Chemical and Enzymatic Degradation of Ganciclovir Prodrugs: Enhanced Stability of the Diadamantoate Prodrug Under Acid Conditions

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We report the chemical and enzymatic hydrolysis of two hydrophobic prodrugs of ganciclovir (3 = dipropionate ester; 4 = diadamantoate ester). Both prodrugs undergo hydrolysis showing a pH dependence of $k_{obs} = k_{H+}a_{H+} + k_o + k_{HO-}a_{HO-}$ and a pH of maximum stability near pH 5. Only 4 exhibited a shelf life (t_{90}) greater than 2 years. Compound 4 reacts significantly slower than ganciclovir in acidic media, even though the adamantyl esters provide additional reaction sites (which would be expected to increase the rate of degradation) that are distally removed from the guanine ring system, offering negligible steric or electronic substituent effects. Both 3 and 4 hydrolyzed in tissue homogenate, where k_{obs} followed liver > intestine ≫ skin. Based on these findings of chemical stability, lipophilicity, and acceptable rate of enzymatic cleavage by skin esterases, 4 meets several of the criteria required for the topical sustained delivery of ganciclovir.

KEY WORDS: nucleoside analogues; topical sustained release; enzymatic degradation; hydrolysis; chemical stability; adamantoate ester; DHPG.

INTRODUCTION

Prodrugs have been widely used for altering compound hydrophobicity (1). Typically, prodrugs are designed to increase aqueous solubility, resulting in improved dissolution and oral bioavailability (2). Alternatively, water soluble or polar drugs have been modified with lipophilic moieties to retard dissolution for sustained release or to improve transport across biomembranes (3,4). A well-designed lipophilic prodrug has the following characteristics: (i) adequate aqueous solubility, (ii) chemical and physical stability of 2 years or more in the final formulation at room temperature, and (iii) *in vivo* cleavage of the prodrug moiety at a pharmacologically significant rate. Adamantoate esters are known to exhibit these characteristics and have been used previously for modifying biologically active nucleosides for drug delivery (5–7).

Ganciclovir (1) is a selective antiviral agent structurally similar to acyclovir (Zovirax; 2) (8) but possessing a greater potency and broader activity against herpes virus (9). Ganciclovir has been shown to be active against herpes simplex I and II, cytomegalovirus, varicella zoster, and Epstein–Barr virus. Topically applied lipophilic prodrugs of ganciclovir may be more effective for the treatment of topical viral infections than ganciclovir given orally (10) because they may penetrate the stratum corneum resulting in direct delivery to the site of infection. Further, delivery of ganciclovir using a topically applied prodrug should reduce the systemic level of ganciclovir, thus reducing the side effects of gonadal toxicity and neutropenia (11). Although early experiments demonstrated that 3 is enzymatically labile (12,13), 3 exhibits a limited chemical stability in aqueous solution at room temperature, thus hampering formulation development. Recently the diadamantoate ester of ganciclovir 4 was prepared (14) and now we report its chemical and *in vitro* stability, as well as its novel stability under acid conditions.

MATERIALS AND METHODS

Chemicals

9-[(1,3-Dihydroxy-2-propoxy)methyl]guanine (ganciclovir, 1), 9-[1,3-di-(1-propylcarboxy)-2-propoxy)methyl]guanine (3), 9-[1,3-di(l-adamantylcarboxy)-2-propoxymethyl]guanine (4), and 9-[1,(l-adamantylcarboxy)-2-propoxymethyl-3-hydroxyl]guanine (5) were synthesized at the Institute of Bioorganic Chemistry (Syntex Research) (15). Acyclovir (2) and guanine (6) were obtained from Sigma and Aldrich Chemicals, respectively. 9-[1,3-Di-(l-propylcarboxy)-2-propoxy)methyl]guanine [1,3-propyl-^{3}H] (or ^{3}H-labeled 3) and 9-[1,3-di(l-adamantylcarboxy)-2-propoxymethyl]guanine [8-^{14}C] (or ^{14}C-labeled 4) were synthesized at the Institute of Organic Chemistry (Syntex Research). HPLC mobile phase was prepared from HPLC-grade acetonitrile and methanol (Burdick and Jackson) and doubly distilled water (Nanopure). All other chemicals were of analytical grade.

