) serum efficiently converted the tetrapeptide amide prodrug **3** to ACV, and the tripeptide ester prodrug **4** to Val-ACV. In the former case, no traces of intermediate ValPro-ACV were observed in the hydrolysis process. The anchoring of the peptide promoiety to either the amino or the hydroxyl group of ACV led to successful amide or ester prodrugs. The stability results indicate that the tetrapeptide amide prodrug of ACV exhibit faster release of the parent drug compared to peptide ester prodrugs of acyclovir. Both types of prodrugs afforded an efficient anti-herpetic activity in cell culture.

4. Experimental section

4.1. Chemistry

Microanalyses were obtained with a Heraeus CHN-O-RAPID instrument. Electrospray mass spectra were measured on a quadropole mass spectrometer equipped with an electrospray source (Hewlett–Packard, LC/MS HP 1100). Spectra were recorded with Varian Inova-300 or Varian Inova-400 spectrometers operating at 300 or 400 MHz for ^{1}H NMR with Me_4Si as internal standard. Analytical thin-layer chromatography (TLC) was performed on silica gel 60 F_{254} (Merck). Separations on silica gel were performed by flash column chromatography with silica gel 60 (230–400 mesh) (Merck) or preparative centrifugal circular thin layer chromatography (CCTLC) on a Chromatotron^R (Kieselgel 60 PF_{254} gipshaltig (silica gel containing gypsum) (Merck)), layer thickness of 1 mm, flow rate of 5 mL/min. Liquid chromatography was performed using a force flow (flash chromatography) Horizon HPFG System (Biotage) with Flash 25 or 40 silica gel cartridges.

The coupling reactions on solid phase were carried out using microwave radiation in a Biotage Initiator reactor in a 5 mL vial. Excluding the coupling reaction on the microwave reactor, the rest of the SPPS reactions were stirred using an IKA-100 orbital shaker. After cleavage, the acidic crudes were sedimented in Et_2O on a Hettlich Universal 320R centrifuge at 5000 rpm. All the crude and samples were lyophilized using mixtures water/acetonitrile on a Telstar 8-80 instrument. The monitoring of the reactions was performed by HPLC/MS through a HPLC-waters 12695 connected to a Waters Micromass ZQ spectrometer.

Chemicals and reagents were obtained from commercial sources and were used without further purification. 2-chlorotrityl resin (PS) and HCTU were purchased from GL Biochem (Shanghai) Ltd. Aminomethyl resin (PS) and dihydropyrane resin were purchased from Novabiochem and aminomethyl resin (PEG) and 2-chlorotrityl resin (PEG) from Chematrix. Acyclovir was purchased from Ark Pharm Inc. (USA) and valacyclovir from Sigma. The dipeptide derivative Cbz-Val-Pro-OH and the tripeptide Boc-Val-Pro-Val-OH were purchased from Bachem Feinchemikalien and Peptide Protein Research Ltd, respectively. The 5′-*O*-acetyl derivative of acyclovir (**5**) [31] and the tetrapeptide Cbz-Val-Pro-Val-Pro-OH and the tripeptide Cbz-Val-Pro-Val-OH were synthesized as previously described [12].

4.1.1. Solution-phase synthesis of acyclovir prodrugs

*4.1.1.1. 9-[(2-Acetoxyethoxy)methyl]-2-N-[N-(bencyloxycarbonyl)-valyl-prolyl]guanine (**6**).* To a solution of dry 9-[(acetoxyethoxy) methyl]guanine **5** [31] (0.10 g, 0.37 mmol) in dry DMF (2 mL) was successively added Cbz-Val-Pro-OH (0.47 g, 1.87 mmol), TFFH (0.49 g, 1.87 mmol) and 2,4,6-collidine (0.25 mL, 1.87 mmol). The reaction mixture was stirred at 80 °C overnight and the solvent was evaporated to dryness. The residue was dissolved in ethyl acetate (100 mL) and washed with 10% aqueous citric acid (3 × 20 mL), 10% $NaHCO_3$ (3 × 20 mL), H_2O (3 × 20 mL) and brine (3 × 20 mL). The organic layer was dried (Na_2SO_4), filtered and evaporated to dryness. The crude was purified by flash column chromatography and CCTLC on the Chromatotron (CH_2Cl_2/MeOH, 30:1) to afford **6** (125 mg, 53%) as a white foam. ^{1}H NMR (400 MHz, acetone-d_6): δ 0.90 (d, 3H, γ-CH_3, Val, J = 6.4 Hz), 0.95 (d, 3H, γ-CH_3, Val, J = 6.4 Hz), 1.91–1.95 (m, 7H, OCO$\underline{CH_3}$, β-CH, Val, β-CH_2, γ-CH_2, Pro), 2.20 (m, 1H, β-CH_2, Pro), 3.49–3.53 (m, 2H, δ-CH_2, Pro), 3.68 (m, 2H, O$\underline{CH_2}$), 3.83 (m, 1H, α-CH, Val), 4.07 (m, 2H, $\underline{CH_2}$OCO), 4.55 (m, 1H, α-CH, Pro), 5.02 (dd, 2H, CH_2, Z, J = 12.4 Hz, J = 14.9 Hz), 5.49 (bs, 2H, N$\underline{CH_2}$O), 7.35 (m, 5H, Ar, Z), 7.53 (d, 1H, NH, Val, J = 7.9 Hz), 8.15 (s, 1H, H-8), 11.89 (bs, 1H, NH-1), 11.99 (bs, 1H, 2-NH). MS (ESI$^+$) 598.3 [M + H]$^+$. Anal. Calcd. for $C_{28}H_{35}N_7O_8$: C, 56.27; H, 5.90; N, 16.41. Found: C, 56.42; H, 6.10; N, 14.33.

