The Physicochemical Properties, Plasma Enzymatic Hydrolysis, and Nasal Absorption of Acyclovir and Its 2′-Ester Prodrugs

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A series of 2′-(*O*-acyl) derivatives of 9-(2-hydoxyethoxymethyl)guanine (acyclovir) was synthesized by acid anhydride esterification. Aqueous solubilities in isotonic phosphate buffer (pH 7.4), partition coefficients in 1-octanol/phosphate buffer, and hydrolysis kinetics in rat plasma were determined. The ester prodrugs showed consistent increases in lipophilicity with corresponding decreases in aqueous solubility as a function of side-chain length. The bioconversion kinetics of the prodrugs appear to depend on both the apolar and the steric nature of the acyl substituents. When perfused through the rat nasal cavity using the *in situ* perfusion technique, acyclovir showed no measurable loss from the perfusate. Nasal uptake of acyclovir prodrugs, on the other hand, were moderately improved. Furthermore, the extent of nasal absorption appears to depend on the lipophilicity of the prodrugs in the descending order hexanoate > valerate > pivalate > butyrate. Simultaneous prodrug cleavage by nasal carboxylesterase was also noted in the case of hexanoate.

KEY WORDS: acyclovir; aqueous solubility; 2′-ester prodrugs; lipophilicity; nasal delivery; plasma bioconversion.

INTRODUCTION

Intranasal delivery has attracted attention because of its noninvasive nature and ease of administration. While low molecular weight lipophilic compounds are generally well absorbed through the nasal epithelium, hydrophilic and macromolecular species are poorly absorbed (1). To overcome the nasal absorption barriers, absorption enhancers have been developed which act by various mechanisms, e.g., by acting directly on the nasal mucosa, thereby increasing its fluidity, by transiently inhibiting nasal enzyme activity, and/or by altering the aggregation state of the solute for better paracellular diffusion (2–7). However, a universally applicable absorption adjuvant has not yet been identified. In addition, most of the effective absorption enhancers cause nasal mucosal damage (8–11), which raises serious concerns over their long-term safety (12).

Another strategy would be the design of derivatives with high partition coefficients, thereby thermodynamically favoring solute partitioning into the nasal membrane. The rate and extent of nasal drug uptake depend strongly on the lipophilicity of the penetrant (13–16). Hence, linkage of biocleavable lipophilic moieties to a molecule has been widely used to improve oral bioavailability. Ester prodrug design involves simple esterification of hydroxyl or free carboxylic groups to form derivatives with an enhanced octanol/water partition coefficient. These ester prodrugs will then be cleaved to regenerate the parent compound in the blood because of the presence of high esterase activity (17,18).

In this report, a series of aliphatic prodrugs of acyclovir was synthesized to investigate their effectiveness in enhancing its transport characteristics across the rat nasal mucosa. Acyclovir, a synthetic purine nucleoside analogue derived from guanine, is clinically used in the treatment of herpes simplex viruses (HSV), varicella-zoster virus, Epstein–Barr virus, and cytomegalovirus (19). The drug's oral absorption is poor and variable, whereas its valyl conjugate appears to afford a better bioavailability (20). It was found to be nonabsorbable through the nasal pathway (21) and, thereby, can serve as a good model compound for the study of nasal absorption enhancement. The nasal absorption and metabolism of acyclovir butyrate, pivalate, valerate, and hexanoate were studied using the *in situ* perfusion technique and the significance of this ester prodrug approach for enhanced nasal uptake is discussed.

MATERIALS AND METHODS

Chemicals

Acyclovir, [9-(2-hydroxyethoxymethyl)guanine], was a gift from Burroughs Wellcome Company (Research Triangle Park, NC). Heptanesulfonic acid, acid anhydrides, and 4-dimethylaminopyridine (DMAP) were obtained from Aldrich Chemical Company (Milwaukee, WI). 1-Octanol and dimethyl formamide (DMF) were provided by Fisher Scientific Co. (Fair Lawn, NJ). Other reagents were of analytical grade and were used as received.