HPLC Conditions

All samples, except those containing skin homogenate, were analyzed simultaneously for diester, monoester, 1, and 6 using a dual-column HPLC system consisting of a Spectra Physics Model SP 8700 pump system, a Micromeritics Model 725 autoinjector, a Schoffel Model 770 spectrophotometric detector, and a Spectra Physics 4000 computing integrator. HPLC separation of 3 from its degradation products was carried out as reported previously (12); chromatographic separation of 4 from its products was carried out similarly using a Partisil SCX 10 μm, 25 × 0.46-cm-i.d. (Phenomenex) column connected in series with a C-6 5 μm, 15 × 0.46-cm-i.d. (Alltech) column. Mobile phase was composed of 0.015 *M* ammonium phosphate buffer adjusted to pH 2.0 (H_3PO_4) in acetonitrile (50:50); the flow rate was 1.0 mL/min (1600 psi). The column effluent was monitored at 254 nm (0.02 auf). The injection loop size was 100 μL; the amount injected was ~1 μg/mL. Typical retention volumes were as follows: 5, 6 min; 1, 8 min; 4, 11 min; and 6 (guanine), 13 min. The following reversed-phase (RP) HPLC method was

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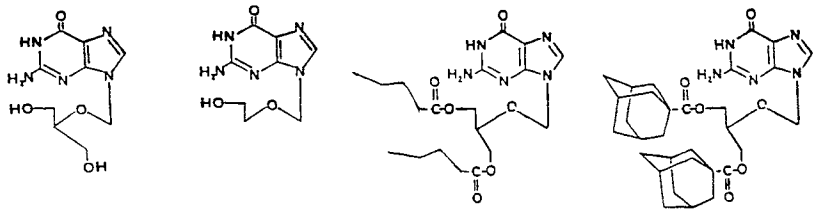


1 2 3 4

Scheme I

used to assay samples containing skin homogenates for 1: 5-μm Whatman ODS-3 column, 25 × 0.46 cm i.d.; mobile phase, water containing 0.1% phosphoric acid; internal standard, acyclovir (2).

Chemical Kinetics and Product Identification

The degradation of 3 (100 μg/mL) was studied using 0.01 *M* buffer solutions (μ = 0.10 *M*) at 25, 40, 60, and 80°C. The degradation of **4** (1 μg/mL) was studied using 0.01 *M* buffer solutions (μ = 0.10 with NaCl) containing 25% acetonitrile (v:v) at 60, 80, and 100°C. pH's were determined at the reaction temperature except for 100°C, where they were extrapolated from linear plots of pH (at 21–80°C) versus the reciprocal of absolute temperature. The buffers used are given in Tables I and II. For strongly acidic or alkaline solutions at 80 and 100°C, the hydronium and hydroxide ion activities were calculated from published activity coefficients (16) or acidity function H_0 values (17). In a typical experiment, 2-mL aliquots of reaction solution containing **4** were transferred to pretreated ampoules, flame-sealed, and stored at 60, 80, or 100°C. Several of these ampoules were also refrigerated immediately after flame-sealing and were later used as controls for the initial time points. At known time intervals, up to <100 days, ampoules were removed and refrigerated until 6–10 samples for each kinetic run were taken. Typically, kinetic runs were followed for several half-lives; however, at lower temperatures or in the neutral pH range, some runs were followed for less than one half-life. Upon removal of the last samples, the stored solutions were allowed to warm to room temperature and then all samples were analyzed by HPLC on the same day. Peak area integration values were used directly in first-order fits of the data; nonlinear least-squares analysis (18) was used to obtain the best-fit secondary rate constants from the pH-rate profiles. UV spectra of the major RP-HPLC peaks were obtained by monitoring the HPLC effluent on an HP-8450A spectrophotometer equipped with an 8-μL flow cell. For the reaction of **4**, large-scale separation and collection of the major peaks afforded the reaction products **1**, **5**, and **6**. These were confirmed by comparison of UV spectra and HPLC retention times with authentic samples.

