Bile Salt–Fatty Acid Mixed Micelles as Nasal Absorption Promoters. III. Effects on Nasal Transport and Enzymatic Degradation of Acyclovir Prodrugs

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The absorption enhancement and presystemic degradation kinetics of a homologous series of acyclovir 2′-ester prodrugs were investigated in rats using the *in situ* nasal perfusion technique in the presence of bile salt–fatty acid mixed micells. *In vitro* incubation studies indicated that nasal perfusate containing a mixed micellar solution generated higher ester-cleaving activity than isotonic phosphate buffer washings. Inhibitor screening and substrate specificity studies demonstrated the enzyme to be most likely carboxylesterase rather than true cholinesterase. The extent of prodrug cleavage by the carboxylesterase appears to correlate well with the substrate lipophilicity for esters with linear acyl chains. On the other hand, branching of the acyl side chain significantly retards acyclovir prodrug breakdown. To estimate the nasal epithelial membrane and cytoplasmic damaging effect caused by sodium glycocholate (NaGC)–linoleic acid (15 m*M*:5 m*M*) mixed micelles, the release profiles of 5′-nucleotidase (5′-ND), lactate dehydrogenase (LDH), and carboxylesterase in the nasal perfusate were measured as a function of time. The results indicated that the activities of all three enzymes resulting from the mixed micellar solution appeared to be significantly higher than those caused by 15 m*M* NaGC alone. The apparent nasal absorption rate constants of acyclovir and its butyrate, valerate, pivalate, and hexanoate ester prodrugs in mixed micellar solutions containing an esterase inhibitor (1 m*M* phenylmethylsulfonyl fluoride) were individually calculated. Without an inhibitor, lengthening of the linear acyl side chain of the prodrug resulted in greatly accelerated degradation coupled with moderate absorption improvement. The solubilities and micellar binding constants of acyclovir prodrugs were also determined. Mixed micelles composed of 15 m*M* NaGC and 5 m*M* linoleic acid are incapable of incorporating these esters into the micellar cavity, although NaGC micelle alone can actively solubilize them in a concentration-dependent manner.

KEY WORDS: absorption; bile salts; carboxylesterase degradation; ester prodrugs; lactate dehydrogenase; linoleic acid; mixed micelles; nasal drug delivery; 5′-nucleotidase.

INTRODUCTION

Intranasal drug delivery has attracted attention because of its ease of administration, rapid drug absorption, avoidance of hepatic first-pass metabolism, and noninvasive nature (1). Despite the fact that significant progress has been made in this area, several important drug delivery issues are still facing pharmaceutical scientists. For instance, hydrophilic organic compounds, peptides, and peptidomimetics which can be considered as desirable drug candidates for noninvasive delivery mostly show a very limited nasal bioavailability, and no therapeutically acceptable modes of their noninvasive delivery to systemic circulation are currently available.

In order to improve the mucosal systemic bioavailability of these compounds from nasal administration, two approaches are commonly sought, i.e., structural modification and formulation manipulation. Physicochemical properties such as the aqueous-to-lipid partition coefficient, pK_a, and molecular weight all significantly affect transnasal drug uptake (2–4); derivatization of a drug to its bioreversible prodrug form appears to serve as a valuable tool. Such an approach has met some success in enhancing oral, ocular, and transdermal drug transport. The strategy to overcome physical and enzymatic barriers of nasal mucosa involves manipulation of the formulation variables, including the use of chemical enhancers.

In the preceding paper (5), the physicochemical properties, plasma enzymatic bioconversion, and *in situ* rat nasal absorption kinetics of a homologous series of acyclovir ester prodrugs are detailed. A combined strategy involving both structural modification and incorporation of absorption enhancers is scrutinized in this investigation. Possible mechanisms responsible for improved acyclovir nasal uptake are postulated by individually examining the physical barrier integrity, enzymatic barrier function, and contribution of micellar solubilization. Furthermore, the degradative enzyme binding specificity is characterized.

MATERIALS AND METHODS

Materials

Acyclovir [9-(2-hydroxyethoxymethyl)guanine] was a gift from Burroughs Wellcome Company (Research Triangle Park, NC). Its aliphatic ester prodrugs were synthesized using an anhydride esterification method (5). For easy comparison, the chemical structures of acyclovir and its ester prodrugs are illustrated in Scheme I. Sodium glycocholate, linoleic acid, sodium heptanesulfonate, thymine, caffeine, and benzamide were obtained from Sigma Chemical Company (St. Louis, MO). All other reagents were of analytical grade and used as received.

