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Biodegradation characteristics of acyclovir 2′-esters by respiratory carboxylesterases: Implications in prodrug design for intranasal and pulmonary drug delivery

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Abstract

Eight aliphatic and one aromatic 2′-ester prodrugs of 9-(2-hydroxyethoxymethyl)guanine (acyclovir) were incubated with rat nasal mucosal homogenate in vitro to characterize their degradation kinetics by carboxylesterase. Lineweaver-Burk plots revealed that prodrugs with longer linear carboxylic side chains possess higher V_{max} and smaller K_m values than esters with shorter side chains, probably as a result of improved lipophilicity. Under subsaturating conditions, the rate and extent of prodrug degradation assume a direct relationship with acyl side chain length. Lengthening of linear carboxylic chains from three to eight carbons resulted in a more than 600-fold increase in first-order degradation rate constants. Esters with branched acyl chains are more resistant to nasal carboxylesterase-mediated cleavage. Acyclovir benzoate, on the other hand, did not afford specific protective effect against nasal carboxylesterase, although such an effect was found in plasma. Comparison of the second-order rate constants for binding and catalysis (V_{max}/K_m) indicated that the catalytic affinity of nasal carboxylesterase differs significantly from that of ester hydrolase in plasma. Comparison of hexanoate degradation kinetics in tracheal, pulmonary parenchymal, and nasal homogenates suggested that nasal mucosa possesses the highest carboxylesterase activity in the respiratory tract. The uniquely rich presence of this enzyme in the nasal cavity forms a remarkable enzymatic barrier to prodrug design for systemic delivery.

Keywords: Acyclovir; Carboxylesterase; 2′-Ester prodrug; Lipophilicity; Metabolism; Nasal delivery; Respiratory tract

1. Introduction

Development of new intranasal delivery devices and dosage forms for biotherapeutics has been under extensive investigation in the past decade. This mode of drug administration offers several advantages over the parenteral route, i.e., ease of delivery, rich vasculature for rapid drug uptake, avoidance of hepatic first-pass metabolism, less rigid mucosal environment, etc. (Chien et al., 1989). Despite much progress being made in this area, the most ideal candidates for nasal

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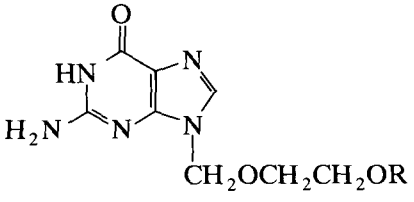
systemic delivery, i.e., peptidomimetics, proteins, and nucleosides, are found to traverse the nasal mucosa poorly, partly due to their rather unfavorable physicochemical properties, i.e., large molecular dimension and low water-to-lipid partition coefficient.

In order to improve nasal drug uptake, several formulation strategies are currently being pursued. One of the most common strategies involves the coadministration of absorption enhancers which structurally belong to a wide range of chemical classes. Nonionic and ionic surfactants (Hirai et al., 1981a,b), bile salts (Gordon et al., 1983), medium chain fatty acids (Mishima et al., 1987), bile salt-fatty acids (Tengamnuay and Mitra, 1990), fusidic acid derivatives (Longenecker et al., 1987; Deurloo et al., 1989; Baldwin et al., 1990), cyclodextrins and derivatives (Merkus et al., 1991; Shao et al., 1992), and chelating agents (Adjei et al., 1992) are the most common choices. One serious disadvantage with these agents lies in the fact that they all appear to cause nasal mucosal damage (Shao and Mitra, 1992), to a greater or lesser extent. Among several important physiological functions of the nose, any deleterious effect exerted by a chemical promoter on the olfactory region raises serious doubts over a formulation's long-term applicability (Kissel et al., 1992) and may preclude the incorporation of such chemical enhancers. Another strategy involves retardation of the nasal mucociliary clearance mechanism, aimed at extending the contact time of penetrant molecules with the nasal mucosal surface. The use of DEAE-dextran, serum albumin, and degradable starch microspheres or hydrophilic polymers encompasses such an endeavor (Duchêne and Ponchel, 1993).

Recently, we have pursued a different approach, i.e., chemical structural modification to improve nasal nucleoside uptake (Shao and Mitra, 1994; Shao et al., 1994). Simple bioreversible ester prodrugs were synthesized to increase substrate lipophilicity for better membrane partitioning. This methodology may open up an additional diffusional pathway for a drug candidate, a transcellular route, in addition to paracellular diffu-



$CH_2OCH_2CH_2OR$

R =	
H	acyclovir
$C(=O)-CH_2CH_3$	acyclovir propionate
$C(=O)-CH(CH_3)_2$	acyclovir isobutyrate
$C(=O)-CH_2CH_2CH_3$	acyclovir butyrate
$C(=O)-C(CH_3)_3$	acyclovir pivalate
$C(=O)-(CH_2)_3CH_3$	acyclovir valerate
$C(=O)-(CH_2)_4CH_3$	acyclovir hexanoate
$C(=O)-(CH_2)_5CH_3$	acyclovir heptanoate
$C(=O)-(CH_2)_6CH_3$	acyclovir octanoate
$C(=O)-C_6H_5$	acyclovir benzoate