Tissue Homogenate Preparation and Enzyme Kinetics

Sections of intestine, liver, and skin were obtained from male cynomolgus monkeys. Tissues were rinsed thoroughly with ice-cold 0.02 *M* potassium phosphate buffer of pH 7.4 containing 1.15% KCl. Excess subcutaneous fat was removed from the skin before pulverization in liquid nitrogen and cell disruption with a Polytron homogenizer (2 × 10-sec bursts at maximum speed) (19). Portions of the liver and intestine were homogenized in the same phosphate buffer-HCl solution at 4°C with a Potter–Elvehjem glass tube and a Teflon pestle. The final concentration of the hepatic and intestinal homogenate was 16.7% (w/v), whereas the final concentration of the skin homogenate was 25%. The homogenates were centrifuged at 10,000*g* for 20 min at 5°C and then used as the enzyme source on the same day they were prepared.

Reactions wtih hepatic and intestinal homogenates were initiated by adding enzyme-containing supernatant to a 0.02 *M* phosphate, pH 7.4, buffer containing ^{3}H-labeled 3 or ^{14}C-labeled **4**. The final concentrations of substrate and homogenate were 120 μ*M* and 1.6% (w/v), respectively. A reaction mixture containing aliquots of heat-denatured supernatant was used as the control. The samples were incubated in a Dubnoff shaking incubator at 37°C. At various time intervals

Table I. Summary of Rate Constants for the Degradation of 3 in Aqueous Solution

Buffer	25°C		40°C		60°C		80°C	
	pH[a]	$10^7 k_{obs}$ (sec^{-1})[c]	pH[a]	$10^7 k_{obs}$ (sec^{-1})[c]	pH[a]	$10^6 k_{obs}$ (sec^{-1})[c]	pH[a]	$10^6 k_{obs}$ (sec^{-1})[c]
0.01 *M* HCl	1.97	28.4	1.96	93.3	1.98	39.9	1.99	1.50
0.01 *M* choloroacetate	2.93	2.9	2.98	8.9	3.0	3.8	3.07	12.9
0.01 *M* formate	3.71	0.55	3.74	1.7	3.78	0.81	3.84	2.3
0.01 *M* acetate	4.73	0.16	4.75	0.33	4.77	0.17	4.83	0.65
0.01 *M* phosphate	6.83	1.8	6.83	5.4	6.83	4.64	6.88	25.8
0.01 *M* phosphate	7.55	4.8	7.54	22.9	—[b]	—	7.62	122

[a] pH's were determined at the temperature indicated.
[b] Not determined.
[c] The relative error in the rate constants was always 5% or less.

Table II. Summary of Rate Constants for the Degradation of 4 in Aqueous Solution Containing 25% Acetonitrile (v:v)

Buffer	60°C		80°C		100°C	
	pH[a]	$10^8 k_{obs}$ (sec^{-1})[c]	pH[a]	$10^7 k_{obs}$ (sec^{-1})[c]	pH[b]	$10^7 k_{obs}$ (sec^{-1})[c]
1 *M* HCl			0.6	250		
0.014 *M* HCl	1.9	31	1.9	11	1.9	39
0.01 *M* acetate	4.4	0.063	4.4	0.078	4.4	0.24
0.01 *M* acetate	4.9	0.08	4.8	0.034	4.8	0.23
0.01 *M* acetate	5.5	0.12	5.4	0.084	5.4	0.51
0.01 *M* phosphate	6.6	0.63	6.5	0.62	6.5	4.6
0.01 *M* phosphate	7.2	3.2	7.1	2.3	7.1	15
0.01 *M* phosphate	7.7	9.0	7.7	6.9	7.7	49
0.01 *M* carbonate	10.3	2800	10.1	2300		

[a] pH's of 25% acetonitrile solutions were determined at the temperatures indicated. It is noted that these are only approximate pH values because of the medium effect with added acetonitrile.

[b] The pH values at 100°C were determined from the least-squares line ($r > 0.99$) of a plot of $1/T(K^{-1})$ vs pH using pH values determined at 25, 40, 60, and 80°C.