Preparation of Mixed Micellar Solutions

Sodium glycocholate was dissolved in isotonic phosphate-buffered saline solution (PBS), pH 7.4, consisting of 14 m*M* KH_2PO_4, 57 m*M* Na_2HPO_4, and 70 m*M* NaCl. Linoleic acid was then added and the solution was subsequently sonicated for 5 min at room temperature.

In Situ Nasal Perfusion Method

The rat *in situ* nasal perfusion technique developed by Hirai *et al.* (6) and Huang *et al.* (3) was used in this investigation because of the fairly simple nature of the experimental setup and good reproducibility of the method. Male Spra-

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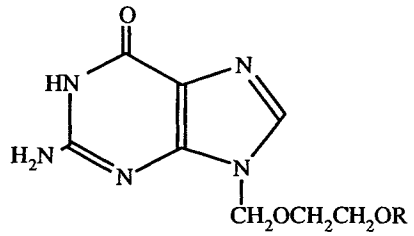

R =		
	H	acyclovir
	$C-CH_2CH_2CH_3$ (O)	acyclovir butyrate
	$C-C(CH_3)_3$ (O)	acyclovir pivalate
	$C-(CH_2)_3CH_3$ (O)	acyclovir valerate
	$C-(CH_2)_4CH_3$ (O)	acyclovir hexanoate

Scheme I. Structures of acyclovir and its ester derivatives.

gue–Dawley rats weighting 250 to 350 g were fasted for about 14 to 18 hr prior to use 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 peristaltic 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 a slightly modified HPLC method of Land and Bye (7). The mobile phase composition was then modified appropriately to render adequate retention of acyclovir esters. The mobile phase compositions, flow rates, and internal standards used have been reported previously (5).

Aliquots of 50 μL samples were withdrawn periodically from the reservoir and immediately mixed with 100 μL of acetonitrile containing an internal standard. The samples were vigorously vortexed for 30 sec and centrifuged at 10,000 rpm for 15 min 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. Thymine (20 μg/mL) and benzamide (0.2 m*M*) were used as the internal standards for acyclovir and acyclovir hexanoate, while caffeine served as the internal standard for acyclovir butyrate, valerate, and pivalate esters. Peak height ratios of the compounds to internal standard were used for the quantitation of analytes.

In vitro Enzymatic Degradation of Acyclovir Ester Prodrugs in Rat Nasal Washings

PBS solution, pH 7.4, with or without mixed micellar components was perfused though the rat nasal cavity for 90 min as described previously. The perfusate at the end of the experiment was collected and stored at −20°C until further use. Aliquots (200 μL) of prewarmed nasal perfusate were then mixed with equal volumes of 50 μ*M* ester stock solutions (37°C) and vortexed. A zero-time sample (100 μL) was taken immediately and mixed with 2 vol of acetonitrile, containing an internal standard, to arrest the enzyme activity. The mixture was then incubated for 90 min at 37°C and a 100-μL sample was taken again and subjected to the same treatment. After centrifugation at 8000 rpm for 10 min, the ester concentrations in the supernatant of both samples were determined by HPLC analysis.

Various enzyme inhibitors were evaluated with respect to their relative effectiveness in inhibiting nasal carboxylesterase. Stock solutions of each inhibitor were prepared in water or ethanol, and 10 μL was added to 190 μL of nasal perfusate and incubated for 15 min at 37°C prior to prodrug addition.

Solubility of Acyclovir Prodrugs in Micellar Solutions and Binding Constant Determinations

Micellar solutions of sodium glycocholate in isotonic phosphate buffer, pH 7.4, were prepared to contain 0, 2, 4, 6, 10, 20, 30, 40, and 50 m*M* NaGC. Excess amounts of ester prodrugs were added to these micellar solutions and incubated at 37°C for 2 days until equilibrium was reached. The suspension was filtered through a 0.45-μm membrane and the prodrug concentration determined by HPLC.

A pseudophase model is considered for the calculation of binding constants in which a micellar solution is composed of two separate phases, i.e., the bulk aqueous phase and the micellar phase. The micellar binding constant K_{eq} can be calculated from the following equation (8):

$$\frac{S_t - S_0}{S_0} = K_{eq}\,[C - \mathrm{CMC}]$$

The term S_t represents the total substrate concentration, S_0 denotes the unbound substrate concentration, C is the total bile salt concentration, and CMC is the critical micellar concentration. The slope of the plot of $(S_t - S_0)/S_0$ versus C,

therefore, generates the micellar equilibrium binding constant K_{eq}.

To investigate the displacing effect of linoleic acid on prodrug micellar solubilization, the solubilities of acyclovir hexanoate in mixed micellar solutions containing 30 m*M* NaGC and 0, 10, 20, 30, and 40 m*M* linoleic acid were similarly measured.