Synthesis of Acyclovir Ester Prodrugs

An acid anhydride esterification method (22) was used for synthesizing acyclovir ester prodrugs as shown in Scheme I. Acyclovir (1.0 mmol) was treated with the appropriate acid anhydride (10–20 mmol) and 4-dimethylaminopyridine (0.2 mmol) in DMF (10 mL) at room temperature for 2–3 days. Ethanol (1 mL) was then added and the solution stirred for another 15 min. The solvent was subsequently removed with a rotary evaporator under reduced pressure. The final products were purified by silica gel chromatography using a chloroform–methanol mixture as eluent. Purity was monitored by TLC, HPLC, elemental analysis, and melting point determinations. Structural confirmation was made by ^{1}H-NMR and FAB-MS.

I. Acyclovir ^{1}H-NMR (MeSO-d_6): δ 3.34 (2H, s, CH_2), 3.36 (4H, d, CH_2CH_2), 5.33 (2H, s, NH_2), 7.80 (1H, s, CH). FAB-MS (DTT/DTE) *m/e* 226 (*m* + 1).

II. Acyclovir butyrate ^{1}H-NMR (MeSO-d_6): δ 0.83 (3H, t, J = 7.4 Hz, CH_3), 1.50 (2H, sextet, CH_2), 2.19 (2H, t, J = 7.4 Hz, CH_2), 3.31 (2H, s, CH_2), 3.68 (2H, t, J = 4.6 Hz, CH_2), 4.13 (2H, t, J = 4.6

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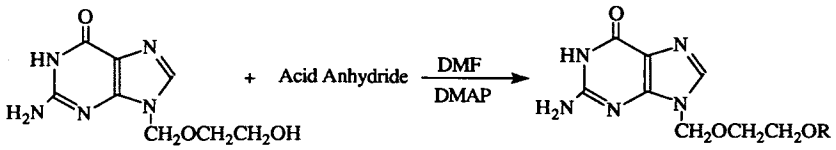

I. R = H acyclovir
II. R = $C(=O)-CH_2CH_2CH_3$ acyclovir butyrate
III. R = $C(=O)-C(CH_3)_3$ acyclovir pivalate
IV. R = $C(=O)-(CH_2)_3CH_3$ acyclovir valerate
V. R = $C(=O)-(CH_2)_4CH_3$ acyclovir hexanoate

Scheme I. Structures of acyclovir esters synthesized in this study.

Hz, CH_2), 5.36 (2H, s, NH_2), 7.84 (1H, t, CH). FAB-MS (DTT/DTE) *m/e* 296 (*m* + 1).

III. Acyclovir pivalate ^{1}H-NMR (MeSO-d_6): δ 1.07 (9H, s, $3CH_3$), 3.31 (2H, s, CH_2), 3.65 (2H, t, *J* = 4.6 Hz, CH_2), 4.10 (2H, t, *J* = 4.6 Hz, CH_2), 5.32 (2H, s, NH_2), 7.81 (1H, s, CH). FAB-MS (DTT/DTE) *m/e* 310 (*m* + 1).

IV. Acyclovir valerate ^{1}H-NMR (MeSO-d_6): δ 0.85 (3H, t, *J* = 7.4 Hz, CH_3), 1.23 (2H, sextet, *J* = 7.5 Hz, CH_2), 1.43 (2H, quintet, *J* = 7.5 Hz, CH_2), 2.22 (2H, t, *J* = 7.4 Hz, CH_2), 3.29 (2H, s, CH_2), 3.65 (2H, t, *J* = 4.6 Hz, CH_2), 4.09 (2H, t, *J* = 4.6 Hz, CH_2), 5.34 (2H, s, NH_2), 7.81 (1H, s, CH). FAB-MS (DTT/DTE) *m/e* 310 (*m* + 1).

