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Original article

Structure–activity relationships for dipeptide prodrugs of acyclovir: Implications for prodrug design

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ABSTRACT

A series of water-soluble dipeptide ester prodrugs of the antiviral acyclovir (ACV) were evaluated for their chemical stability, cytotoxicity, and antiviral activity against several strains of Herpes Simplex-1 and -2, vaccinia, vesicular stomatitis, cytomegalovirus and varicella zoster viruses. ACV dipeptide esters were very active against herpetic viruses, independently of the rate at which they liberate the parent drug. Their minimum cytotoxic concentrations were above 100 μM and the resulting MCC/EC_{50} values were lower than those of ACV. When comparing the reactivity of Phe-Gly esters and amides (ACV, zidovudine, paracetamol, captopril and primaquine) in pH 7.4 buffer it was found that the rate of drug release increases with drug's leaving group ability. Release of the parent drug from Phe-Gly in human plasma is markedly faster than in pH 7.4 buffer, thus suggesting that the dipeptide-based prodrug approach can be successfully applied to bioactive agents containing thiol, phenol and amine functional groups.

1. Introduction

Herpetic viruses (HV) include about 130 different species that are widespread in nature and are responsible for a large number of viral diseases affecting humans. Generally, HV have the ability to not only cause disease when they are active, but also remain silent in a latent state over long periods until they are reactivated. The *Herpetoviridae* family comprises 25 viral subfamilies of which 8 affect humans with some regularity: Herpes Simplex-1 (HSV-1), Herpes Simplex-2 (HSV-2), Cytomegalovirus (CMV), Varicella Zoster Virus (VZV) and, less frequently, Vesicular Stomatitis Virus (VSV), Epstein–Barr Virus (EBV), Human Herpes Virus-6 (HHV-6) and Human Herpes Virus-8 (HHV-8) [1]. Moreover, a significant percentage of patients with acquired immunodeficiency syndrome (AIDS) develop slight–fatal infections that are caused by HV like CMV, VZV, HSV-1 and HSV-2 [2]. Great progress in the field of anti-HV therapy has been brought about by acyclovir (ACV, 9-[(2-hydroxyethoxy)methyl]guanine; **1**, Scheme 1), whose mechanism of action involves its conversion into the corresponding *O*-triphosphate (ACV-3P), promoted by the viral enzyme thymidine kinase (TK), and then ACV-3P acts by both competitive inhibition of the viral DNA polymerase and incorporation in, and subsequent chain termination of the growing viral DNA strand [3]. However, ACV is only active against HV belonging to the α-subfamily, as those from the β-subfamily, such as CMV, produce a different TK that fails to phosphorylate the drug [4]. This obstacle can be surpassed by the use of gancyclovir (GCV) that is phosphorylated by TKs from both subfamilies, but GCV is less safe than ACV as it is more prone to incorporation into the host's cell DNA [4].

ACV-based therapies have some serious drawbacks imposed by both the drug's limited solubility in water [5] and its low oral and intravenous bioavailabilities [6]. This led to the development of ACV analogues [7,8] as well as to a number of potential ACV prodrugs, most of them based on amino acid and dipeptide esters of ACV [2,5,6,9–11]. From a clinical viewpoint, this research so far culminated in the L-valyl ester prodrug of ACV (Valacyclovir, $R^1 = CHMe_2$ in **2**, Scheme 1). The key role of amino acid and dipeptide-based esters as prodrugs for ACV and structurally similar drugs (gancyclovir, saquinavir) is mainly due to the considerably higher water-solubility of those esters at physiological pH and especially due to their excellent transepithelial and transcorneal permeabilities mediated by, respectively, the intestinal and the corneal hPEPT1 oligopeptide transporters [11–17], which are responsible for improved oral pharmacokinetics in rats [18] and ocular pharmacokinetics in rabbits [19].

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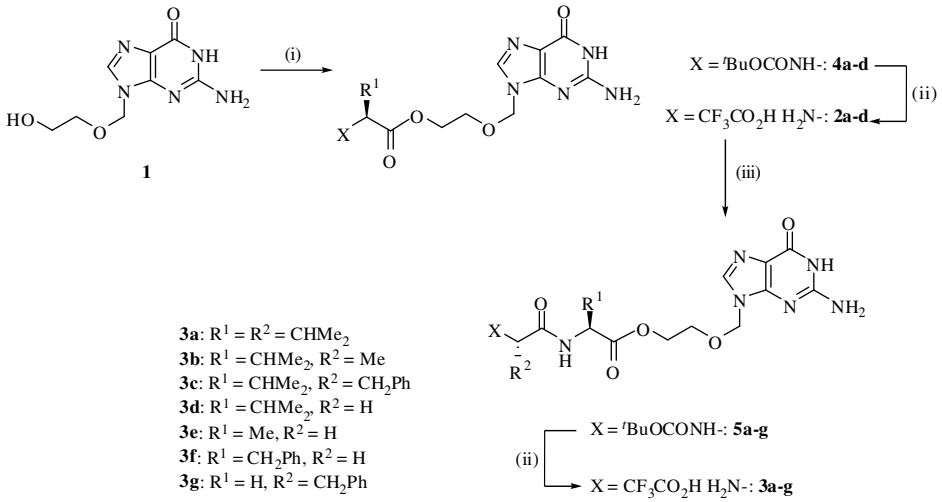


Scheme 1. Synthesis of ACV *O*-dipeptide esters **3a**–**g**: (i) BocAA[1]OH (1 mol eq), DCCI (1 +1 mol eq), DMAP (0.1 mol eq), pyr/DCM, 0 °C (2 h) → r.t. (48 h); (ii) neat TFA, DCM, r.t., 30 min; (iii) BocAA[2]OH (1 mol eq), DCCI (1 +1 mol eq), DMAP (0.1 mol eq), TEA (1.2 mol eq), DCM, 0 °C (2 h) → r.t. (48 h).