[c] The relative error in the rate constants shown was always 5% or less.

after initiation of the reaction, 1-mL aliquots of the reaction mixture were removed and mixed with 4 mL of ice-cold methanol to terminate the enzymatic hydrolysis reaction. The methanolic mixtures were centrifuged at 2000 rpm for 10 min. The supernatants were analyzed simultaneously by HPLC for diester, monoester, and 1. The eluent from the HPLC column was collected in scintillation vials at 30-sec intervals for 20 min and each vial was assayed for radioactivity using a Packard Tricarb Model 3330 liquid scintillation counter.

The reactions with skin homogenate were carried out similarly except that a 22.5% skin homogenate was used in the reaction mixture and that nonradioactive 3 and 4 were used. In this case, only ganciclovir was followed by extraction from the reaction mixture as described previously (12) and quantitation by HPLC using UV detection with 2 as the internal standard.

RESULTS AND DISCUSSION

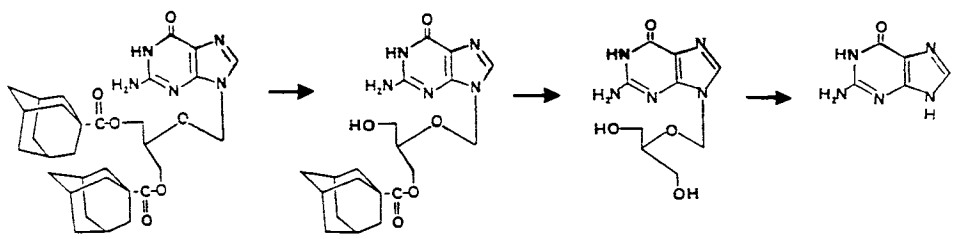
Herein we report the chemical and enzymatic hydrolysis of the dipropionate (3) and diadamantoate (4) prodrugs of ganciclovir. We are interested in such *in vitro* studies because (i) they provide a model system for determining factors governing the shelf life of ganciclovir analogues in water-based topical formulations, (ii) the monoester degradation products are also prodrugs of ganciclovir and so their formation rates are germane, and (iii) these results strengthen our earlier observation (12) that the size of the ester prodrug moiety is of little consequence in determining esterase activity, even when a very large group such as the adamantoate moiety is used. Further, we have discovered that the diadamantoate derivative of ganciclovir is much more stable in acid than predicted—indeed more stable than ganciclovir itself.

Degradation of 3 and 4

The degradation pathway for 4 is shown in Scheme II. In what follows, the rate constant subscripts denote the catalytic species; for example, $k_{H+}a_{H+}$, $k_{HO-}a_{HO}$, and k_o are the rate contributions by specific hydronium ion catalysis, specific hydroxide ion catalysis, and spontaneous (or water-catalyzed) hydrolysis, respectively. The solution activities for hydronium ion and hydroxide ion are denoted a_{H+} and a_{HO-} and are approximately equal to [H+] and [HO−] at low ionic strength in aqueous solution. Under all reaction conditions, hydrolysis of 3 and 4 initially gave the monoester and small amounts of guanine, followed by the formation of 1. At acidic pH's and longer reaction times, guanine was the major product formed, presumably by the further breakdown of 1 and 5.

Effect of pH and Temperature

Apparent first-order kinetics were observed in all chemical degradation experiments, and the pH dependence on the degradation of 3 and 4 is given in Tables I and II. The pH–



4 5 1 Guanine

Scheme II

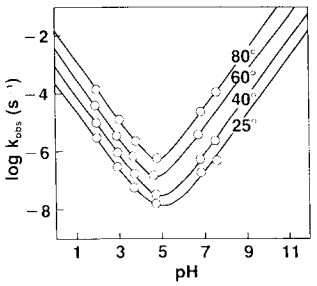


Fig. 1. pH–rate profile for the chemical degradation of 3 in aqueous solution.

rate profiles are shown in Figs. 1 and 2, where the best-fit lines in these figures were generated using Eq. (1).