V. Acyclovir hexanoate ^{1}H-NMR (MeSO-d_6): δ 0.82 (3H, t, *J* = 7.4 Hz, CH_3), 1.21 (4H, m, CH_2CH_2), 1.48 (2H, m, CH_2), 2.18 (2H, t, *J* = 7.4 Hz, CH_2), 3.30 (2H, s, CH_2), 3.65 (2H, t, *J* = 4.6 Hz, CH_2), 4.09 (2H, t, *J* = 4.6 Hz, CH_2), 5.34 (2H, s, NH_2), 7.81 (1H, s, CH). FAB-MS (DTT/DTE) *m/e* 324 (*m* + 1).

Determination of Aqueous Solubility

The solubility of acyclovir and its ester prodrugs was determined in pH 7.4 isotonic phosphate buffer (PBS) at 37°C. Excess amount of acyclovir or an ester was added to 10 mL phosphate buffer in screw-capped glass scintillation vials and subsequently agitated in a shaker bath at 37 ± 0.5°C for 48 hr. The suspension was then filtered through a 0.45-μm membrane filter to remove undissolved particles. The adsorption of acyclovir and its prodrugs by the membrane was found to be negligible. Nevertheless, the first 2 mL of the filtrate was discarded to eliminate any adsorptive effect by the membrane. After proper dilution with acetonitrile, the supernatant was subjected to HPLC analysis.

Determination of 1-Octanol/PBS Partition Coefficient

Apparent partition coefficients were determined by the shake flask method using mutually saturated 1-octanol and pH 7.4 istonic phosphate buffer at 37°C. An aliquot (5 mL) of 1-octanol saturated phosphate buffer containing known concentrations of a compound was mixed with an equal volume of 1-octanol. The two phases were then allowed to equilibrate at 37°C for 24 hr. The concentration of the compound in the aqueous phase was determined by HPLC and the partition coefficient (PC) was calculated using the following equation:

$$PC = \frac{C_{aq} - C_{eq}}{C_{eq}}$$

C_{aq} is the initial aqueous acyclovir or prodrug concentration at the start and C_{eq} is the aqueous concentration at equilibrium. The prodrugs in this system were found to be chemically stable during the course of the partition experiment.

In Situ Nasal Perfusion Method

The rat *in situ* nasal perfusion technique developed by Hirai *et al.* (23) and Huang *et al.* (13) was used in this investigation because of its simplicity and good reproducibility. Male Sprague–Dawley rats weighing 250 to 350 g were fasted for about 14 to 18 hr prior to an experiment but water was allowed ad libitum. The rats were anesthetized with an intraperitoneal injection of 0.1 mL/100 g body wt of a ketamine (90 mg/mL) and xylazine (10 mg/mL) mixture followed by an additional 0.1 mL/rat every 30 to 45 min to maintain the anesthetic state. After an incision was made in the neck, the trachea was cannulated with a polyethylene tube (PE-200, Intramedic, Clay Adams, NY) to maintain respiration. Another PE-200 tube was inserted through the esophagus toward the posterior part of the nasal cavity and ligated. The passage of the nasopalatine tract was sealed with an adhesive agent (Instant Jet, Cal Goldberg Models Inc., Chicago, IL) to prevent drainage of the solution from the nasal cavity to the mouth. The cannula served to deliver the solution to the nasal cavity. The perfusion medium was circulated by means of a perstaltic pump (Buchler Instruments, Lenexa, KS) at a flow rate of 2 mL/min and recollected into a reservoir. The temperature of the reservoir was maintained at 37 ± 0.5°C during the course of an experiment. A constant perfusate volume of 5 mL was maintained throughout with constant stirring and an aliquot (50 μL) was sampled every 15 min for 1.5 hr.

Analytical Procedures

The concentration of acyclovir remaining in the nasal perfusate was determined by slightly modified HPLC method of Land and Bye (24). The mobile phase composition was then modified appropriately in order to render adequate retention of acyclovir esters. For easy comparison, the mobile phase compositions, flow rates, and internal standards are listed in Table I.