*4.1.1.2. 9-[(2-Acetoxyethoxy)methyl]-2-N-[N-(bencyloxycarbonyl) prolyl]guanine (**10**).* According to the coupling procedure described for compound **6**, a solution of dry 9-[(acetoxyethoxy)methyl]guanine **5** [31] (0.20 g, 0.74 mmol) in dry DMF (4 mL) was successively reacted with Cbz-Pro-OH (0.94 g, 3.74 mmol), TFFH (0.98 g, 3.74 mmol) and 2,4,6-collidine (0.50 mL, 3.74 mmol) at 80 °C overnight. The final residue was purified by CCTLC on the Chromatotron (CH_2Cl_2/MeOH, 30:1) to afford **10** (244 mg, 65%) as a white foam. ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.58–2.21 (m, 7H, $OCOCH_3$, β-CH_2, γ-CH_2, Pro), 3.42–3.66 (m, 4H, δ-CH_2, Pro, O$\underline{CH_2}$), 4.04 (m, 2H, $\underline{CH_2}$OCO), 4.46 (m, 1H, α-CH, Pro), 4.87–5.08 (m, 2H, CH_2, CBz), 5.44 (s, 2H, N$\underline{CH_2}$O), 7.05–7.34 (m, 5H, Ar, Cbz), 8.13 (s, 1H, H-8), 11.87 (bs, 1H, NH-1), 11.93 (bs, 1H, 2-NH). MS (ESI$^+$) 499.2 [M + H]$^+$. Anal. Calcd. for $C_{23}H_{26}N_6O_7$: C, 55.42; H, 5.26; N, 16.86. Found: C, 55.71; H, 5.21; N, 16.68.

*4.1.1.3. 9-[(2-Acetoxyethoxy)methyl]-2-N-[N-(bencyloxycarbonyl)-valyl-prolyl-valyl-prolyl]-guanine (**9**). Method A.* A solution of *N*-protected prolyl derivative **10** (0.065 g, 0.144 mmol) in methanol (5 mL) containing Pd/C (10%) (40% wt/wt) was hydrogenated at 25 psi at room temperature for 2 h. The reaction mixture was filtered and the filtrate was evaporated to dryness under reduced pressure. The residue (*N*-deprotected prolyl intermediate) was dissolved in dry DMF (2 mL) and was successively treated with PyBOP (0.112 g, 0.216 mmol) and triethylamine (50 μL, 0.36 mmol). The reaction mixture was stirred at room temperature for 2 h and an extra amount of PyBOP (0.112 g, 0.216 mmol) and triethylamine (50 μL, 0.36 mmol) were added. The reaction mixture was stirred at room temperature overnight and the solvent was evaporated to dryness. The residue was dissolved in ethyl acetate **26** (50 mL), washed with 10% aqueous citric acid (3 × 20 mL), 10% aqueous $NaHCO_3$ (3 × 20 mL), water (3 × 20 mL) and brine (3 × 20 mL). The organic layer was dried (Na_2SO_4), filtered and evaporated to dryness. The final residue was purified by CCTLC on the Chromatotron (CH_2Cl_2/MeOH, 40:1) to afford **9** (27 mg, 24% overall yield from **10**) as a white foam. ^{1}H NMR (400 MHz, DMSO-d_6): δ 0.83–0.92 (d, 12H, γ-CH_3, Val), 1.60–1.99 (m, 13H, OCO$\underline{CH_3}$, β-CH, Val, β-CH_2, γ-CH_2, Pro), 3.44–3.73 (m, 5H, δ-CH_2, Pro, O$\underline{CH_2}$), 3.99–4.20 (m, 4H, α-CH, Val, $\underline{CH_2}$OCO), 4.42–4.52 (m, 2H, α-CH, Pro), 4.92–5.08 (m, 2H, CH_2, Cbz), 5.48 (s, 2H, N$\underline{CH_2}$O), 7.31–7.47 (m, 6H, Ar, Cbz, NH), 7.88 (d, 1H, NH, Val, J = 9.1 Hz), 8.10 (d, 1H, NH, Val, J = 8.3 Hz), 8.15 (s, 1H, H-8), 11.89 (bs, 1H, NH-1), 11.99 (bs, 1H, 2-NH). MS (ESI$^+$) 794.5 [M + H]$^+$. Anal. Calcd. for $C_{38}H_{51}N_9O_{10}$: C, 57.49; H, 6.48; N, 15.88. Found: C, 57.70; H, 6.21; N, 15.63.