$$k_{obs} = k_{H+}a_{H+} + k_o + k_{HO-}a_{HO-} \quad (1)$$

The secondary rate constants derived from nonlinear least-squares analysis of the data are given in Table III. (Note that there is not a pK_a term for the ionization of the guanine ring in Eq. (1); as shown later it is not warranted, as the primary reaction center is distally removed from the guanine ring.) At pH 5, k_{obs} is minimized and the rate contributions by hydronium ($k_{H+}a_{H+}$) and hydroxide ion ($k_{HO-}a_{HO-}$) are approximately equal. The spontaneous reaction makes only a small contribution at pH 5; for example, the calculated k_{obs} for 4 using Eq. (1) is 7.6×10^{-10} sec^{-1}; without the k_o term the calculated k_{obs} is only 2.6×10^{-10} sec^{-1}, or three times less. Other stable esters such as ethylacetate (20) also show a relatively small contribution by the water-catalyzed reaction at the pH of maximum stability. Inspection of the rate data in Fig. 1 shows that 3 does not exhibit a 2-year shelf life at room temperature because of the facile reaction of the propionate ester group. Although the reaction of 3 was carried out in wholly aqueous solution and 4 in 25% acetonitrile (added for increased drug solubility), it is unlikely that these large rate differences are due to a change in the solvent, as evidenced by smaller solvent effects in other organic cosolvent studies (21). Thus, the adamantoate ester is one of the most stable esters known and is of comparable stability to ethyl acetate (20).

Stabilization of 1 by the Adamantoate Ester Moiety

Comparison of k_{H+} for ganciclovir and 4 shows that ganciclovir is more reactive than 4 in acidic media. For example, k_{H+} at 80°C (with added acetonitrile to increase drug solubility) for 4 is 0.9×10^{-4} M^{-1} sec^{-1}, whereas for ganciclovir $k_{H+} = 2.0 \times 10^{-4}$ M^{-1} sec^{-1}. Although this rate difference may not seem large at first, it should be noted that 4 has two additional sites—the ester groups—that predominate in the reactivity of 4 (vide infra). To sort out the reaction contributions by ester hydrolysis and guanine ring degradation, we studied the complete acid-catalyzed degradation of 4 by following the prodrug 4, the monoadamantyl hydrolysis product 5, 1, and guanine. The kinetically competent reaction pathway in Scheme III accounts for the reaction profile in Fig. 3.

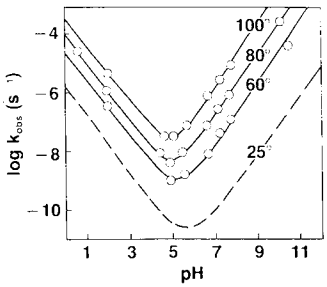
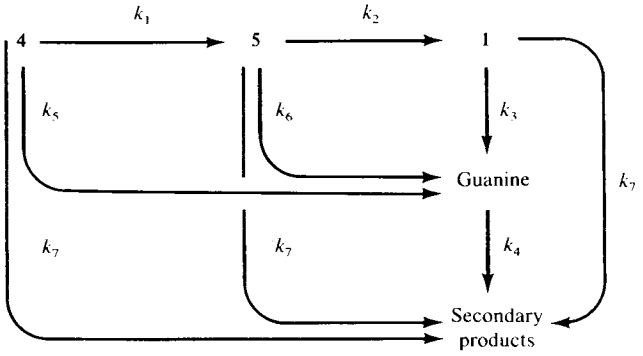


Fig. 2. pH–rate profile for the chemical degradation of 4 in aqueous solution containing 25% acetonitrile (v:v).

Table III. Secondary Rate Constants for the Reaction of 1, 3, and 4[a]

Substrate	Temp (°C)	$10^4 k_{H+}$ (M^{-1} sec^{-1})	$10^8 k_o$ (sec^{-1})	k_{HO-} (M^{-1} sec^{-1})
1	80	2.0		
3	25	2.4	1.1	1.8
	40	8.5	1.4	2.5
	60	39	6.7	6.8
	80	150	21	12
4	25	0.015[b]	0.0019[b]	0.00083[b]
	60	0.25	0.050	0.015
	80	0.96	0.21	0.063
	100	3.1	11	0.21

[a] Reaction of 3 was carried out in aqueous buffers; 1 (calculated from data obtained in dilute HCl solutions containing 25% acetonitrile; data not shown) and 4 were studied in 25% acetonitrile.
[b] Calculated from the Arrhenius parameters derived from the 60–100°C data.