Aliquots (50 μL) were withdrawn periodically from the reservoir and immediately mixed with 100 μL acetonitrile containing an internal standard as listed in Table I. The samples were vigorously vortexed for 30 sec and centrifuged at 10,000 rpm for 15 min in order to precipitate any proteins prior to sample injection onto the HPLC column. The HPLC system was equipped with a Waters Model 510 solvent delivery system, a Rheodyne injector, a Waters Lambda-Max Model 481 multiwavelength UV detector, and a Fisher Recordall Series 5000 strip-chart recorder. Samples (10 μL) were injected onto an Alltech Econosil 10-μm spherical C_{18} reversed-phase column (250 × 4.6 mm) at ambient temperature. The wavelength for detection was set at 254 nm. Peak height ratios of the compounds to internal standards were used for quantitative purposes.

Enzymatic Hydrolysis in Rat Plasma

Rat blood was collected through the jugular vein cannula and centrifuged at 2000*g* for 10 min. The heparinized plasma was then stored in aliquots at −20°C to avoid frequent freezing and thawing. Twenty-five microliters of acyclovir ester stock solution in ethanol (1 m*M*) was added to 475 μL of prewarmed rat plasma to generate an initial drug concentration of 50 μ*M* and the mixture was incubated at 37°C. Samples were withdrawn at appropriate time intervals and mixed with 3 vol of ice-cold acetonitrile to arrest the enzymatic activity. The supernatant was analyzed by HPLC after the mixture was centrifuged at 8000*g* for 10 min.

RESULTS AND DISCUSSION

Physicochemical Properties

The chemical structures of acyclovir and its four aliphatic esters, i.e., butyrate, pivalate, valerate, and hexanoate, are depicted in Scheme I. The physicochemical properties were determined and are listed in Table II, together with the elemental analysis results. As expected, the melting points of the ester prodrugs decreased as a result of lengthening of the carboxylic side chain, a well-recognized phenomenon attributed to the weakening of intermolecular hydrogen bonding. Similarly, the aqueous solubility measurements revealed consequential decreases, whereas the 1-octanol/phosphate buffer partition coefficients exhibited steady increases as the homologous series was ascended. The pivalate ester had a slightly higher melting point than the valerate, however, exhibiting a lower solubility. The partition coefficients of the two prodrugs appear to be comparable in magnitude.

The lipophilicity of derivatives, represented by their partition coefficients, correlates positively with the chromatographic capacity factor (k') on a reversed-phase column (25). To test this generality, k' values for the four derivatives were determined on the HPLC system using 28% acetonitrile: 72% buffer II as the mobile phase. Indeed, a good correlation was established between the logk' and the logPC values, with a linear correlation coefficient of 0.989. Therefore, proper selection of an ester side chain may provide the desired lipophilicity for optimal nasal absorption.

Enzymatic Hydrolysis of Acyclovir Esters by Rat Plasma

The hydrolysis of acyclovir ester prodrugs in rat plasma was studied and the degradation profiles are plotted in Fig. 1. Decreases in acyclovir ester peak height are accompanied by corresponding increases in acyclovir peak height, suggesting the presence of carboxylesterase activity in the rat plasma. Acyclovir, on the other hand, was found to be completely stable in rat plasma during the course of study. Further, the hydrolysis of the four ester prodrugs appears to follow apparent first-order kinetics, as shown in Fig. 1. The observed first-order hydrolytic rate constants and the corresponding half-lives of acyclovir prodrugs are listed in Table I. Apparently, increases in the side-chain length and lipophilicity led to facilitated cleavage of the ester bond in the order hexanoate > valerate > butyrate, a phenomenon established for many types of ester prodrugs previously (26). This pattern is also indicative of possibly enhanced binding of the substrate to a hydrophobic pocket at the active center of carboxylesterase. Branching of the side chain, on the other hand, resulted in much slower hydrolysis of acyclovir piv-

Table I. Summary of HPLC Analytical Conditions, Enzymatic Degradation Rate Constants and Half-Lives, Apparent First-Order Rat Nasal Absorption Rate Constants, and Percentages of Absorption in 90 min of Acyclovir and Its Ester Prodrugs