To the best of our knowledge a comprehensive study on the reactivity of ACV dipeptide esters in aqueous solution and antiviral efficacy against several relevant species of HV has not been reported so far. Recent studies have been focused on amino acid and dipeptide esters of gancyclovir (GCV) [20,21] rather than on acyclovir. We now report the synthesis of several dipeptide esters of ACV (**3a**–**g**, Scheme 1) and the study of their reactivity in aqueous media. Prodrugs **3a** (Val-Val-ACV) and **3d** (Gly-Val-ACV) have been explored by Mitra and co-workers also in terms of their chemical reactivity, so they were included in the present work as reference compounds. The in vitro cytotoxicity and antiviral activity of compounds **3a**–**g** were also evaluated against different strains of HSV-1, HSV-2, CMV, VZV, VSV and vaccinia virus (VV), including thymidine kinase-deficient (TK^-) mutants of HSV-1 and VZV, as well as reference anti-herpetic drugs such as the parent ACV, GCV, brivudin, ribavirin and cidofovir. Finally, in order to assess the applicability of the dipeptide-based prodrug approach to other functional groups other than alcohols such as ACV, we studied the chemical and plasma activation of Phe-Gly derivatives of captopril (i.e. a thiol prodrug) and primaquine (i.e. an amine prodrug).

2. Results and discussion

2.1. Chemistry

The synthetic pathway to the ACV prodrugs is illustrated in Scheme 1 and is based on the stepwise introduction of the amino acid residues, with slight changes with respect to a previously reported methodology for the synthesis of these compounds [10]. Briefly, ACV was firstly suspended in pyridine (pyr) and then reacted with appropriate N^{α}-Boc-protected amino acid (BocAA[1]OH), *N*,*N*′-dicyclohexylcarbodiimide (DCCI) as coupling agent and 4-(*N*,*N*-dimethyl)aminopyridine (DMAP) as auxiliary nucleophile, all dissolved in dichloromethane (DCM). The corresponding N^{α}-Boc-protected amino acid esters (**4a**–**d**) were isolated, Boc removed with neat trifluoroacetic acid (TFA) and the resulting trifluoroacetates (**2a**–**d**) were then reacted with the second N^{α}-Boc-protected amino acid (BocAA[2]OH) in the presence of triethylamine (TEA) to yield the protected dipeptide esters **5a**–**g** that were converted to the final products (**3a**–**g**; Scheme 1) again with neat TFA. The structures of the target compounds were all confirmed by electrospray ionization mass spectrometry (ESI-MS), proton (^{1}H) and carbon (^{13}C) nuclear magnetic resonances (NMR). Spectral data of known compounds (**3a**, **3d**) were identical to those previously reported [10] and those of the novel compounds (**3b**,**c** and **3e**–**g**) are given in Section 4.

2.2. Kinetics and products of hydrolysis

The ACV esters **3a**–**g** were incubated in pH 7.4 phosphate isotonic buffer at 37 °C and the reaction progress monitored by HPLC. The pseudo-first order rate constants, k_{obs}, thus determined are presented in Table 1. HPLC quantitative product analysis showed that degradation of compound **3** leads to quantitative formation of ACV. For the dipeptide ester Phe-Gly-ACV, **3g**, product analysis (Fig. 1A) revealed the formation of diketopiperazine cyclo(Phe-Gly) (**6** in Scheme 2), which is consistent with the expected intramolecular acyl transfer reaction (c.f., pathway ***a***, Scheme 2) that has been reported as the major degradation pathway for simple dipeptide alkyl and aryl esters [22–24]. However, it is clear from Fig. 1A that cyclo(Phe-Gly) does not account for all degradation of **3g**, suggesting that direct ester hydrolysis is also operating as a drug releasing pathway (c.f., pathway ***b***, Scheme 2). This is consistent with the identification of Phe-Gly as a minor degradation product of **3g** using a different HPLC method. Thus, the overall rate constant k_{obs} can be expressed by:

$$k_{obs} = k_1 + k_2 \quad (1)$$

where k_1 and k_2 are the first-order rate constants for the cyclization process and the hydrolysis of the ester group, respectively. The rate constants were obtained from the best computer fit to the experimental data of the parallel first-order reaction model depicted in Scheme 2 and represented by Eqs. (2)–(4).

Table 1
Log *P* values and pseudo-first order rate constants for degradation of dipeptide prodrugs **3a**–**g** in pH 7.4 phosphate buffer at 37 °C

Compound	R[1a]	R[2a]	log *P*[b]	$10^5 k_{obs}$ (s^{-1})
3a, Val-Val-ACV	$CHMe_2$	$CHMe_2$	−0.51	0.144
3b, Ala-Val-ACV	Me	$CHMe_2$	−1.08	0.394
3c, Phe-Val-ACV	CH_2Ph	$CHMe_2$	0.04	0.154
3d, Gly-Val-ACV	H	$CHMe_2$	−1.29	0.547
3e, Gly-Ala-ACV	H	Me	−1.71	19.1
3f, Gly-Phe-ACV	H	CH_2Ph	−0.57	5.86
3g, Phe-Gly-ACV	CH_2Ph	H	−0.52	5.10

[a] Side chains in compounds **3a**–**g**, c.f. Scheme 1.
[b] Obtained using ALOGPS 2.1 (available at VCCLAB, Virtual Computational Chemistry Laboratory, http://www.vcclab.org); experimental log *P* for ACV is −1.56.