Method B. A solution of *N*-protected prolyl derivative **10** (0.065 g, 0.144 mmol) in methanol (5 mL) containing Pd/C (10%) (40% wt/wt) was hydrogenated at 25 psi at room temperature for 2 h. The reaction mixture was filtered and the filtrate was evaporated to dryness under reduced pressure. The residue (*N*-deprotected prolyl intermediate) was dissolved in dry DMF (2 mL) and was successively reacted with Cbz-Val-Pro-Val-OH (0.098 g, 0.22 mmol), HATU (0.084 g, 0.22 mmol), HOAt (0.03 g, 0.22 mmol) and DIEA (30.1 μL, 0.22 mmol). The reaction mixture was stirred at room temperature for 2 h and an extra amount of HATU (0.084 g, 0.22 mmol), HOAt (0.03 g, 0.22 mmol) and DIEA (30.1 μL, 0.22 mmol) were added. The reaction mixture was stirred at room temperature overnight and the solvent was evaporated to dryness. The residue was dissolved in ethyl acetate (50 mL), washed with 10% aqueous citric acid (3 × 20 mL), 10% aqueous $NaHCO_3$ (3 × 20 mL), water (3 × 20 mL) and brine (3 × 20 mL). The organic layer was dried (Na_2SO_4), filtered and evaporated to dryness. The final residue was purified by

CCTLC on the Chromatotron (CH_2Cl_2/MeOH, 40:1) to afford **9** (10 mg, 9% overall yield from **10**) as a white foam.

*4.1.1.4. 9-[(2-Hydroxyethoxy)methyl]-2-N-[N-(bencyloxycarbonyl)-valyl-prolyl-valyl-prolyl]-guanine (**11**).* A solution of protected prodrug **9** (0.100 g, 0.126 mmol) was reacted with methanol saturated with ammonia (10 mL) at room temperature overnight and the solvent was evaporated to dryness. The residue was purified by CCTLC (CH_2Cl_2/MeOH, 20:1). The fastest moving fractions gave **11** (30 mg, 32%) as a white foam. ^{1}H NMR (400 MHz, DMSO-d_6): δ 0.80–0.93 (d, 12H, γ-CH_3, Val), 1.60–2.26 (m, 10H, β-CH, Val, β-CH_2, γ-CH_2, Pro), 3.24–3.72 (m, 8H, δ-CH_2, Pro, O$\underline{CH_2}$, $\underline{CH_2}$OCO), 4.00–4.22 (m, 2H, α-CH, Val), 4.49, 4.68 (2m, 2H, α-$\underline{CH}$, Pro), 4.93–5.17 (m, 2H, CH_2, Cbz), 5.48 (s, 2H, N$\underline{CH_2}$O), 7.09–7.46 (m, 6H, Ar, Cbz, NH), 7.39 (d, 1H, NH, Val, J = 8.3 Hz), 7.90 (d, 1H, NH, Val, J = 9.0 Hz), 8.14 (s, 1H, H-8), 11.92 (bs, 2H, NH-1, 2-NH). MS (ESI$^+$) 752.5 [M + H]$^+$. Anal. Calcd. for $C_{36}H_{49}N_9O_{10}$: C, 57.79; H, 6.50; N, 16.89. Found: C, 57.51; H, 6.57; N, 16.77.

From the slowest moving fractions of 2-*N*-[*N*-(bencyloxycarbonyl)-valyl-prolyl-valyl-prolyl]guanine (30 mg, 26%) were isolated due to partial rupture of the C–N bond of the acyclovir prodrug. ^{1}H NMR (400 MHz, DMSO-d_6): δ 0.80–0.95 (d, 12H, γ-CH_3, Val), 1.62–2.29 (m, 10H, β-CH, Val, β-CH_2, γ-CH_2, Pro), 3.20–3.78 (m, 4H, δ-CH_2, Pro), 4.12–4.26 (m, 2H, α-CH, Val), 4.51, 4.75 (2m, 2H, α-CH, Pro), 4.90–5.19 (m, 2H, CH_2, Cbz), 7.00–7.48 (m, 6H, Ar, Cbz, NH), 7.45 (d, 1H, NH, Val, J = 8.3 Hz), 7.95 (d, 1H, NH, Val, J = 9.0 Hz), 8.19 (s, 1H, H-8), 12.00 (bs, 2H, NH-1, 2-NH), 13.00 (bs, 1H, NH7/9). MS (ESI$^+$) 679.5 [M + H]$^+$. Anal. Calcd. for $C_{33}H_{43}N_9O_7$: C, 58.48; H, 6.40; N, 18.60. Found: C, 58.61; H, 6.57; N, 18.87.