Enzyme Activity Measurements

The activity of a nasal membrane-bound marker enzyme, 5′-nucleotidase (5′-ND; EC 3.1.3.5) in the perfusate was analyzed using Sigma Diagnostic Procedure 265. The activity of a nasal epithelial intracellular enzyme, lactate dehydrogenase (LDH; EC 1.1.1.27), was determined by Sigma Diagnostic Procedure 500.

Nonspecific carboxylesterase (EC 3.1.1.1) activity in the nasal perfusate was measured by a modification of several previously reported procedures (9–11). *p*-Nitrophenyl butyrate was used as a standard substrate and a stock solution of 0.2 *M* in acetone was first prepared. The final concentration of *p*-nitrophenyl butyrate used in this study was 0.2 m*M* dissolved in 0.1 *M* sodium phosphate buffer, pH 7.8. The volume fraction of acetone in this solution was, therefore, 1%, at which no inhibitory effect on carboxylesterase activity was detected (11). All solutions of *p*-nitrophenyl butyrate were prepared immediately prior to use to minimize chemical hydrolysis. After the addition of 10 μL perfusate into 1.5 mL *p*-nitrophenyl butyrate solution, an increment in absorbance at 400 nm was followed with a Beckman DU-7 spectrophotometer. The slopes of absorbance versus time plots from 1 to 5 min post-sample addition were averaged and utilized thereafter to calculate carboxylesterase activity. A molar absorption coefficient of 17,000 M^{-1} was used for this purpose, and 1 U was defined as the activity required to hydrolyze 1.0 μmol of the substrate/min at pH 7.8.

RESULTS

Nasal Absorption Enhancement of Acyclovir by Sodium Glycocholate and Mixed Micellar Solutions

The transnasal absorption kinetics of acyclovir from isotonic PBS, 15 m*M* NaGC in PBS, and mixed micellar solutions composed of 15 m*M* NaGC and 5 m*M* linoleic acid in PBS was first investigated. No measurable loss of acyclovir was noted in PBS in the absence of any absorption enhancers. The incorporation of 15 m*M* NaGC in PBS significantly improved acyclovir disappearance from the perfusate such that 39 ± 5% (mean ± SD; $n = 3$) of the acyclovir was lost from the reservoir in 90 min. Solubilization of 5 m*M* linoleic acid in 15 m*M* NaGC further increased acyclovir absorption, to 58 ± 5%, in 90 min. All three profiles (not shown) were well fitted by the apparent first-order kinetics and the absorption rate constants are 0, 0.0056 ± 0.0009, and 0.0097 ± 0.0013 min^{-1} ($n = 3$), respectively. Therefore, mixed micelles exhibit a synergistic effect in enhancing nasal drug transport, which is in good agreement with our previous reports (12,13). Mixed micellar components were used subsequently in all studies involving acyclovir ester prodrug transport.

In Vitro Hydrolysis of Acyclovir Ester Prodrugs in Rat Nasal Washings

Although nasal drug delivery has the advantage of circumventing hepatic first-pass metabolism, many degradative enzymes are still present in the nasal mucus and respiratory and olfactory mucosal cells. To clarify whether any nasal ester hydrolase activity was present during perfusion, an *in vitro* incubation study was undertaken as suggested by Faraj *et al.* (14). Table I summarizes the ester degradative activities present in both phosphate buffer perfusate and mixed micellar perfusate. PBS washing caused only slight hydrolysis of acyclovir hexanoate, a prodrug possessing the highest octanol/water partition coefficient in this series. Mixed micellar washing, on the other hand, appears to release strong carboxylesterase activity such that only 4% hexanoate remained after a 90-min incubation. The order of substrate suscepibility to mixed micellar solubilized enzymes follows: hexanoate > valerate > butyrate > pivalate. To characterize the nature of the nasal enzyme(s) responsible for ester prodrug cleavage, various chemical inhibitors have been incorporated into the rat nasal washings prior to the addition of prodrug substrates. These enzyme inhibitors are phenylmethylsulfonyl fluoride (PMSF; 1 m*M*), neostigmine bromide (1 m*M*), acetazolamine (0.1 m*M*), and *p*-chloromercuribenzoate (0.1 m*M*). PMSF is a nonspecific serine protease inhibitor which binds very efficiently to the specific ester binding pocket of the enzyme due to its high hydrophobicity (15). The other three inhibitors act as a cholinesterase inhibitor, a carbonic anhydrase inhibitor, and an arylesterase modulator, respectively (16).