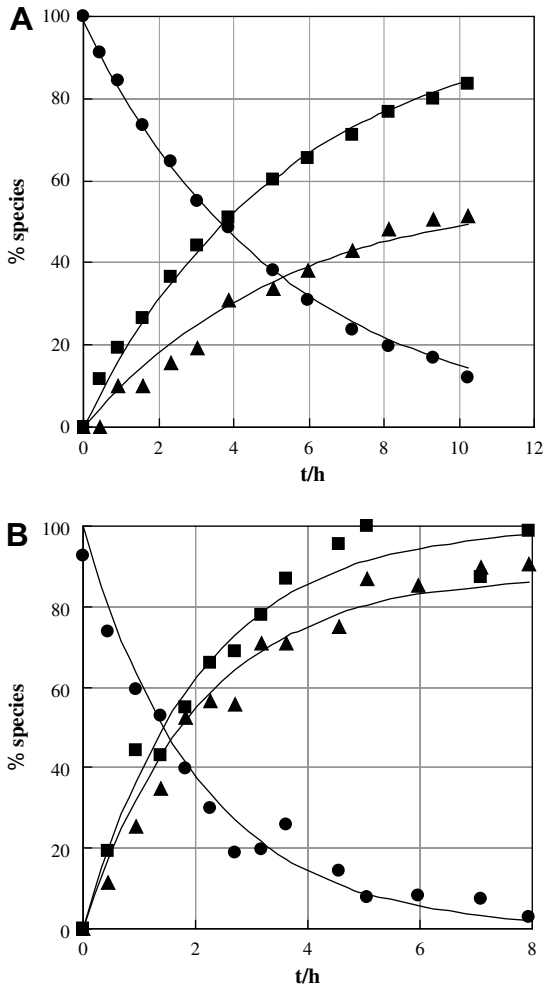
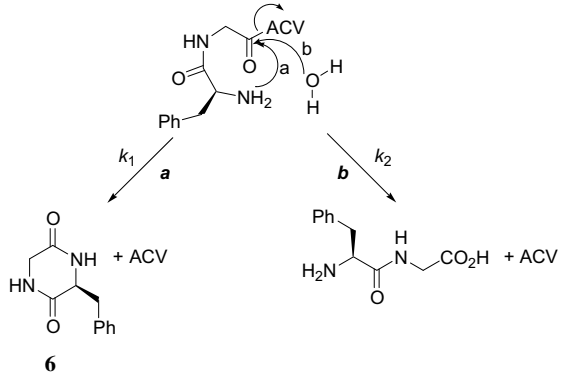


Fig. 1. Product analysis for the degradation of Phe-Gly-ACV, **3g**, and Phe-Gly-AZT in pH 7.4 buffer at 37 °C. (A): (●) **3g**; (■) ACV; (▲) cyclo(PheGly); (B): (●) **8**; (■) AZT; (▲) cyclo(PheGly).

$$[3g] = [3g]_0 e^{-(k_1+k_2)t} \quad (2)$$

$$[ACV] = [3g]_0\left(1 - e^{-(k_1+k_2)t}\right) \quad (3)$$

$$[6] = [3g]_0 \frac{k_2}{k_1+k_2}\left(1 - e^{-(k_1+k_2)t}\right) \quad (4)$$



Scheme 2. ACV release through intramolecular cyclization and ester hydrolysis of dipeptide prodrug **3g** in pH 7.4 buffer.

The good agreement between the overall rate constant (5.31×10^{-5} s^{-1}) calculated by the sum of the obtained k_1 (3.10×10^{-5} s^{-1}) and k_2 (2.21×10^{-5} s^{-1}), and k_{obs} (5.10×10^{-5} s^{-1}) determined from the slope of the linear plot of the logarithm of intact ester **3g** against time suggests that the degradation pathway presented in Scheme 2 adequately describes the degradation kinetics of dipeptide esters of **3**.

2.3. Effect of carrier structure on reactivity

From the data presented in Table 1 one can conclude that the rate of ACV release from **3** depends on the size of the side chains of the peptide carrier. This is further confirmed by the good correlation between log k_{obs} values and the sum of the Charton parameter, ν, for R^1 and R^2 substituents of each amino acid residue (Fig. 2). Slope of −1.8 indicates that the rate of degradation is significantly reduced when sterically hindered amino acids are incorporated into the dipeptide backbone. This effect is more pronounced when bulky β-branched amino acids (i.e. Val) are present as C-terminal residues, i.e. directly linked to the drug. For example, the order of k_{obs} for the Gly-AA^1-ACV series is Gly-Ala, **3e** > Gly-Phe, **3f** ≫ Gly-Val, **3d**, with **3d** being ca. 35 times less reactive than **3e**. This is consistent with the findings on similar dipeptide ester prodrugs of zidovudine (AZT), with Gly-Val-AZT being ca. 35 times less reactive than its Gly-Gly counterpart in pH 7.4 buffer [25]. In what concerns the role of the N-terminal residue on drug release rate, the data for the AA^2-Val-ACV series presented in Table 1 show that a similar trend exists, i.e. the bulkier (especially, β-branched) the N-terminal amino acid side chain, the slower is ACV release, but such effect is now more discrete, as Gly-Val-ACV, **3d**, is only 4 times more reactive than Val-Val-ACV, **3a**. Again, the same difference in reactivity was recently observed between the Gly-Val and Val-Val dipeptide esters of AZT and GCV at pH 7.4 [25,26] and saquinavir at pH 7.2 [27]. Overall, these results suggest that the degradation of dipeptide esters of ACV in pH 7.4 buffer is particularly sensitive to the size of the amino acid present at the C-terminus [28].

2.4. In vitro antiviral activity and cytotoxicity of dipeptide esters of acyclovir

Compounds **3a**–**g** were evaluated for inhibition of the cytopathic effects in human embryonic lung (HEL) cells of HSV-1 (KOS, TK$^-$ KOS

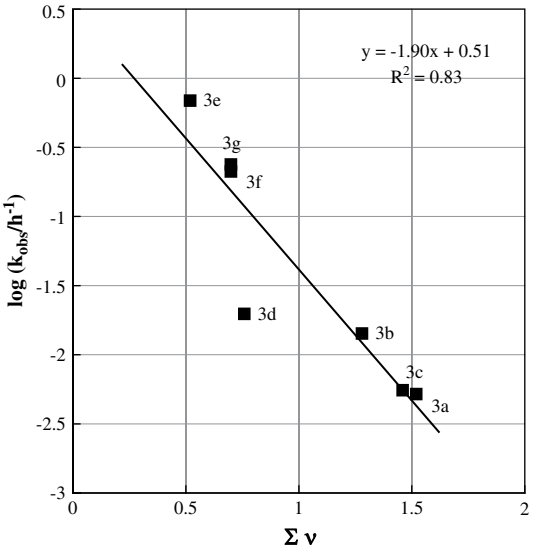


Fig. 2. Taft plot for the decomposition of dipeptide esters of acyclovir, **3**, in pH 7.4 buffer at 37 °C.