Drug moiety	Mobile phase composition	Flow rate (mL/min)	Internal standard & concentration	k_{deg} (min^{-1}) × 10^{3a}	$t_{1/2}$ (min)	k_{abs} (min^{-1}) × 10^{3b}	% uptake in 90 min[b]
I	2% ACN in buffer I[c]	1	Thymine, 20 μg/mL	—	—	0	0
II	22% ACN in buffer II[d]	2	Caffeine, 0.1 m*M*	27.07	25.60	1.25 ± 0.30	9.66 ± 3.90
III	28% ACN in buffer II	2	Caffeine, 0.1 m*M*	6.60	105.02	1.66 ± 0.12	14.82 ± 0.45
IV	28% ACN in buffer II	2	Caffeine, 0.1 m*M*	42.89	16.16	1.95 ± 0.49	17.17 ± 4.44
V	35% ACN in buffer II	2	Benzamide, 0.2 m*M*	192.20	3.61	3.79 ± 1.08	30.08 ± 3.57

[a] The total protein concentration in rat plasma is 64 mg/mL.
[b] Data represent means ± SD of three determinations.
[c] Buffer I contains 1 m*M* heptanesulfonic acid sodium salt and 10 m*M* ammonium acetate.
[d] Buffer II contains 0.1 m*M* heptanesulfonic acid sodium salt and 10 m*M* ammonium acetate. The pH of both buffer I and buffer II was adjusted to 5.0 with glacial acetic acid.

Table II. Physicochemical Properties of Acyclovir and Its Ester Prodrugs

Compound	Formula	Melting point (°C)	Solubility (mM ± SD)[a]	PC[b]		Elemental analysis (%) Calculated	Found
I	$C_8H_{11}N_5O_3$	244–246	11.18 ± 0.47	0.06 ± 0.01	C	42.62	42.44
					H	4.88	5.08
					N	31.08	30.79
II	$C_{12}H_{17}N_5O_4$	225–230	4.57 ± 0.07	0.83 ± 0.04	C	48.76	48.05
					H	5.76	5.26
					N	23.70	23.47
III	$C_{13}H_{19}N_5O_4$	215–217	1.49 ± 0.04	2.01 ± 0.09	C	50.43	50.99
					H	6.14	5.66
					N	22.63	22.48
IV	$C_{13}H_{19}N_5O_4$	205–207	1.64 ± 0.03	2.35 ± 0.23	C	50.43	50.40
					H	6.14	6.35
					N	22.63	23.47
V	$C_{14}H_{21}N_5O_4$	200–202	0.71 ± 0.03	8.58 ± 0.10	C	51.96	51.99
					H	6.49	6.29
					N	21.65	21.58

[a] In pH 7.4 isotonic phosphate buffer at 37°C.
[b] 1-Octanol/phosphate buffer at 37°C.

alate. This is attributed to the steric hindrance of the substituent, thus inhibiting its binding to the active center of the enzyme. Therefore, both the apolar and the steric natures of the substituent play an important role in determining the rate of enzymatic cleavage of acyclovir ester prodrugs in plasma.

Nasal Transport and Simultaneous Hydrolysis of Acyclovir Ester Prodrugs

Acyclovir and its ester prodrugs, all dissolved in pH 7.4 isotonic phosphate buffer at an initial concentration of 50 μM, were perfused through the rat nasal cavity in order to demonstrate the effect of improved lipophilicity on nasal uptake. Since plasma hydrolysis data suggest possible breakdown of prodrugs simultaneously with nasal uptake, both the prodrug and the resulting acyclovir concentrations in the perfusate were subsequently measured. Acyclovir does not appear to be transported across the nasal mucosa to any measurable extent (Fig. 2). Its accumulation in the perfusion medium thus indicates the event of nasal mucosal cleavage of the prodrugs. Simultaneous nasal hydrolysis of other types of esters has been documented, and a parallel approach was proposed by Huang *et al.* (14) in order to estimate individually the nasal absorption rate constant (k_{abs}) and hydrolysis rate constant (k_{hyd}) by virtue of the following equation: $k_{dis} = k_{abs} + k_{hyd}$, where k_{dis} denotes the overall rate constant of prodrug disappearance.