The relative efficacies of these inhibitors on nasal acyclovir ester-hydrolyzing activity are listed in Table I. All enzyme inhibitors except PMSF were only marginally effective. PMSF, however, completely arrested the enzyme activity at a concentration of 1 m*M*. In addition to performing inhibitor screening experiments, acetylcholine chloride was tested as a potential substrate. However, only minimal true acetylcholinesterase activity was detected from mixed micellar nasal washings (5.3 ± 0.2 Rappaport U/mL). These results suggest that the nasal ester hydrolytic activity is due mainly to carboxylesterase rather than true cholinesterase.

Table I. Effect of Different Inhibitors on the Carboxylesterase Activity Present in Rat Nasal Perfusate[a]

	% of acyclovir esters remaining after 90 min of incubation at 37°C						
	Mixed micellar perfusate					PBS perfusate	
Substrate	None	I	II	III	IV	None	I
Hexanoate	4	102	5	11	5	94	100
Valerate	69	100	—	—	—	99	100
Butyrate	92	100	—	—	—	100	—
Pivalate	97	100	—	—	—	100	—

[a] The rat nasal cavity was washed with either mixed micellar solution or isotonic PBS buffer at 37°C for 90 min and was then used for incubation with acyclovir esters. I, 1 m*M* PMSF; II, 1 m*M* neostigmine bromide; III, 0.1 m*M* acetazolamine; IV, 0.1 m*M* *p*-chloromercuribenzoate.

The participation of a serine residue at the active site of carboxylesterase which is known to be inhibited by organophosphorus- and fluorine-containing compounds has been well recognized. Since the hydrophobic nature of a substrate also plays an important role in the carboxylesterase–substrate binding step, neither positively charged nor negatively charged hydrophilic compounds can be hydrolyzed by this enzyme (17). Therefore, carboxylesterase and cholinesterase activity of the nasal mucosa can be easily discerned.

Release of 5′-ND, LDH, and Carboxylesterase from Rat Nasal Epithelia

Since a considerable amount of carboxylesterase activity was released from the *in vitro* nasal incubation studies, experiments were directed toward identifying the extent of nasal mucosal damaging effects from the mixed micellar formulation. The membrane-bound enzyme, 5′-ND, the cytosolic enzyme, LDH, and carboxylesterase activities were measured at different time points during a perfusion run.

Figure 1 schematically depicts the activity–time profiles of these three marker enzymes. The release profiles of 5′-ND and LDH following PBS and 15 m*M* NaGC in PBS perfusion have been reported previously from this laboratory (18). Therefore, only mixed micellar perfusion results are now presented. Perfusion with mixed micellar adjuvants apparently caused more severe nasal membrane damage than that caused by NaGC alone. At 90 min of perfusion time, the activities of 5′-ND were found to be 145 ± 12 and 88 ± 12 U/L, respectively, in the presence of mixed micelles and NaGC alone. LDH assay provided more insights into the intracellular-damaging mechanisms. The LDH release profiles in the perfusate reached 3.93 ± 0.20 KU/mL at 90 min, representing a significantly higher value than that of 15 m*M* NaGC alone (1.04 ± 0.40 KU/mL).

The release profiles of carboxylesterase following nasal perfusion of PBS, 15 m*M* NaGC, and mixed micelles containing 15 m*M* NaGC and 5 m*M* linoleic acid are also depicted in Fig. 1. PBS washing up to 90 min did not result in any significant release of this enzyme, with a mean activity of only 9.4 ± 4.2 U/L. Perfusion with 15 m*M* NaGC, however, resulted in a greatly elevated extent of carboxyesterase release, such that 266 ± 107 U/L was detected at 90 min. Mixed micellar perfusion further increase carboxylesterase release to 1545 ± 147 U/L in a 90-min perfusion interval. Statistically significant differences in carboxylesterase activities were found among the three treatment conditions as shown by analysis of variance (ANOVA) with $P < 0.05$.