ACV^r, F and McIntyre strains), HSV-2 (G, 196 and Lyons strains), CMV (AD-169 and Davis strains), VZV (TK$^+$ OKA and TK$^-$ 07/1 strains), VV and VSV. Compound-induced cytotoxicity was also measured in HEL cells parallel to antiviral activity. Results are presented in Tables 2 and 3 and reveal the following interesting features. First, the scope of activity of dipeptide esters **3a**–**g** is similar to that of the parent drug: both ACV and its dipeptide esters were active against all HSV-1, HSV-2 and VZV strains, except those that are TK-deficient (TK$^-$), and inactive against VV, VSV and CMV. Second, *all* compounds **3** are more active than ACV against HSV-1 KOS, HSV-1 F, HSV-2G and HSV-2 Lyons strains, as the MICs for esters **3a**–**g** are ca 2–15 times lower than those of the parent drug. Third, compound **3g** is the only dipeptide derivative more active than ACV against the VZV TK$^+$ OKA strain (EC_{50} ACV $\approx 5 \times EC_{50}$, **3g**). Fourth, compounds of **3** are not cytotoxic against HEL cells, displaying MCC/EC_{50} values lower than that of the parent drug. Overall, these results suggest that chemical reactivity is not the only factor affecting the activity profiles of prodrugs **3a**–**g**, as their MCC/EC_{50} values do not correlate with the corresponding k_{obs} values. This is a somewhat surprising observation since the majority of these compounds release the parent drug with half-lives that fall within the time-frame of the biological assays. The exceptions are Val-Val-ACV, **3a**, and Phe-Val-ACV, **3c**, which present $t_{1/2}$ values higher than 5 days. Previous findings by Mitra and co-workers show that **3a** does represent a marked improvement on the pharmacokinetics and on the in vivo oral efficacy of the active drug [11,18,27]. In this context, Phe-Val-ACV, **3c**, emerges as an interesting candidate as (i) it presents improved lipophilicity when compared to ACV and **3a** (Table 1) and (ii) the corresponding MCC/EC_{50} values are lower than those of ACV and similar to those of Val-Val-ACV, **3a**.

2.5. Effect of the drug's leaving group on the reactivity of dipeptide prodrugs

Dipeptide-based prodrugs are receiving increasing attention as a strategy to target peptide membrane transporters. In most cases, the drug functional group coupled to the dipeptide is an alcohol, as in ACV (e.g. compounds **3**), zidovudine (AZT) or GCV. However, it is of obvious importance to predict the relative rates of degradation for dipeptide derivatives based on other drug functionalities such as thiols and amines. In order to evaluate the effect of the nature of the drug functional group bound to the dipeptide carrier on the chemical and enzymatic reactivities of the resulting prodrug, the kinetic data for the degradation of Phe-Gly-ACV, **3g**, in pH 7.4 buffer and human plasma were compared to those of Phe-Gly derivatives of paracetamol, **7**, AZT, **8**, captopril methyl ester, **9**, and primaquine (PQ), **10**, counterparts (Table 4).

The pseudo-first order rate constants, k_{obs}, for the degradation of compounds **7**–**9** in pH 7.4 phosphate buffer are presented in Table 4. Compound **10** was stable in these conditions, with no degradation detected when incubated for 5 days. Inspection of data presented in Table 4 shows that the most reactive compound is the thiol ester **9**, which degrades ca. 2 times more rapidly than phenol ester **7** and ca. 5–10 times more rapidly than AZT, **8**, and ACV, **3g**, esters, respectively. This result suggests that the rate of prodrug decomposition, k_{obs}, increases with the drug's leaving group ability. For the paracetamol and captopril methyl ester derivatives, **7** and **9**, respectively, the formation of cyclo(Phe-Gly) and corresponding parent drug was quantitative. In contrast, degradation of **8** did not lead to quantitative formation of cyclo(Phe-Gly), indicating that direct ester hydrolysis is also operating as a drug releasing pathway (c.f., pathway ***b***, Scheme 2). However, the cyclization pathway for **8** to form cyclo(Phe-Gly) is much more efficient ($k_1 = 1.18 \times 10^{-4}$ s^{-1}; $k_2 = 1.62 \times 10^{-5}$ s^{-1}; Table 4; Fig. 1B) when compared to that of Phe-Gly-ACV, **3g** ($k_1/k_2 = 7.3$ for **8** versus $k_1/k_2 = 1.4$ for **3g**). A possible explanation for this observation is that the less sterically hindered alcohol moiety of ACV favours ester hydrolysis.

Compounds **3g**, **7**–**10** were incubated in 80% human plasma and their degradation monitored by HPLC. The resulting first-order rate constants, k_{obs}, presented in Table 4, show that these prodrugs are markedly more susceptible to plasma hydrolysis than to chemical degradation in pH 7.4 buffer. Quantitative analysis of incubation mixtures by HPLC revealed that dipeptide esters **3g**, **8**, **7** and thiol ester **9** released directly the corresponding parent drug and Phe-Gly, while dipeptide amide **10** hydrolyzed with formation of the corresponding amino acid amide, Gly-PQ, as a reaction intermediate. These results are consistent with enzymatic degradation of dipeptide prodrugs occurring via (i) stepwise removal of the amino acid residues and (ii) direct cleavage of the dipeptide carrier (Scheme 3), depending on the nature of drug's leaving group. Examples of product analysis are presented in Fig. 3 for the hydrolysis of Phe-Gly-ACV, **3g**, and Phe-Gly-PQ, **10**, where the solid lines represent the best computer fit to the experimental data of the model derived from Scheme 3 and represented by Eqs. (5)–(7).

$$C_{1,t} = C_{1,0} e^{-(k_{12}+k_{13})t} \tag{5}$$

$$C_{2,t} = C_{1,0} \frac{k_{12}}{k_{23} - k_{12} - k_{13}} \left(e^{-(k_{12}+k_{13})t} - e^{-k_{23}t} \right) \tag{6}$$

$$C_{3,t} = C_{1,0} \left[\left(\frac{k_{12}k_{23} - k_{13}(k_{12} + k_{13} - k_{23})}{k_{12} + k_{13}} e^{-(k_{12}+k_{13})t} - k_{12} e^{-k_{23}t} \right) \Big/ (k_{12} + k_{13} - k_{23}) + \frac{k_{12}k_{23} + k_{13}k_{23}}{(k_{12} + k_{13})k_{23}} \right] \tag{7}$$

Table 2
Activity (MIC) of compounds **3a**–**g**, ACV and other control drugs against HSV-1, HSV-2, VV and VSV