As expected, when hexanoate ester was perfused through the rat nasal cavity, measurable amounts of acyclovir accumulation were detected. As shown in Fig. 3, approximately 6% molar equivalence of acyclovir was formed at the end of a 90-min perfusion procedure. While an overall first-order rate constant of disappearance was found to be $5.03 \times 10^{-3} \pm 9.93 \times 10^{-4}$ min^{-1} (mean ± SD; $n = 3$), a k_{abs} of $3.79 \times 10^{-3} \pm 1.08 \times 10^{-3}$ min^{-1} was obtained after subtracting the contribution of hydrolysis. Therefore, a value of 1.24×10^{-3} min^{-1} can be calculated for hexanoate k_{hyd}.

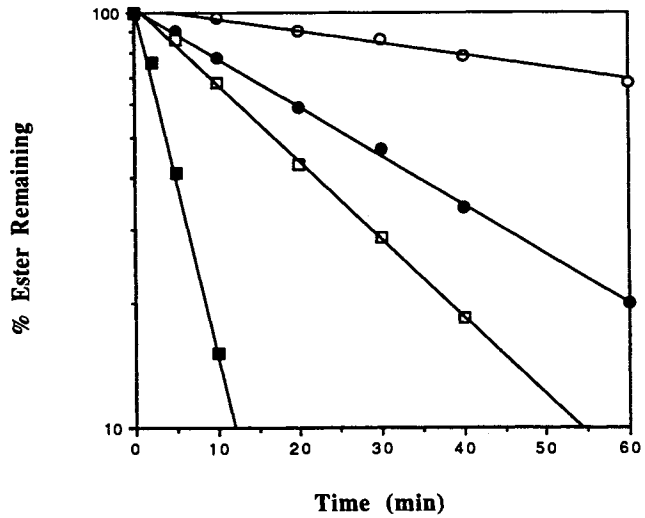

Fig. 1. Hydrolytic profiles of acyclovir ester prodrugs in rat plasma at 37°C as a function of time. (○) pivalate; (●) butyrate; (□) valerate; (■) hexanoate.

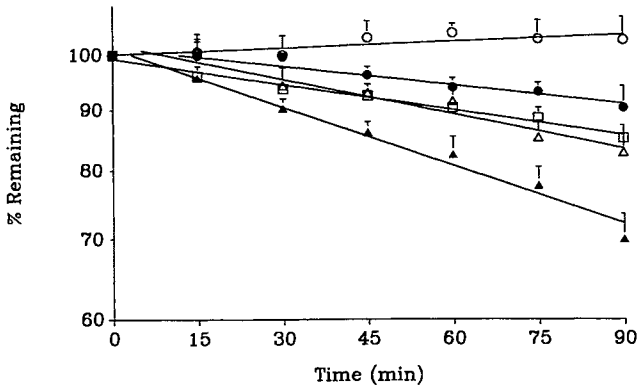

Fig. 2. Disappearance of acyclovir and its ester prodrugs from the nasal perfusate. The initial concentration of all compounds was 50 μM. (○) Acyclovir; (●) acyclovir butyrate; (□) acyclovir pivalate; (△) acyclovir valerate; (▲) acyclovir hexanoate.