*4.1.1.5. 9-[(2-Hydroxyethoxy)methyl]-2-N-[valyl-prolyl-valyl-prolyl] guanine (**3**).* A solution of the *N*-protected tetrapeptide prodrug derivative **11** (0.025 g, 0.033 mmol) in methanol (1 mL) containing Pd/C (10%) (40% wt/wt) was hydrogenated at 25 psi at room temperature for 2 h. The reaction mixture was filtered and the filtrate was evaporated to dryness under reduced pressure. The residue was dissolved in water and lyophilized to give the final deprotected conjugate **3** (17 mg, 83%) as a white foam. ^{1}H NMR (300 MHz, DMSO-d_6): δ 0.78–1.01 (m, 12H, 2γ-CH_3, Val_1 y Val_2), 1.70–2.15 (m, 10H, 2β-CH, Val_1 y Val_2, 2β-CH_2, Pro_1 y Pro_2, 2γ-CH_2, Pro_1 y Pro_2), 3.31–3.72 (m, 6H, 2δ-CH_2, Pro_1 y Pro_2, OCH_2), 3.93–4.26 (m, 4H, α-CH, Pro, α-CH, Val_2, $\underline{CH_2}$OH), 4.36–4.52 (m, 2H, 2α-CH, Val_1, α-CH, Pro), 5.38 (s, 2H, N$\underline{CH_2}$O), 6.67 (bs, 1H, NH-1), 7.99–8.05 (m, 4H, H-8, NH_3^+), 8.52 (m, 1H, NH, Val_2), 10.86 (bs, 1H, NH-2). EM (ESI$^+$): 619.2 [M + H]$^+$. Anal. Calcd. for $C_{28}H_{43}N_9O_7$: C, 54.44; H, 7.02; N, 20.41. Found: C, 54.77; H, 6.89; N, 20.24.

*4.1.1.6. 9-[2-((N-Bencyloxycarbonyl)valyl-prolyl-valyloxyethoxy) methyl]guanine (**14**).* To a solution of VACV (**13**) (0.10 g, 0.27 mmol) in dry DMF (2 mL), was successively added pyBOP (0.198 g, 0.38 mmol), Cbz-Val-Pro-OH (0.13 g, 0.38 mmol) and TEA (90.3 μL, 0.65 mmol). The reaction mixture was stirred at room temperature for 24 h and the solvent was evaporated to dryness. The residue was dissolved in ethyl acetate (50 mL) and washed with 10% aqueous citric acid (3 × 20 mL), 10% $NaHCO_3$ (3 × 20 mL), H_2O (3 × 20 mL) and brine (3 × 20 mL). The organic layer was dried (Na_2SO_4), filtered and evaporated to dryness. The final residue was purified by flash column chromatography (ethyl acetate/MeOH, 4:1) to afford **14** (150 mg, 81%) as a white foam. ^{1}H NMR (300 MHz, DMSO-d_6): δ 0.86 (d, 12H, γ-CH_3, Val), 1.73–2.01 (m, 6H, β-CH, Val, β-CH_2, γ-CH_2, Pro), 3.39–3.70 (m, 4H, O$\underline{CH_2}$, δ-CH_2, Pro), 4.02 (m, 1H, α-CH, Val), 4.10 (m, 1H, α-CH, Val), 4.20 (m, 2H, $\underline{CH_2}$OCO), 4.44 (m, 1H, α-CH, Pro), 5.01 (dd, 2H, CH_2, Cbz, J = 12.4 Hz, J = 14.9 Hz), 5.34 (bs, 2H, N$\underline{CH_2}$O), 6.49 (bs, 2H, 2-NH_2), 7.34 (m, 5H, Ar, Cbz), 7.42 (d, 1H, NH, Val, J = 8.3 Hz), 7.66 (s, 1H, H-8), 8.09 (d, 1H, NH, Val, J = 8.3 Hz), 10.61 (s, 1H, NH-1). MS (ESI$^+$) 655.3 [M + H]$^+$. Anal. Calcd. for $C_{31}H_{42}N_8O_8$: C, 56.87; H, 6.47; N, 17.11. Found: C, 56.96; H, 6.61; N, 17.23.

*4.1.1.7. 9-[2-(Valyl-prolyl-valyloxyethoxy)methyl]guanine (**4**).* A solution of **14** (0.08 g, 0.12 mmol) in MeOH:H_2O:THF (5:2:2 mL) containing Pd/C (10%) (0.04 g) was hydrogenated at 1 atm at room temperature for 3 h. The reaction mixture was filtered and the filtrate was washed with MeOH (20 mL) and was evaporated to dryness under reduced pressure to give **4** (40 mg, 67%) as a white foam. ^{1}H NMR (300 MHz, DMSO-d_6): δ 0.82–0.97 (d, 12H, γ-CH_3, Val), 1.73–2.04 (m, 6H, β-CH, Val, β-CH_2, γ-CH_2, Pro), 3.19–3.37–3.68 (m, 4H, O$\underline{CH_2}$, δ-CH_2, Pro), 4.09–4.54 (m, 5H, $\underline{CH_2}$OCO, 2α-CH, Val, α-CH, Pro), 5.34 (bs, 2H, N$\underline{CH_2}$O), 6.57 (bs, 2H, 2-NH_2), 7.80 (s, 1H, H-8), 8.07 (d, 1H, NH, Val, J = 8.4 Hz), 8.44 (d, 1H, NH, Val, J = 8.4 Hz), 10.61 (s, 1H, NH-1). MS (ESI$^+$) 521.3 [M + H]$^+$. Anal. Calcd. for $C_{23}H_{36}N_8O_6$: C, 53.06; H, 6.97; N, 21.52. Found: C, 52.89; H, 7.13; N, 21.64.