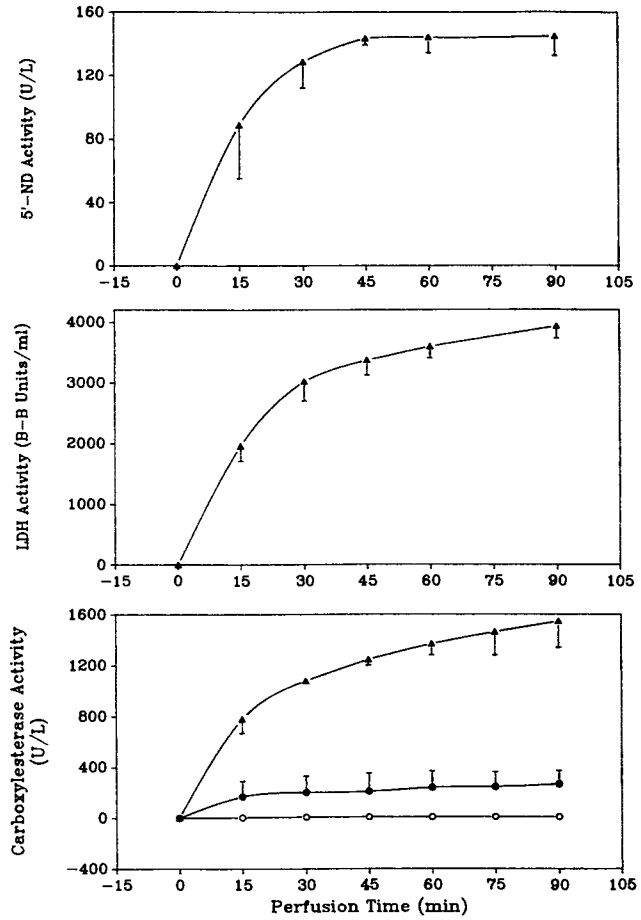

Fig. 1. The activity profiles of 5′-ND, LDH, and carboxylesterase released in the perfusate in the presence of PBS (○), 15 m*M* NaGC (●), and mixed micelles composed of 15 m*M* NaGC and 5 m*M* linoleic acid (▲).

Micellar Solubilization of Acyclovir Ester Prodrugs and Binding Constant Determinations

The solubilities of acyclovir hexanoate in PBS at 37°C in the presence of various concentrations of NaGC are shown in Fig. 2. Below the CMC of NaGC (9 m*M*), the solubility of hexanoate increased only slightly with increasing NaGC concentration. However, above the CMC, the solubility increased linearly as a function of bile salt concentration. Subsequently, the solubilities of acyclovir, butyrate, and valerate esters were determined at 0, 20, 30, 40, and 50 m*M* NaGC. The values of $(S_t - S_0)/S_0$ were calculated and plotted against the bile salt concentration. The micellar binding constants, obtained from the slopes of these lines, are 1.2, 3.2, 12.0, and 36.7 M^{-1} for acyclovir, butyrate, valerate, and hexanoate esters, respectively. It is evident that with a longer ester side-chain length, the 1-octanol/PBS partition coefficient is enhanced and the micellar binding efficiency also increases in a stepwise manner. Good correlation exists between the micellar binding constants and the corresponding 1-octanol/PBS partition coefficients ($r^2 = 0.998$).

Since mixed micellar solutions are used as absorption enhancers of acyclovir ester prodrugs, it is necessary to determine the incorporation efficiency of these compounds into the cylindrical bile salt micellar cores partially filled with linoleic acid molecules. Figure 3 illustrates the solubility of acyclovir hexanoate in the presence of 30 m*M* NaGC and 0, 10, 20, 30, and 40 m*M* linoleic acid. Without linoleic acid, 30 m*M* NaGC increased the solubility of acyclovir hexanoate from 0.71 to 1.21 m*M*, representing an entrapment efficiency of 41%. Solubilization of linoleic acid, however, appears to inhibit micellar incorporation of the hexanoate ester competitively, leading to an almost-linear reduction in its total solubility. Least-squares regression of the linear segment of the plot generated a slope of −0.0162 with a corre-