Compound	Minimum inhibitory concentration (MIC)[a] (μM)								
	HSV-1 KOS	HSV-1 TK$^-$ KOS ACV^r	HSV-1 F	HSV-1 McIntyre	HSV-2G	HSV-2 196	HSV-2 Lyons	VV	VSV
3a	2.9	>100	0.96	2.9	1.3	6.4	0.96	>200	>200
3b	1.3	>100	0.32	0.96	1.3	2.9	1.3	>200	>200
3c	2.9	>100	0.64	2.9	1.6	4.5	1.3	>200	>200
3d	2.9	>100	0.64	0.64	1.3	1.3	0.96	>200	>200
3e	0.96	>100	0.96	0.96	0.96	1.3	0.96	>200	>200
3f	0.96	>100	0.96	0.96	4.5	1.3	0.64	>200	>200
3g	0.80	>100	0.80	0.80	0.48	0.48	0.80	>100	>200
ACV	8.2	>200	2.2	1.4	6.8	6.8	6.2	>1000	>1000
GCV	0.38	6.3	0.38	0.35	0.63	1.2	0.38	>300	>300
Brivudin	0.26	>600	0.24	0.051	>1000	>1000	>1000	>100	>1000
Ribavirin	>1000	>1000	>1000	>500	>1000	>1000	>1000	>1000	>1000

[a] Required to reduce virus-induced cytopathogenicity by 50% in HEL cell culture (average values from two independent assays).

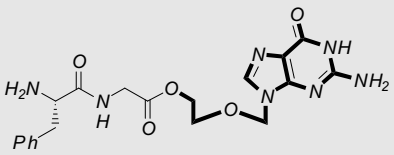
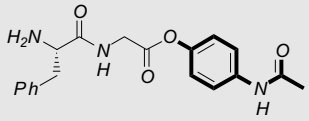
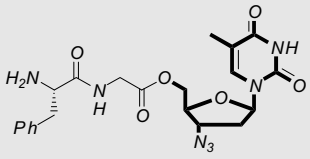
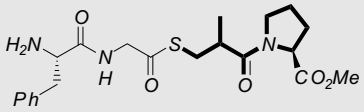
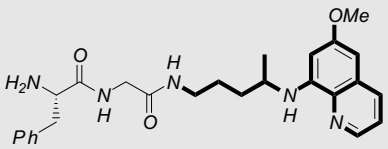
Table 3
Cytotoxicity and antiviral activity (EC_{50}) of compounds **3a–g**, ACV and other control drugs against CMV and VZV

Compound	Antiviral activity (EC_{50})[a] (μM)				Cytotoxicity (μM)	
	CMV AD-169	CMV Davis	TK^+ VZV OKA	TK^- VZV 07/1	MCC[b]	CC_{50}[c]
3a	>100	>100	13	>100	>100	>50
3b	>100	>100	12	>100	>100	>50
3c	>100	>100	12	>100	>100	>50
3d	>100	>100	16	>100	>100	>50
3e	>100	100	8	>100	>100	>50
3f	>100	100	8	>100	>100	>50
3g	73	–	0.56	>100	>100	>50
ACV	–	–	2.9	73	>1778	667
Brivudin	–	–	0.020	>300	>1200	502
GCV	3.2	6.9	–	–	>1575	370
Cidofovir	0.56	1.3	–	–	>1270	150

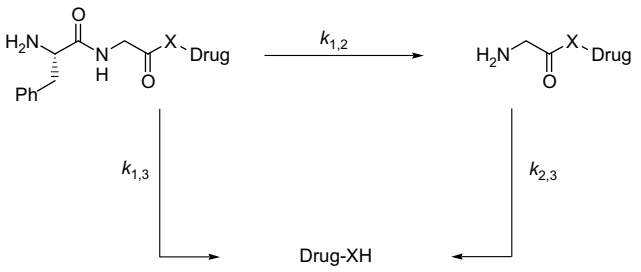
[a] Effective concentration required to reduce virus plaque formation by 50% in HEL cell culture (average values from two independent assays); virus input was 20 plaque forming units (PFU).
[b] Minimum cytotoxic concentration that causes a microscopically detectable alteration of HEL cell morphology.
[c] Cytotoxic concentration required to reduce HEL cell growth by 50%.

In Eqs. (5)–(7), C_1 stands for the concentration of the dipeptide substrate, C_2 is the concentration of the amino acyl intermediate (e.g. Gly-PQ from **10**) and C_3 is the concentration of the parent drug; $k_{1,2}$ is the first-order rate constant for the loss of the N-terminal amino acid (i.e. Phe), $k_{1,3}$ is the first-order rate constant for the loss of Phe-Gly and $k_{2,3}$ is the first-order rate constant for the hydrolysis of the amino acid intermediate. $k_{1,2}$, $k_{1,3}$ and $k_{2,3}$ values thus determined for prodrugs containing the Phe-Gly carrier are presented in Table 4. The data reveal some interesting features. First, the $k_{1,3}$ values (i.e. removal of Phe-Gly) for compounds containing a phenol and thiol leaving group (**7** and **9**, respectively) are at ca 3–10 times higher than those for prodrugs of ACV and AZT (**3g** and **8**, respectively), suggesting that the presence of a good leaving group in the drug moiety also affects the reactivity towards plasma enzymes. Second, hydrolysis of dipeptide amide **10** in human plasma involves stepwise removal of Phe and Gly residues but no direct loss of the Phe-Gly carrier (i.e. $k_{1,3} = 0$). Third, the hydrolysis of **10** to give Gly-PQ is ca. 30 times more rapid than the hydrolysis of Gly-PQ to primaquine. This presumably reflects a requirement of the aminopeptidases for N-terminal and penultimate residues with the L-configuration and an hydrophobic side chain in one of these residues [29].