4.1.2. Solid-phase synthesis of acyclovir prodrugs

*4.1.2.1. DHP resin attachment of acyclovir (**20**).* A suspension of acyclovir (2.5 g, 11.25 mmol) in dry DMF (3 mL) was stirred at 80 °C to achieve maximum dissolution. Then, this suspension was transferred into a 5-ml glass tube containing DHP resin (2.5 g, 2.25 mmol) previously swollen in DCM/DMF/DCM/DMF (4 × 0.5 min) and pyridinium *p*-toluenesulfonate (PPTS) (1.13 g, 4.50 mmol) was added. The mixture was irradiated in a Biotage Initiator MW reactor for 8 h at 80 °C. Then, the reaction mixture was transferred to a polypropylene cartridge and the resin was washed with DMSO (3×), DMF (3×) and DCM (3×) and dried under reduced pressure for 24 h. The loading level of acyclovir onto the resin was determined according to the following procedure. Resin **20** (216 mg) was treated with 95:5 TFA/H_2O (2 mL) for 20 min and washed thoroughly with DMF/DCM (3×) and the combined filtrates concentrated under reduced pressure. In this way, 41 mg of acyclovir were recovered which corresponds to a resin loading of 0.843 mmol/g.

*4.1.2.2. Support bound 9-[(2-hydroxyethoxy)methyl]-2-N-[N^{α}-(9-fluorenylmethoxycarbonyl)-prolyl]guanine (**21**).* To a solid phase syringe, acyclovir on DHP-resin (**20**) (0.071 g, 0.056 mmol) was added. The resin was swollen with DCM, DMF, DCM and DMF (3 × 30 s per solvent). Then, Fmoc-Pro-OH (23 mg, 0.067 mmol), HCTU (28 mg, 0.067 mmol) and DIPEA (0.023 mL, 0.134 mmol) were added to the resin. The mixture was transferred to a microwave vial and irradiated in a Biotage Initiator MW reactor for 10 min at 40 °C. Then, the vial was opened, the supernatant removed and new coupling mixture added. This process was repeated 5 times in total (5 × 10 min). Finally, the Fmoc-Pro-acyclovir on DHP-resin **21** was transferred to a fritted syringe, drained and washed extensively (DMF/DCM/DMF/DCM, 5 × 0.5 min). To follow the progress of the reaction, a cleavage of a small portion of the resin 21 with TFA/H_2O (95:5 (v/v), 1 mL) for 20 min was carried out. The resin was thoroughly washed with DMF and DCM and the filtrates were evaporated to dryness. The product was lyophilized from CH_3CN/H_2O and analysed by HPLC-MS. In the chromatogram a 1.5:1 mixture of 16 (t_R = 13.03 min, 545.15 [M + H]$^+$, 1089.37 [2M + H]$^+$) and acyclovir (t_R = 1.32 min, 226.14 [M + H]$^+$) was detected.

*4.1.2.3. 9-[(2-Hydroxyethoxy)methyl]-2-N-[valyl-prolyl-valyl-prolyl] guanine (**3**).* To a solid phase syringe, Fmoc-Pro-acyclovir on DHP-resin **21** (0.21 mmol) was added. The resin was swollen with DCM, DMF, DCM and DMF (3 × 30 s per solvent). Then, Boc-Val-Pro-Val-OH (0.104 g, 0.252 mmol), HCTU (0.104 g, 0.252 mmol) and DIPEA (0.086 mL, 0.504 mmol) were added to the resin. The mixture was

transferred to a microwave vial and irradiated in a Biotage Initiator MW reactor for 10 min at 40° C. Then, the vial was opened, the supernatant was removed and new coupling mixture was added. This process was repeated 5 times in total (5 × 10 min). Finally, the compound was cleaved from the resin 26 with TFA/H_2O (95:5 (v/v), 3 mL) for 20 min. The resin was thoroughly washed with DMF and DCM and the filtrates were evaporated to dryness. The product was lyophilized from CH_3CN/H_2O and purified by reverse-phase chromatography on a *Biotage Isolera* instrument using a reverse phase column (KP-C18-HS 12 g) to give target prodrug **3** (25 mg, 20% overall yield). As mobile phase, mixtures of A:B were used, where A = 0.05% TFA water and B = acetonitrile with a flow rate of 7 mL/min. The prodrug **3** was purified using a gradient from 0% of B to 100% of B in 30–45 min and was detected at 254 nm. All the characterization data were identical to those of prodrug **3** obtained from the solution-phase strategy.