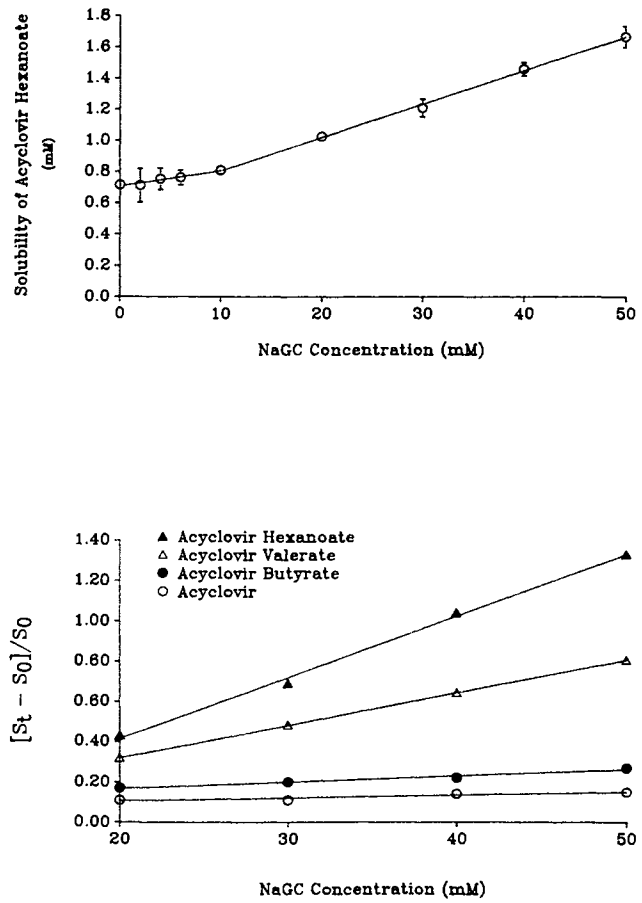

Fig. 2. Solubilization of acyclovir and its ester prodrugs in sodium glycocholate (NaGC) micelles. Top: Solubility of acyclovir hexanoate expressed as a function of NaGC concentration. Above the CMC of the system (9 m*M*), increases in the ester solubility correlate linearly with the bile salt concentration. Bottom: Micelle/PBS partition coefficients of acyclovir and esters plotted as a function of bile salt concentration. The slopes give the binding constants of the micelle for acyclovir and its prodrugs.

lation coefficient of 0.993. Therefore, linoleic acid replaces entrapped acyclovir hexanoate in proportionate manner such that each 100 molecules of linoleic acid replaces 1.6 molecules of hexanoate.

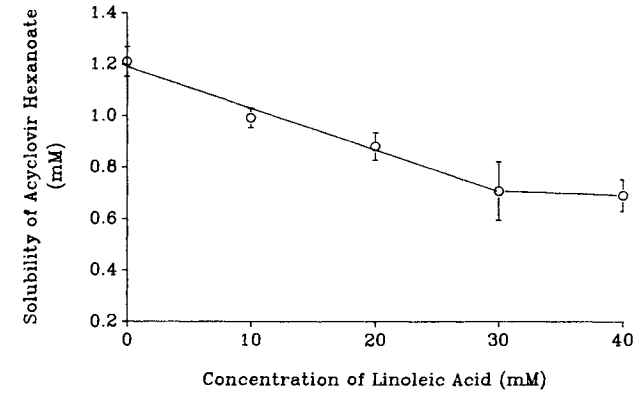

Fig. 3. Displacement of acyclovir hexanoate by linoleic acid incorporated in the core of cylinder-type micelles.

The mixed micellar solution, therefore solubilizes substrate molecules at a much lower efficiency than the bile salt micellar solution alone. With this information in mind, we determined the solubilities of acyclovir and its ester prodrugs in mixed micelles composed of 15 m*M* NaGC and 5 m*M* linoleic acid. The molar solubilities (37°C) in this mixed micellar solution are 11.57 ± 0.70, 4.42 ± 0.07, 1.75 ± 0.04, and 0.72 ± 0.09 m*M* for acyclovir, butyrate, valerate, and hexanoate ester, respectively, values very similar to their solubilities in PBS, i.e., 11.18 ± 0.47, 4.57 ± 0.07, 1.64 ± 0.03, and 0.71 ± 0.03 m*M*, suggesting that there is no significant incorporation of these compounds into mixed micellar cavities.

Nasal Absorption and Degradation of Acyclovir Ester Prodrugs in the Presence of Mixed Micelles

In situ nasal perfusion of acyclovir and its butyrate, pivalate, valerate, and hexanoate esters was performed with an initial 50 μ*M* drug concentration. Mixed micelles consisting of 15 m*M* NaGC and 5 m*M* linoleic acid were used as the permeation enhancer component, with and without the incorporation of 1 m*M* PMSF as the nasal carboxylesterase inhibitor. The percentage loss–time profiles are illustrated schematically in Fig. 4 and the apparent first-order rate constants of disappearance are listed in Table II. In addition to analyzing the ester prodrug content as a function of time, the appearance of regenerated acyclovir was followed. In the absence of the esterase inhibitor PMSF, the concentration of acyclovir regenerated was found to increase in the perfusates, indicating parallel degradation of the prodrugs. In the presence of 1 m*M* PMSF, no measurable acyclovir concentration in the perfusate could be detected. The serine protease inhibitor efficiently inhibited the nasal carboxylesterase activity for at least 90 min. Based on this observation, the apparent rate constants of disappearance in mixed micellar solutions can be subdivided into two independent processes, i.e., absorption and degradation. This parallel mechanism has been proposed previously by Huang *et al.* (19) to determine the contribution of the individual processes to the overall disappearance rate constants. The following equation was derived: $k_{dis} = k_{obs} + k_{deg}$, where k_{dis} is the overall apparent first-order rate constant of nasal drug disappearance, k_{obs} denotes the observed drug absorption rate constant, and k_{deg} is the rate constant for hydrolytic degradation. Therefore, k_{hyd} could be approximated from the k_{dis} and k_{obs} values. For comparative purposes, the calculated k_{deg}'s and the ratios of k_{deg}/k_{dis} in terms of percentages are also listed in Table II.