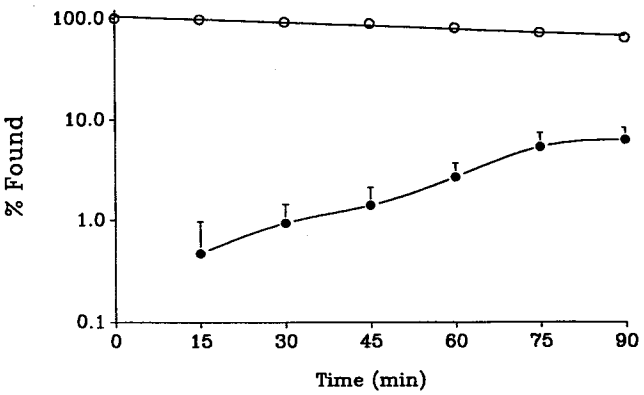

Fig. 3. Disappearance of acyclovir hexanoate (○) and accumulation of acyclovir (●) following rat nasal perfusion.

Knowing the concentrations of both acyclovir and the intact hexanoate ester at each sampling point, the contribution of nasal transport could be calculated. The percentage of total acyclovir equivalence remaining is plotted semilogarithmically against time as shown in Fig. 2. Other acyclovir prodrugs, i.e., butyrate, pivalate, and valerate, exhibited minimal hydrolysis during perfusion and the percentage loss was attributed solely to nasal uptake. The results are also presented schematically in Fig. 2. The pseudo-first-order absorption rate constants were obtained through linear regression and are listed in Table I, together with the respective percentages of absorption in 90 min.

Transnasal absorption of acyclovir can indeed be improved by the prodrug approach, although only to a moderate extent. Proper manipulation of the side-chain length and configuration may improve lipophilicity while minimizing mucosal hydrolysis. Branched-chain aliphatic esters may meet both prerequisites. Indeed, these types of prodrugs can in fact improve nucleoside transport across the blood–brain barrier (27).

The importance of nonpeptide substrate lipophilicity on intranasal absorption is well documented. Huang *et al.* (13) found that nasal absorption of benzoic acid exhibited a pH-dependent behavior which could be attributed, at least in part, to its ionizable nature. Studies by the same research group on a series of barbiturates also revealed moderate absorption improvement, as a result of a 50-fold difference in the partition coefficient. Our data resemble their findings in that a more than 140-fold increase in 1-octanol/phosphate buffer partition coefficient resulted only in a 30% uptake of acyclovir in 90 min, coupled with an additional 6% hydrolysis. The magnitude of nasal uptake of the hexanoate ester, though, closely resembles the oral acyclovir availability. A series of progesterone derivatives, possessing the same basic steroid nucleus but differing in the number and location of hydroxy groups, were studied to correlate the rate and extent of *in vivo* absorption with penetrant lipophilicity (15,16). The findings of Corbo *et al.* (15) indicate that both the rate and the extent of nasal uptake of steroidal hormones are sufficiently influenced by penetrant lipophilicity. A hyperbolic relationship between the systemic bioavailability and the nasal mucosal partition coefficients of the drugs was also established.

Nasal systemic drug delivery avoids the hepatic first-pass metabolism. However, Sarkar (28) raised the issue of "pseudo-first-pass effect" to account for the enzymatic barrier of the nasal mucosa. Indeed, metabolism of both protein and nonproteinaceous compounds by nasal enzymes, including Phase I, conjugative Phase II, and proteolytic enzymes, can be extensive. While aminopeptidases are responsible for the enzymatic deactivation of peptide and protein drugs, carboxylesterases cleave ester compounds efficiently. The specific activity of nasal esterase is comparable to that of the liver and greater than that of the kidney, lung, or blood (29). The presence of such carboxylesterase activity challenges ester prodrug design aimed at improving penetrant lipophilicity for better nasal mucosal uptake. Our perfusion data indicated that only the hexanoate ester (PC $\approx$ 8) is prone to nasal carboxylesterase cleavage. Further lengthening of the side chain may prove to be less beneficial. Such a conclusion becomes more evident when absorption enhancers are used and carboxylesterase activity is removed/exposed to act on these prodrugs (30).

In conclusion, ester-type prodrug design may prove useful in enhancing nasal drug uptake. However, simultaneous hydrolysis of the ester bond prior to absorption hampers this effort. Prodrugs with branched aliphatic side chains should be synthesized to overcome this problem.

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