4.2. Water-solubility studies

Water-solubility of the prodrugs **3** and **4** and the parent compound was determined by HPLC analysis. The HPLC runs were carried out on a Waters 484 System using Novapack C18 reverse phase column. Flow rate: 1 mL/min. Detection: UV 254 nm. Gradient solvent system A/B (acetonitrile/water): initial 15% A + 85% B; 5 min linear gradient to 25% A + 75% B; 5 min linear gradient to 35% A + 65% B; 10 min linear gradient to 45% A + 55% B; 5 min linear gradient to 60% A + 40% B and 5 min linear gradient to 100% A. Excess amount of the prodrug or the parent drug was suspended in deionized water (pH = 5.5), sonicated for 10 min at room temperature, and then equilibrated overnight at room temperature. The samples were centrifuged at 14,000 rpm in an eppendorf microcentrifuge for 1.5 min at room temperature. An aliquot of the clear supernatant was removed and diluted to a concentration within the range of a five-point standard curve. Water-solubility was calculated from each peak area of the solution by HPLC analysis compared with the sample dissolved in dimethylsulfoxide as the standard, the exact concentration of which is known.

4.3. Biological methods. Compounds and enzymes

Human soluble CD26 was purified as described [27] or obtained from Sigma (St. Louis, MO). Fetal bovine serum (FBS) was obtained from Integro (Dieren, The Netherlands) and human serum was provided by the Blood Bank, Leuven, Belgium. Vildagliptin was custom synthesized by GLSynthesis Inc. (Worcester, MA, USA).

4.3.1. Conversion of the prodrugs of ACV to the corresponding parent compound

The test compounds have been evaluated for their substrate activity against purified CD26, human serum (HS), and bovine serum (BS) in Eppendorf tubes. The 400 μL reaction mixtures contained 50 μM test compound (amide and ester prodrugs of ACV **3** and **4**) in PBS (containing 0.1% DMSO). The reaction was started by the addition of purified CD26 (1.5 mU) or 20% (final concentration) of HS (in PBS) or 20% (final concentration) of BS (in PBS) at 37 °C. At different time points (as indicated in the figures) 100 μL was withdrawn from the reaction mixture, added to 200 μL of cold methanol, and put on ice for 10 min. Then the mixtures were centrifuged at 13,000 rpm for 5 min at 4 °C and 250 μL supernatant was analysed by HPLC on a reverse phase RP-8 column (excitation wavelength: 340 nm; envision wavelength: 415 nm), using following buffers and gradients. Buffer A: 50 mM NaH_2PO_4 + 5 mM heptanesulfonic acid, pH 3.2. Buffer B: acetonitrile. Gradient: 90% A + 10% B; 12 min linear gradient to 75% A + 25% B; 13 min linear gradient to 65% A + 35% B; 8 min linear gradient to 60% A + 40% B; 7 min linear gradient to 50% A + 50% B; 5 min 90% A + 10% B; 5 min equilibration at 90% A + 10% B.

Test prodrugs (25 μM) were exposed to CD26, 20% HS or 20% BS (as described above) in the presence or absence of different concentrations of the DPPIV/CD26 inhibitor Vildagliptin (ranging between 50 and 0.05 μM). Conversion of the prodrugs to the parent compounds was evaluated after 15, 30, 45 and 60 min, and analysed as described above.

4.3.2. Anti-HSV-1 and -HSV-2 activity assay

The ACV prodrugs were evaluated against herpes simplex virus type 1 (HSV-1) strain KOS, and herpes simplex virus type 2 (HSV-2) strain G. The antiviral assay was based on inhibition of virus-induced cytopathicity in human embryonic lung (HEL) fibroblasts. Confluent cell cultures in microtiter 96-well plates were inoculated with 100 $CCID_{50}$ of virus (1 $CCID_{50}$ being the virus dose to infect 50% of the cell cultures) in the presence of varying concentrations of the test compounds. Viral cytopathicity was recorded as soon as it reached completion in the control virus-infected cell cultures that were not treated with the test compounds. Antiviral activity was expressed as the EC_{50} or compound concentration required to reduce virus-induced cytopathicity by 50%.

Acknowledgements

We thank Ria Van Berwaer and Leentje Persoons for excellent technical assistance. We also thank the Spanish MEC/MICINN (Project SAF2009-13914-C02 and SAF2012-39760-C02), the Comunidad de Madrid (BIPEDD-2-CM ref S-2010/BMD-2457), and the KU Leuven (GOA No. 10/014) for financial support. A Juan de la Cierva contract to S.d.C. (JDC-MICINN) from the Spanish Ministry of Science and Innovation is also gratefully acknowledged. We very much appreciated the helpful discussions with Dr. Judit Tulla-Puche and Prof. Fernando Albericio (IRB Barcelona, Spain).

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2013.10.001.