DISCUSSION

Because of its undesirable physicochemical properties (both low aqueous and low lipid solubility), the gastrointestinal absorption of acyclovir is limited, exhibiting both large intersubject and interspecies variations (20). Studies on alternative routes of administrations such as transdermal (21) and transnasal (18) pathways all indicated negligible acyclovir transport in the absence of any promoters. This poor transmembrane permeation is attributable at least partly to its low octanol/water partition coefficient.

Using acyclovir as a model compound, we found that a

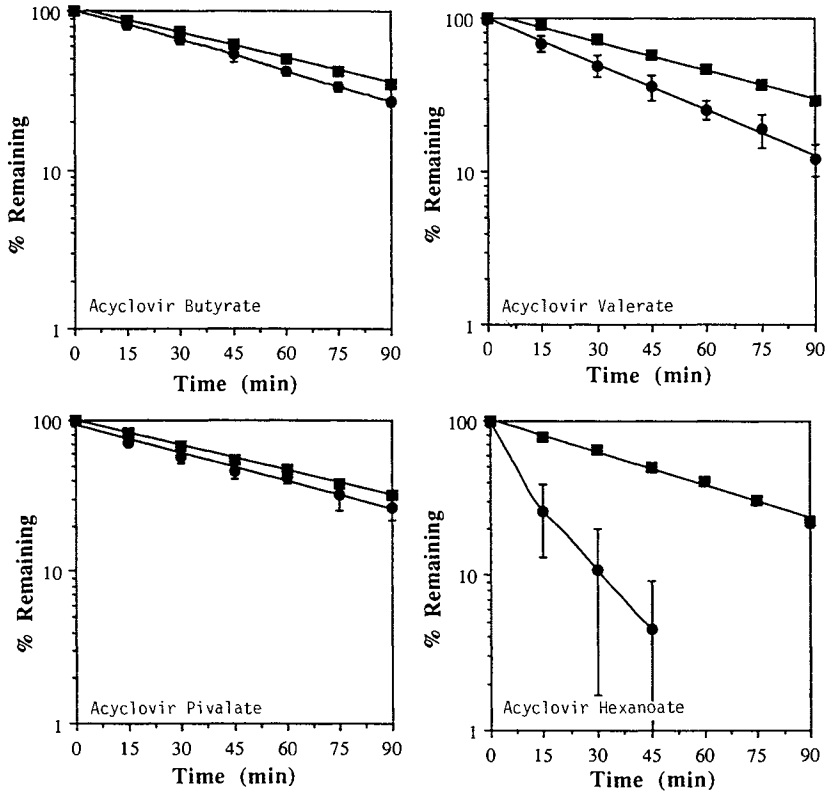

Fig. 4. Disappearance of acyclovir ester prodrugs from the perfusate in the absence (●) and presence (■) of the carboxylesterase inhibitor PMSF (1 m*M*).

two-component micellar system consisting of 15 m*M* NaGC and 5 m*M* linoleic acid, which has been extensively studied by us and other research groups (12,13,18), significantly promoted nasal acyclovir uptake. The underlying mechanisms of bile salt-induced nasal absorption enhancement have been proposed to be a combination of several modes (22), i.e., a direct membrane damaging effect ranging from dilation of the paracellular junction to extensive bilayer destruction, inhibition of nasal degradative enzymes thus reducing presystemic cleavage, and micellar solubilization of the penetrant. The last mode of action leads to two distinctly opposite consequences: (i) increased total solubility of a hydrophobic substrate and creation of a higher juxtamembrane concentration gradient and (ii) reduced thermodynamic activity of the penetrant due to partial incorporation into the micellar interior. Each of these mechanisms was, therefore, assessed with respect to its contribution to the nasal absorption of these rather labile ester prodrugs.

First, the nasal mucosal damaging effect of a mixed micellar system was characterized by means of a biochemical approach which utilizes the measurement of marker enzymes release from the nasal cavity as a toxicity index (18). The release of two marker enzymes, i.e., 5′-ND and LDH, represents the relative extent of nasal membrane versus cytoplasmic damaging effect during a perfusion experiment. As shown in Fig. 1, a combination of bile salt–fatty acid mixed micelles exerts more severe nasal mucosal damage than the bile salt alone. The solubilization of linoleic acid by bile salt has made this lipid more available at the membrane surface and the disturbance of such polar unsaturated fatty acids on

Table II. Apparent First-Order Nasal Absorption and Enzymatic Degradation Rate Constants of Acyclovir and Its Ester Prodrugs[a]