Table 4
Log *P* values and kinetic data for the hydrolysis of compounds **3a–g**, **7–10** in pH 7.4 phosphate buffer and in human plasma at 37 °C

Compound	Structure	log *P*[a]	pH 7.4 buffer			Human plasma			
			$10^4\ k_{obs}\ (s^{-1})$[b]	$10^4\ k_1\ (s^{-1})$	$10^4\ k_2\ (s^{-1})$	$10^4\ k_{obs}\ (s^{-1})$[b]	$10^4\ k_{1,2}\ (s^{-1})$	$10^4\ k_{1,3}\ (s^{-1})$	$10^4\ k_{2,3}\ (s^{-1})$
3g		−0.52	0.510	0.310	0.221	4.84[c]	0	4.84	0
7		1.36	2.96	2.96	0	>50[c]	ND	ND	ND
8		1.05	1.28[d]	1.18	0.162	7.90[c,d]	0	7.90	0
9		1.24	5.65	5.65	0	21.0[c]	0	21.0	0
10		2.87	NR	NR	NR	0.398	0.412	0	0.0109

NR: no reaction; ND: not determined.
[a] Obtained using ALOGPS 2.1 (available at VCCLAB, Virtual Computational Chemistry Laboratory, http://www.vcclab.org).
[b] Pseudo-first order rate constants for disappearance of dipeptide prodrugs **3g**, **7–10**.
[c] Direct release of the parent drug (no intermediate amino acyl derivatives detected).
[d] From Ref. [25].



Scheme 3. Pathways for drug release from dipeptide derivatives Phe-Gly-X-Drug in human plasma.

3. Conclusions

Taken together, the present findings show that dipeptide esters of ACV are worthy of being further explored for potential clinical applications as ACV prodrugs, as they allow for variation of the dipeptide carrier structure, which enables modulation of lipophilicity, prodrug stability and antiviral activity. The present work clearly indicates that while a Val residue directly linked to ACV is of outmost importance to improve chemical stability, the N-terminal residue can be used for further fine tuning of the prodrug's properties. Compounds **3** are generally more active than ACV against HSV-1 and HSV-2 sensitive strains and present an improved MCC/EC_{50} ratio when compared to the parent drug. A decrease in MCC/EC_{50} caused by coupling a dipeptide carrier has also been described for dipeptide diester prodrugs of GCV whose aqueous stability at pH 7.4 is similar to or lower than those of compounds **3** and such was interpreted as indicative of the good prospects of such diesters as GCV prodrugs [20]. Importantly, the differences found between ACV and its dipeptide esters **3a**–**g**, and even among these esters, show that the antiviral activities observed are not simply the outcome of the prompt release of the parent drug in the course of the in vitro assays. Drug release from dipeptide derivatives **3** occurs by cyclization–elimination and addition–elimination (i.e. ester hydrolysis) pathways. When one considers the possibility to use the dipeptide-based prodrug approach to other functional groups, it is important to notice that the rate at which a drug is released from the dipeptide pro-moiety increases sharply with the drug's leaving group ability. This is a very rapid process for leaving groups with $pK_a < 10$ (e.g. phenols and thiols). The results herein presented suggest that dipeptide derivatives of drugs containing alcohol or amine functional groups present an adequate balance of chemical and enzymatic reactivities to be used as prodrugs. Finally, future resort to non-coded amino acids [30] and dipeptide isosters [31] can lead to clinically useful ACV dipeptide ester prodrugs with increased enzymatic stability and enhanced affinity towards oligopeptide transporters.

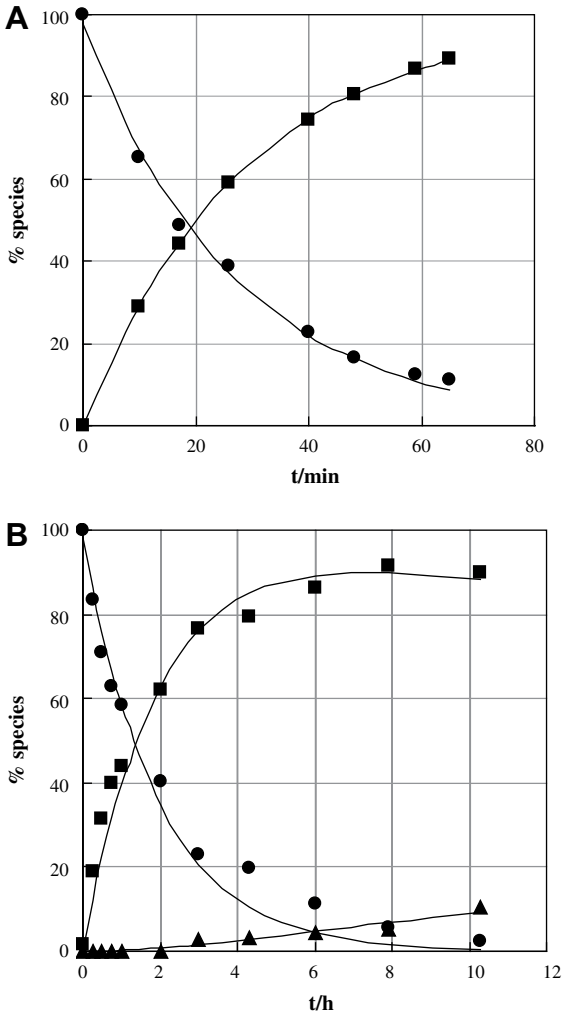


Fig. 3. Product analysis for the degradation of Phe-Gly-ACV, **3g**, and Phe-Gly-PQ, **10**, in 80% human plasma at 37 °C. (A): (●) **3g**; (■) ACV; (B): (●) **10**; (■) Gly-PQ; (▲) PQ.

4. Experimental

4.1. Materials

Boc-amino acids were obtained from Bachem, solvents were all of p.a. quality from Merck and were dried over pre-activated 3 Å molecular sieves prior to utilization. All remaining reactants were from Sigma–Aldrich and silica gel 60 plates (Merck F_{254}) were used for thin-layer chromatography. Nuclear magnetic resonance (NMR) spectra were acquired on a Bruker AMX 300 spectrometer from solutions of the compounds in hexadeuterated dimethylsulfoxide (DMSO-d_6) having tetramethylsilane (TMS) as internal reference. ^{1}H and ^{13}C chemical shifts are given in parts per million (ppm) and proton–proton coupling constants in Hertz (Hz); ^{1}H NMR peak multiplicity is indicated by br s (broad singlet), s (singlet), d (doublet), dd (double doublet), t (triplet), q (quartet), m (unresolved multiplet). Mass spectra (MS) were acquired by the matrix-assisted laser desorption ionization-time-of-flight (MALDI-TOF) technique on a Bruker Biflex II spectrometer.