References

[1] D.A. Fox, R.E. Hussey, K.A. Fitzgerald, O. Acuto, C. Poole, L. Palley, J.F. Daley, S.F. Schossman, E.L. Reinhert, Ta1, a novel 105 kDa human T cell activation antigen defined by a monoclonal antibody, J. Immunol. 133 (1984) 1250–1256.
[2] I. De Meester, G. Vanhoof, A.-M. Lambeir, S. Scharpe, CD26, let it cut or cut it down, Immunol. Today 20 (1999) 367–375.
[3] A.-M. Lambeir, C. Durinx, S. Scharpe, I. De Meester, Dipeptidyl-peptidase IV from bench to bedside: an update on structural properties, functions and clinical aspects of the enzyme DPPIV, Crit. Rev. Clin. Lab. Sci. 40 (2003) 209–294.
[4] A. Yaron, F. Naider, Proline-dependent structural and biological properties of peptides and proteins, Crit. Rev. Biochem. Mol. 28 (1993) 31–81.
[5] R. Mentlein, Dipeptidyl-peptidase IV (CD26)-role in the inactivation of regulatory peptides, Regul. Pept. 85 (1999) 9–24.
[6] R. Mentlein, Cell-surface peptidases, Int. Rev. Cytol. 235 (2004) 165–202.
[7] J. Balzarini, E. Andersson, D. Schols, P. Proost, J. Van Damme, B. Svennerholm, P. Horal, A. Vahlne, Obligatory involvement of CD26/dipeptidyl peptidase IV in the activation of the antiretroviral tripeptide glycylprolylglycinamide (GPG-NH_2), Int. J. Biochem. Cell Biol. 36 (2004) 1848–1859.
[8] C. García-Aparicio, M.-C. Bonache, I. De Meester, A. San-Félix, J. Balzarini, M.-J. Camarasa, S. Velázquez, Design and discovery of a novel dipeptidyl-peptidase IV (CD26)-based prodrug approach, J. Med. Chem. 49 (2006) 5339–5351.
[9] G. Vanhoof, F. Goossens, I. De Meester, D. Hendriks, S. Scharpé, Proline motifs in peptides and their biological processing, FASEB J. 9 (1995) 736–744.
[10] (a) J. Balzarini, M.J. Pérez-Pérez, A. San-Félix, D. Schols, C.F. Perno, A.M. Vandamme, M.J. Camarasa, E. De Clercq, 2′,5′-Bis-*O*-(*tert*-butyldimethylsilyl)-3′-spiro-5″-(4″-amino-1″,2″-oxathiole-2″,2″-dioxide)pyrimidine

(TSAO) nucleoside analogues: highly selective inhibitors of human immunodeficiency virus type 1 that are targeted at the viral reverse transcriptase, Proc. Natl. Acad. Sci. U. S. A. 89 (1992) 4392–4396;
(b) M.J. Camarasa, M.J. Pérez-Pérez, A. San-Félix, J. Balzarini, E. De Clercq, 3′-Spironucleosides (TSAO derivatives), a new class of specific human immunodeficiency virus type 1 inhibitors: synthesis and antiviral activity of 3'-spiro-5″-[4″-amino-1″,2″-oxathiole-2″,2″-dioxide]pyrimidine nucleosides, J. Med. Chem. 35 (1992) 2721–2727.

[11] M.J. Camarasa, A. San-Félix, S. Velázquez, M.J. Pérez-Pérez, F. Gago, J. Balzarini, TSAO compounds: the comprehensive story of a unique family of HIV-1 specific inhibitors of reverse transcriptase, Curr. Top. Med. Chem. 4 (2004) 945–963.

[12] A. Diez-Torrubia, C. García-Aparicio, S. Cabrera, I. De Meester, J. Balzarini, M.J. Camarasa, S. Velázquez, Application of the dipeptidyl-peptidase IV (DPPIV/CD26)-based prodrug approach to different amine-containing drugs, J. Med. Chem. 53 (2010) 559–572.

[13] C. García-Aparicio, A. Diez-Torrubia, J. Balzarini, A.-M. Lambeir, S. Velázquez, M.J. Camarasa, Efficient conversion of tetrapeptide-based TSAO prodrugs to the parent drug by dipeptidyl-peptidase IV (DPPIV/CD26), Antivir. Res. 76 (2007) 130–139.

[14] A. Diez-Torrubia, J. Balzarini, G. Andrei, R. Snoeck, I. De Meester, M.J. Camarasa, S. Velázquez, Dipeptidyl-peptidase IV-dependent water-soluble prodrugs of highly lipophilic bicyclic nucleoside analogues, J. Med. Chem. 54 (2011) 1927–1942.

[15] A. Diez-Torrubia, S. Cabrera, A.-M. Lambeir, J. Balzarini, M.J. Camarasa, S. Velázquez, Dipeptidyl peptidase IV (DPPIV/CD26)-based prodrugs of hydroxy-containing drugs, ChemMedChem 7 (2012) 618–628.

[16] A. Diez-Torrubia, S. Cabrera, I. De Meester, M.J. Camarasa, J. Balzarini, S. Velázquez, Dipeptidyl-peptidase IV-activated prodrugs of anti-varicella zoster virus bicyclic nucleoside analogues containing different self-cleavage spacers systems, ChemMedChem 7 (2012) 1612–1622.

[17] H.J. Schaeffer, L. Beauchamp, P. De Miranda, G.B. Elion, D.J. Bauer, P. Collins, 9-(2-Hydroxyethoxymethyl)guanine activity against viruses of the herpes group, Nature 272 (1978) 583–585.

[18] P. de Miranda, M.R. Blum, Pharmacokinetics of acyclovir after intravenous and oral administration, J. Antimicrob. Chemother. 12 (Suppl. B) (1983) 29–37.