Compound	k_{dis} (min^{-1}) × 10^3 in mixed micellar solution	k_{obs} (min^{-1}) × 10^3 in mixed micellar solution containing 1 m*M* PMSF	k_{deg} (min^{-1}) × 10^{3b}	(k_{deg}/k_{dis}) × 100[c]
Acyclovir	9.69 ± 1.32	9.69 ± 1.32	0	0
Butyrate	14.71 ± 0.47	12.04 ± 0.05	2.67	18.15
Pivalate	14.19 ± 2.15	12.60 ± 0.76	1.59	11.20
Valerate	23.07 ± 2.73	14.26 ± 0.47	8.81	38.19
Hexanoate	75.95 ± 2.22	16.38 ± 0.94	59.57	78.43

[a] Values represent the mean ± SD (n = 3).
[b] Obtained as the difference between column 2 and column 3.
[c] Contribution of enzymatic degradation to total drug disappearance from the perfusate.

lipid bilayers has been well illustrated in a review article by Muranishi (23).

Second, the degradative effects of rat nasal washings generated by both phosphate buffer and mixed micelle prewashes were evaluated in an *in vitro* incubation study. Mixed micellar nasal washing appears to possess a much higher prodrug degradative esterase activity. Both inhibitor screening and substrate specificity assessment indicated that the responsible enzyme is most likely carboxylesterase.

From the environmental health standpoint, the important role of nasal carboxylesterase in cleaving glycol ether acetates and acrylate esters, widely used as ingredients in the synthetic, paint, and coating industries, has recently been demonstrated (24). The hydrolyzed acidic metabolites of these compounds have been shown to produce lesions in the olfactory epithelium. In addition, tissue distribution studies indicated that the nasal carboxylesterase activity is equivalent to that of the liver and greater than that of the kidney, lung, or blood in mice. Biochemical quantitation and histochemical localization studies further confirmed that the activity of olfactory mucosal carboxylesterase is three to six times greater than that of the respiratory mucosa.

The influence of carboxylesterase on nasal absorption of drugs containing labile ester bonds had not been systematically studies previously. We have demonstrated the importance of presystemic prodrug cleavage in nasal drug absorption. Although the prodrug approach has proven to be rather successful in ocular and transdermal routes, the extremely high carboxylesterase activity in nasal tissue may discourage ester prodrug development for nasal drug delivery, except for local effect. Unlike aminopeptidase, which has been found to be inhibited by bile salts (25), our *in vitro* incubation and carboxylesterase release experiments did not indicate similar inhibition behavior. Recent studies involving both intestinal aminopeptidases and chymotrypsin also question inhibition spectra of bile salts on other enzymes (26,27).

Micellar solubilization of a penetrant is also recognized as playing an important role in bile salt-mediated permeation enhancement. For protein oligomers, micellar solubilization facilitates dissociation of macromolecular aggregates, resulting in a drastic reduction in the molecular dimension. As a result, improved transmucosal uptake has been observed for nasal, pulmonary conjunctival, and enteral absorption of insulin. For nonproteinaceous small organic compounds, however, the opposite contribution has been found to be true. From the early work of Kakemi *et al.* (28) to the recent results of Poelma *et al.* (29), sufficient evidence can be found to support the fact that intestinal lipophilic drug absorption correlates proportionally with the fraction of free drug in solution. Similarly, investigation of a group of β-blockers across the rat nasal mucosa also indicated that micellar solubilization retards their uptake (30).

With this information in mind, we have also conducted micellar binding experiments to assess the incorporation of these ester prodrugs into the cylindrical core of NaGC micelles. With the increase in the ester-side length, the micellar binding constant (K_{eq}) indeed improved linearly, corresponding to their octanol/PBS partition coefficients. Incorporation of linoleic acid, however, causes displacement of the solubilized penetrant molecules in a concentration-dependent manner. As a result, mixed micellar solutions consisting of 15 m*M* NaGC and 5 m*M* linoleic acid no longer have an affinity for any of these prodrugs, despite the much improved lipophilicity of the penetrant.

Therefore enhanced nasal absorption of acyclovir and its ester prodrugs may be the result of a reduction in the mucosal resistance rather than other mechanisms. The results of the effects of mixed micelles on nasal absorption and degradation of ester prodrugs are summarized in Table II. With linear increases in acyl chain length, the observed drug absorption rate constant ascends moderately. However, the degradation rate constant increases almost exponentially such that 78% of the overall rate constant of disappearance is attributable to presystemic degradation. Branching of the acyl side chain (pivalate) appears to retard the enzymatic activity significantly. Therefore, the design of an ester prodrug with a branched side chain may be favorable for the purpose of nasal delivery with enhanced lipophilicity and minimal presystemic degradation.

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