4.2. Chemical syntheses

4.2.1. Preparation of compounds *3a–d*

Acyclovir (1 eq) was suspended in dry pyridine and the suspension put under magnetic stirring in a water–ice–acetone bath. The relevant $BocAA_1OH$ (1 eq) and DMAP (0.1 eq), previously dissolved in dry DCM, were then added to the mixture, after which a suspension of DCCI (1 eq) in dry DCM was slowly added dropwise. The mixture was stirred at 0 °C for 2 h, then at room temperature for further 24 h. The suspension was again led to 0 °C by immersion in a water–ice–acetone bath and, again, DCCI (1 eq) in DCM was slowly added dropwise, after which the reaction was allowed to proceed at room temperature for further 24 h. The crude mixture was evaporated to dryness, re-suspended in warm acetone and left overnight at 4 °C. *N*,*N*′-Dicyclohexylurea (DIU) fraction precipitated was removed by suction filtration and the filtrate evaporated to dryness and submitted to column chromatography on silica. This procedure allowed the isolation of white "waxes" that were identified by NMR and MS as compound **4** (see Supporting information).

*4.2.2. Preparation of trifluoroacetates **2a–d***

Each compounds **4a–d** was dissolved in neat TFA and the reaction was allowed to reach completion (~30 min) at room temperature. The target trifluoroacetate was precipitated with anhydrous ethyl ether and isolated after centrifugation at 3300 rpm and at −9 °C. Compounds **2a–d** were thus isolated as white hygroscopic solids and their structures confirmed by NMR and MS (see Supporting information).

*4.2.3. Synthesis of compounds **5a–g***

Each trifluoroacetates **2a–d** (1 eq) was dissolved together with TEA (1.2 eq) in dry DCM and the solution put under magnetic stirring for 2 h. The solution was then immersed in a water–ice–acetone bath and the relevant $BocAA_2OH$ (1 eq) was then added together with DMAP (0.1 eq). A suspension of DCCI (1 eq) in dry DCM was slowly added dropwise, and the reaction allowed to proceed at 0 °C for 2 h, after which it went on at room temperature for further 48 h. Procedures onwards were as above described for compound **4**. Compounds of 5 were isolated pure, as whitish "waxes", and their identity confirmed by NMR and MS (see Supporting information).

*4.2.4. Preparation of the target trifluoroacetates **3a–g***

The final compounds were obtained as white hygroscopic solids by acidolysis of their Boc-protected precursors **5a–g**, following the same procedure described above for the preparation of trifluoroacetates **2a–d**. Their structures were confirmed by NMR and EA, as illustrated by the spectral data given below (only for novel compounds).

*4.2.4.1. 9-{[2-(O-Alanylvalyl)hydroxyethoxy]methyl}guanine trifluoroacetic acid salt, **3b**.* Yield, 95%; δ_H: 0.83 (6H, m); 1.32 (3H, m); 1.99 (1H, m); 3.66 (3H, m); 4.16 (3H, m); 5.34 (2H, s); 6.57 (2H, br s); 7.81 (1H, s); 8.10 (3H, br s); 8.55 (1H, d, $J = 7.9$); 10.7 (1H, s); δ_C: 18.04; 19.18; 30.88; 48.76; 58.38; 64.20; 67.84; 72.63; 117.21; 138.49; 152.24; 154.84; 157.61; 170.83; 171.71; *m/z* ($M + H^+_{cation}$) = 396.72 (calcd, 396.68).

*4.2.4.2. 9-{[2-(O-Phenylalanylvalyl)hydroxyethoxy]methyl}guanine trifluoroacetic acid salt, **3c**.* Yield, 94%; δ_H: 0.83 (6H, m); 1.96 (1H, m); 3.01 (3H, m); 4.17 (5H, m); 5.34 (2H, s); 6.57 (2H, br s); 7.28 (5H, m); 7.81 (1H, s); 8.17 (3H, br s); 8.67 (1H, m); 10.7 (1H, s); δ_C: 18.82; 19.55; 30.98; 53.94; 58.33; 64.18; 66.35; 72.64; 117.21; 128.00; 129.34; 130.34; 135.56; 138.47; 152.24; 154.85; 157.58; 169.22; 171.49; *m/z* ($M + H^+_{cation}$) = 472.54 (calcd, 472.77).

*4.2.4.3. 9-{[2-(O-Glycylalanyl)hydroxyethoxy]methyl}guanine trifluoroacetic acid salt, **3e**.* Yield, 99%; δ_H: 1.23 (1H, m); 3.60 (2H, br s); 3.69 (2H, t, $J = 7.1$); 4.29 (2H, m); 4.50 (1H, m); 5.37 (2H, s); 6.72 (2H, s); 7.93 (1H, s); 8.11 (3H, br s); 8.82 (1H, d, $J = 8.2$); 11.0 (1H, s); δ_C: 18.63; 30.88; 58.20; 64.20; 67.33; 72.63; 117.25; 138.50; 152.26; 154.85; 157.66; 167.21; 171.84; *m/z* ($M + H^+_{cation}$) = 354.30 (calcd, 354.48).

*4.2.4.4. 9-{[2-(O-Glycylphenylalanyl)hydroxyethoxy]methyl}guanine trifluoroacetic acid salt, **3f**.* Yield, 78%; δ_H: 3.67 (4H, m); 4.25 (5H, m); 5.35 (2H, s); 6.57 (2H, br s); 7.28 (5H, m); 7.82 (1H, s); 8.17 (3H, br s); 8.63 (1H, m); 10.7 (1H, s); δ_C: 28.76; 38.23; 57.93; 64.47; 66.32; 72.64; 117.21; 127.15; 128.32; 132.46; 135.56; 138.45; 152.86; 155.36; 158.62; 169.19; 171.21; *m/z* ($M + H^+_{cation}$) = 430.30 (calcd, 430.24).

*4.2.4.5. 9-{[2-(O-Phenylalanylglycyl)hydroxyethoxy]methyl}guanine trifluoroacetic acid salt, **3g**.* Yield, 93%; δ_H: 3.53 (4H, m); 4.21 (5H, m); 5.34 (2H, s); 6.57 (2H, br s); 7.29 (5H, m); 7.81 (1H, s); 8.12 (3H, br s); 8.62 (1H, m); 10.8 (1H, s); δ_C: 30.98; 40.23; 58.33; 64.27; 66.43; 72.62; 117.21; 128.10; 129.32; 130.32; 135.52; 138.47; 152.28; 155.85; 157.62; 170.22; 171.29; *m/z* ($M + H^+_{cation}$) = 430.34 (calcd, 430.24).