[19] E. De Clercq, H.J. Field, Antiviral prodrugs-the development of successful prodrug strategies for antiviral chemotherapy, Br. J. Pharmacol. 147 (2006) 1–11.

[20] H. Gao, A.K. Mitra, Synthesis of acyclovir, ganciclovir and their prodrugs: a review, Synthesis-Stuttgart 3 (2000) 329–351.

[21] R. Karaman, K.K. Dajani, A. Qtait, M. Khamis, Prodrugs of acyclovir-a computational approach, Chem. Biol. Drug Des. 79 (2012) 819–834 and references therein.

[22] (a) L. Colla, E. De Clercq, R. Busson, H. Vanderhaeghe, Synthesis and antiviral activity of water-soluble esters of acyclovir [9-[(2-hydroxyethoxy)methyl] guanine], J. Med. Chem. 26 (1983) 602–604;
(b) L.M. Beauchamp, T.A. Krenitsky, Acyclovir prodrugs: the road to valacyclovir, Drugs Future 18 (1993) 619–628.

[23] K.J. Vigil, R.F. Chemaly, Valacyclovir: approved and off-label uses for the treatment of herpes virus infections in immunocompetent and immunocompromised adults, Expert Opin. Pharmacother. 11 (2010) 1901–1913 and references therein.

[24] I. Kim, X. Chu, S. Kim, C.J. Provoda, K. Lee, G.L. Amidon, Identification of a human valacyclovirase: biphenyl hydrolase-like protein as valacyclovir hydrolase, J. Biol. Chem. 278 (2003) 25348–25356.

[25] S. Weller, M.R. Blum, M. Doucette, T. Burnette, D.M. Cederberg, P. de Miranda, M.L. Smiley, Pharmacokinetics of the acyclovir pro-drug valaciclovir after escalating single-and multiple-dose administration to normal volunteers, Clin. Pharmacol. Ther. 54 (1993) 595–605.

[26] (a) Y.E. Nashed, A.K. Mitra, Synthesis and characterization of novel dipeptide ester prodrugs of acyclovir, Spectrochim. Acta Part A 59 (2003) 2033–2039;
(b) R.S. Talluri, S.K. Samanta, R. Gaudana, A.K. Mitra, Synthesis, metabolism and cellular permeability of enzymatically stable dipeptide prodrugs of acyclovir, Int. J. Pharm. 361 (2008) 118–124.

[27] C.R. Santos, R. Capela, C.S.G.P. Pereira, E. Valente, L. Gouveia, C. Pannecouque, E. De Clercq, R. Moreira, P. Gomes, Structure–activity relationships for dipeptide prodrugs of acyclovir: Implications for prodrug design, Eur. J. Med. Chem. 44 (2009) 2339–2346 and references therein.

[28] G. Hristov, I. Stankova, Chemical stability of new acyclovir analogues with peptidomimetics, Sci. Pharm. 79 (2011) 259–264.

[29] H. Gao, A.K. Mitra, Regioselective synthesis of acyclovir and its various prodrugs, Synth. Commun. 31 (2001) 1399–1419.

[30] X. Li, Q. Wu, D. Lv, X. Lin, Controllable synthesis of polymerizable ester and amide prodrugs of acyclovir by enzyme in organic solvent, Bioorg. Med. Chem. 14 (2006) 3377–3382.

[31] P. Herdewijn, R. Charubala, E. De Clercq, W. Pfleiderer, Nucleotides. Part. XXXII. Synthesis of 2′–5′ Connected oligonucleotides. Prodrugs for antiviral and antitumoral nucleosides, Helv. Chim. Acta 72 (1989) 1739–1748.

[32] L.A. Carpino, A. El-Faham, Tetramethylfluoroformamidinium hexafluorophosphate: a rapid-Acting peptide coupling reagent for solution and solid phase, J. Am. Chem. Soc. 117 (1995) 5401–5402.

[33] (a) E. Giralt, R. Eritja, J. Sosa, C. Kuklinski, E. Pedroso, The use of Nbb resin in cyclic dipeptide ("diketopiperazine") synthesis, Synthesis (1985) 181–184;
(b) E. Pedroso, A. Grandas, X. de las Heras, R. Eritja, E. Giralt, Diketopiperazine formation in solid phase peptide synthesis using *p*-alkoxybenzyl ester resins and Fmoc-amino acids, Tetrahedron Lett. 27 (1986) 743–746.

[34] F. García-Martín, M. Quintanar-Audelo, Y. García-Ramos, L.J. Cruz, C. Gravel, R. Furic, S. Côté, J. Tulla-Puche, F. Albericio, ChemMatrix, a poly(ethylene glycol)-based support for the solid-phase synthesis of Complex peptides, J. Comb. Chem. 8 (2006) 213–220.

[35] H. Rosemeyer, F. Seela, Polymer-linked acycloguanosine, Makromol. Chem. 185 (1984) 687–695.

[36] L.A. Thompson, J.A. Ellman, Straightforward and general method for coupling alcohols to solid supports, Tetrahedron Lett. 35 (1994) 9333–9336.