Scheme III

The rate constants are as follows: k_1 = acid-catalyzed ester hydrolysis of 4; k_2 = acid-catalyzed ester hydrolysis of 5; k_3, k_5, and k_6 represent chain cleavage of 1, 4, and 5 to give guanine; k_4 = breakdown of guanine to secondary products; and k_7 represents the degradation of 4, 5, and 1 to secondary products directly. (This analysis is based upon similar rates of secondary product formation for 4, 5, and 1; we have found that this is an acceptable approximation in that the kinetic profiles and k_1–k_5 were nearly identical when a nine-parameter fit was used.) By this mechanism 1 degrades only by chain cleavage and guanine oxidation (k_3 and k_7, respectively), whereas 4 degrades by ester hydrolysis, chain cleavage, and guanine oxidation (k_1, k_5, and k_7, respectively). Reaction scheme analysis of Fig. 3 using the program PROTEUS (22) shows that $k_1 \approx 10(k_5 + k_7)$ or that only 10% of

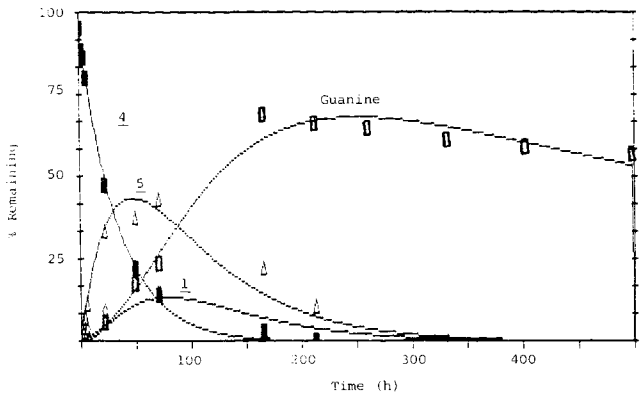


Fig. 3. Reaction profile and best-fit curves for the degradation of 4 (■, 5 (△), 1 (◇), and guanine (□) in 0.1 *M* HCl at 80°C (Scheme III). The best-fit rate constants (sec^{-1}) for $k_1 \rightarrow k_7$ are (6.94 ± 0.28) × 10^{-6}, (3.72 ± 0.20) × 10^{-6}, (10.5 ± 0.5) × 10^{-6}, (0.41 ± 0.15) × 10^{-6}, (0.73 ± 0.05) × 10^{-6}, (0 ± 0.25) × 10^{-6}, and (4.3 ± 4.0) × 10^{-8}

the observed degradation of **4** at low pH is due to chain cleavage or degradation of the guanine moiety (90% of the observed degradation is due to ester hydrolysis). Thus the corrected partial rate constant for the degradation of **4** (omitting ester hydrolysis) is $0.1(0.9 \times 10^{-4}\ M^{-1}\ sec^{-1}) = 9 \times 10^{-6}\ M^{-1}\ sec^{-1}$, or 20 times smaller than the rate constant for the degradation of **1**. Similarly, the degradation of **5** (again omitting ester hydrolysis) is much slower than for **1**. This large reactivity difference between the guanine moiety in **4** and **1** may be due to (i) steric hindrance or a rate-retarding substituent effect caused by the adamantyl groups or (ii) rate accelerating intramolecular catalysis by the hydroxyl group(s) of **1**. As a 20-fold rate retardation by steric hindrance or an inductive substituent effect through five atoms of flexible geometry is unprecedented, intramolecular hydroxyl catalysis (of which there is ample precedent) is a likely alternate explanation. Prodrug modification—especially with esters—usually affords derivatives that are more reactive than the parent compound, not less so. Prodrug **4** presents an interesting exception to this tenet in that **4** is more stable than **1**, even when it has two additional sites (the ester groups) for degradation.