*4.2.5. Synthesis of compounds **7–9***

The synthesis and spectroscopic identification of compounds **7** and **8** have been described elsewhere [25,32]. Compound **9** was prepared in three steps as follows.

*4.2.5.1. Preparation of captopril methyl ester (CapMe), **9a**.* This compound was prepared as previously reported [33]. Briefly, captopril (10 mmol) was dissolved in methanol (2 mL) under magnetic stirring and the solution cooled to 0 °C. Thionyl chloride (200 μL) was then added dropwise, after which the temperature was raised to 60 °C and the reaction allowed to evolve at this temperature for 2 h. The solvent was evaporated and the residue submitted to column chromatography on silica yielding CapMe as a colorless oil (yield, 82%) with correct spectral data, as compared to the literature [33].

*4.2.5.2. Synthesis of S-(N-tert-butyloxycarbonylphenylalanyl)captopril methyl ester (BocPheGlyCapMe), **9b**.* Captopril methyl ester (**9a**, 1.8 mmol) was dissolved in dioxane (10 mL), and to this solution were added Boc-Phe-Gly-OH (1.8 mmol) and DMAP (0.18 mmol), after which the mixture was cooled to 0 °C. Then, DCCI (1.8 mmol) was added to the mixture and the reaction allowed to proceed at room temperature for 72 h. Procedures onwards were as previously described for compounds **4**. The product was isolated as a yellowish oil with correct spectral data. Methyl *N*-{3-[*S*-(*N*-*tert*-butyloxycarbonylphenylalanylglycyl)]mercapto-2-methyl}propanoylprolinate, **9b**: yield, 75%; δ_H: 1.17 (3H, m); 1.33 (9H, s); 1.95 (3H, m); 2.14 (1H, m); 2.72 (1H, m); 3.00 (4H, m); 3.53 (2H, m); 3.65 (3H, s); 4.05 (2H, m); 4.33 (1H, m); 4.43 (1H, m); 4.91 (1H, br s); 6.58 (1H, t, $J = 5.4$); 7.19 (5H, m); δ_C: 16.78; 24.80; 28.21; 29.02; 38.32; 41.17; 46.37; 46.86; 49.04; 52.14; 55.74; 58.63; 80.49; 126.97; 128.67; 129.25; 136.45; 171.65; 172.65; 173.13; 173.49; 196.75; *m/z* ($M + Na^+ - Boc$) = 458.47 (calcd, 458.17).

*4.2.5.3. Preparation of S-(phenylalanyl)captopril methyl ester trifluoroacetic acid salt (PheGlyCapMe, TFA), **9**.* Compound **9b** was dissolved in 5 mL of 40% trifluoroacetic acid in DCM and the reaction was allowed to evolve at room temperature until it reached completion (~1 h). Procedures onwards were as described for compound **2** and the target product, **9**, was isolated as a white hygroscopic solid with correct spectral data. Methyl *N*-[3-(*S*-(phenylalanylglycyl)mercapto-2-methyl]propanoylprolinate trifluoroacetic acid salt), **9**: yield, 99%; δ_H: 1.07 (3H, d, $J = 6.9$ Hz); 1.83 (1H, m); 1.91 (2H, m); 2.16 (1H, m); 2.94 (4H, m); 3.17 (1H, dd, $J = 14.1, 5.1$); 3.54 (2H, t, $J = 6.6$ Hz); 3.59 (3H, s); 4.11 (3H, m); 4.27 (1H, q, $J = 4.5$); 7.32 (5H, m); 8.24 (3H, br s); 9.28 (1H, t, $J = 6.0$); δ_C: 16.29; 24.38; 28.53; 30.72; 36.71; 37.15; 46.40; 48.69; 51.66; 53.32; 58.17; 127.13; 128.50; 129.46; 134.68; 168.77; 172.19; 173.02; 197.07; *m/z* ($M + Na^+$) = 458.60 (calcd, 458.17).

4.3. General procedure for monitoring of drug release by HPLC-UV

A solution of the compound to be assayed (10^{-4} M) in aqueous buffer (pH 7.4) was kept at a constant temperature of 37.0 ± 0.1 °C; aliquots were periodically taken and immediately injected (loop of 100 μL) into the HPLC system, using a LichroCart 250-4 Lichrospher 100 RP-8 reverse phase column (250 × 4 mm, 5 μm); the elution was isocratic, at varying proportions of CH_3CN in aqueous buffer (sodium hexanesulfonate 10 mM, sodium acetate 2.5 mM, phosphoric acid 2.5 mM; pH 6.3) and at a flow rate of 1.0 mL/min, with UV detection at 240 nm. The hydrolyses of prodrugs in 80% (v/v) human plasma were also studied by the HPLC-UV method described above. Plasma was obtained from heparinized blood of healthy donors, pooled, and frozen at −70 °C before use. Prodrugs **3**

were incubated at an initial concentration of 10^{-4} M at 37 °C in human plasma diluted to 80% (v/v) with pH 7.4 isotonic phosphate buffer. At appropriate intervals, 30 μL aliquots were added to 270 μL of acetonitrile in order to quench the reaction and deproteinise the plasma. These samples were centrifuged for 5 min at 13,000 rpm and the supernatant was analyzed by HPLC.

4.4. In vitro biological assays

The methodology of cytotoxicity and antiviral activity determination assays has been previously described [34,35]. Compounds **3** were evaluated for their antiviral activity against HEL cell cultures. The cytotoxicity was verified in mock-infected HEL cells. The antiviral activity assays were based on the inhibition of virus-induced cytopathicity in the above-mentioned culture and the activity thus determined compared with that of standard brivudin, ribavirin, acyclovir and gancyclovir. Briefly, the confluent cell culture in 96-well microliter plates was inoculated with 100 CC1D50 of virus, ICC1D50 being the virus dose required to infect 50% of the cell cultures. After 1 h of virus adsorption period, the residual virus was removed, and the cell cultures were incubated in the presence of varying concentrations of the test compounds. Viral cytopathicity was recorded as soon as it reached completion in the control virus in treated cell cultures.

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Appendix. Supporting information

Supplementary data associated with this article can be found in the online version, at 10.1016/j.ejmech.2008.08.009.

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