Enzyme Kinetics

The enzymatic hydrolysis of **3** and **4** (and other diesters of **1**) is described by Eq. (2). The diesters **3** and **4** are represented by 1−(OR)$_2$ and the monoesters by 1−(OR). The rate constant k_1 was obtained from the best-fit slope of a plot of −ln[% 1−(OR)$_2$] vs time; k_2 was obtained from the best-fit slope of a plot of ln [%**1**(at time = ∞) − %**1**(at time = t)] vs time (Table IV).

$$1-(OR)_2 \xrightarrow{k_1} 1-(OR) \xrightarrow{k_2} 1 \qquad (2)$$

When intestinal homogenate was used, both k_1 and k_2 for the adamantoate esters were larger than for the propionate esters (3.4 and 14 times greater, respectively). Adamantoate esters may be cleaved faster than propionate esters because of increased enzyme binding due to greater hydrophobicity of the adamantoate moiety. The hydrophobic nature of the binding site was also probed by comparing k_2/k_1 for different substrates. For example, the smaller k_2/k_1 ratio for the hydrolysis of the propionate ester (k_2/k_1 = 0.04) compared with the adamantoate ester (k_2/k_1 = 0.18) by intestinal homogenate suggests that the propionate moiety, when it is introduced as a monoester, binds only weakly with the hydrophobic area on the active site of esterases. [This hypothesis is supported by the data of Kawaguchi *et al.* (23), who have estimated the size of the esterase hydrophobic area to be approximately the size of a decyl moiety.] The kinetics of the propionate and adamantoate esters were found to be similar when live homogenate was used (Table IV); for example, k_2 for the hydrolysis of the propionate and adamantoate esters was 5.3 × 10^{-4} and 7.5 × 10^{-4} sec^{-1}, respectively. Our results are consistent with data of Benjamin *et al.* (12), who reported that shorter-chain esters of **1** (such as diacetate or dipropionate) were rapidly hydrolyzed in liver homogenate compared to intestinal homogenate. They also found that the k_2 hydrolysis rate constants for the propionate and hexanoate esters of **1** in monkey liver homogenate were close in value: 5.8 × 10^{-4} vs 8.0 × 10^{-4} sec^{-1}. This is likely caused by liver esterases that selectively attack the shorter-chain esters (C_2–C_4), similar to the preferential reaction of C_2 choline esters by choline esterase (24). Because the mono- and dipropionate esters of **1** are hydrolyzed only moderately by intestinal homogenate, they should be candidates for orally active prodrugs of **1**. Recently this has been confirmed; **3** demonstrated higher oral bioavailability than **1** alone in mice and monkeys, whereas **4** showed a low oral bioavailability, presumably because of its extremely low aqueous solubility (aqueous solubility of **4** = 2.4 × 10^{-5} mg/mL, **3** = 2.1 mg/mL, **1** = 3.7 mg/mL). For example, single oral doses of **1** (5 mg/kg) and **4** (20 mg/kg, i.e., 8.8 mg/kg equivalents of **1**) in a suspension formulation to mice gave AUCs for **1** (as calculated by the trapezoidal rule from 0 to 7 hr) of 1601 and 513 ng · hr · mL^{-1}, respectively (M. Chaplin, unpublished data). Therefore **4** was only 18% as bioavailable orally as **1**. This is an added benefit for the topical delivery of **4**, since low oral bioavailability of **4** is

Table IV. Rate Constants for the Enzymatic Hydrolysis of **3** and **4** in Liver, Intestine, and Skin Homogenates from Cynomolgus Monkeys

	Liver homogenate			Intestine homogenate			Skin homogenate	
Compound	$10^4\ k_1$ (sec^{-1})	$10^4\ k_2$ (sec^{-1})	k_2/k_1	$10^4\ k_1$ (sec^{-1})	$10^4\ k_2$ (sec^{-1})	k_2/k_1	$10^6\ k_2$ (sec^{-1})	k_2/k_1
3	19	5.3	0.28	3.8	0.17	0.045	9.2	—[a]
4	22	7.5	0.34	13	2.3	0.18	7.2	—

[a] Not determined; k_1 was not measured.

desired in the event of accidental oral drug ingestion during the topical treatment of labial herpes simplex viral infections.

In conclusion, the adamantoyl group (i) increases the lipophilicity of ganciclovir and (ii) provides a prodrug **4** that is fairly resistant to chemical hydrolysis (including greater than predicted stability under acid conditions) yet (iii) is still susceptible to enzymatic hydrolysis by skin esterases. These properties make the diadamantoate derivative of ganciclovir a likely prodrug candidate for sustained drug release in the topical treatment of herpes simplex and other viral infections.

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