Scheme 1. Chemical structures of acyclovir 2'-ester prodrugs.

sion. Moderate enhancement in nasal drug absorption was indeed observed. However, simultaneous ester degradation with nasal transport was observed particularly with prodrugs of higher 1-octanol/water partition coefficients (Shao et al., 1994). Presystemic degradation became more pronounced when prodrugs were formulated together with absorption enhancers (Shao and Mitra, 1994). In vitro studies further confirmed that the enzyme responsible for ester prodrug breakdown belongs to the carboxylesterase family (Shao and Mitra, 1994). In this article, in-depth hydrolysis work has been reported to better characterize the catalytic behavior of this enzyme by utilizing nine acyclovir derivatives with varying lipophilicity and ester configuration. This information is deemed essential for designing further studies in the development of carboxylesterase-resistant lipophilic prodrugs for nasal systemic delivery.

2. Materials and methods

2.1. Materials

Acyclovir [9-(2-hydroxyethoxymethyl)guanine] was a gift from Burroughs Wellcome Co. (Research Triangle Park, NC). Heptanesulfonic acid, sodium salt, was obtained from Aldrich Chemical Co. (Milwaukee, WI). HPLC grade acetonitrile was obtained from Mallinckrodt Specialty Chemicals Co. (Paris, KY). Other reagents were of analytical grade and were used as received.

Acyclovir prodrugs were synthesized by an acid anhydride esterification method as reported previously (Shao et al., 1994). The purity of final products was monitored by TLC, HPLC, elemental analysis, and melting point determinations. ^{1}H-NMR and FAB-MS were used for structural conformation purpose. The chemical structures of acyclovir and its nine ester prodrugs have been depicted in Scheme 1. The aqueous solubility in pH 7.4 isotonic phosphate buffer (PBS) and 1-octanol/PBS partition coefficients have also been determined using the same procedures as reported in an earlier publication (Shao and Mitra, 1994).

2.2. Methods

2.2.1. Rat nasal, tracheal, and lung homogenate preparation

Male Sprague-Dawley rats weighing 250–350 g were fasted for about 14–18 h but water was allowed ad libitum. Following intraperitoneal overdose of a ketamine (90 mg/ml) and xylazine (10 mg/ml) mixture and exsanguination, nasal tissues from all regions of the nasal cavity were removed from five rats. Both respiratory and olfactory mucosae were dissected free of bones. Approx. 100 mg of nasal tissues could be obtained from each rat in this manner. The harvested tissue samples were washed three times with isotonic saline solution to remove blood contamination. Homogenization of the nasal mucosa was performed in 0.1 M sodium phosphate buffer, pH 7.8, at a tissue to buffer ratio of 1 : 10 (w/v) using a Polytron homogenizer (Model LS 10–35, Kinemetrica GmbH, Switzerland) set at speed 8 for 4×15 s on ice.

The tracheas of the rats were also removed, washed clean, and homogenized similarly. The parenchymal region of lungs were dissected and pooled together. After saline wash, the lung parenchyma were homogenized and the preparation was saved for experiments.

After homogenization, the tissue preparations were subsequently centrifuged at 5000 rpm for 10 min in a Beckman TL-100 ultracentrifuge (Palo Alto, CA). Aliquots of the homogenates were divided into small aliquots, frozen in a dry ice-acetone mixture, and stored at -20°C until further use.

2.2.2. Determination of Michaelis-Menten parameters

The hexanoate and propionate esters were used to determine their V_{max} and K_m values in nasal carboxylesterase-mediated degradation. 200 μl of 0.05, 0.1, 0.2, and 0.5 mM hexanoate ester solutions were incubated with 20 μl of nasal homogenate (186.6 μg protein). Samples were taken at t_0 and at 5 or 10 min and hexanoate concentrations were measured. For propionate ester, 200 μl of 0.05, 0.2, 0.5, 1, 2, and 5 mM solutions were mixed with 50 μl nasal ho-

mogenate and incubated for 12 h. Ester concentrations at t_0 and 12 h were measured. After calculation of velocity values (V), Lineweaver-Burk plots were constructed using $1/V$ vs 1/concentration (mM). V_{max} and K_m were then obtained from the Y-intercept and slope values.

2.2.3. Hydrolysis study

The degradation characteristics of acyclovir esters by rat mucosal homogenates was studied in vitro. An aliquot of homogenate sample containing either 93.3 or 466.5 μg total protein was pipetted into a 1.0 ml microcentrifuge tube and mixed with 0.1 M phosphate buffer, pH 7.8, to make a volume of 475 μl. The mixture was placed at 37°C in a Dubnoff shaking water bath (Precision Scientific, Chicago, IL). 25 μl of a prodrug stock solution in ethanol (2 mM) was then added followed by vortexing. The final concentration of an acyclovir ester was therefore 0.1 mM. A 50 μl sample was withdrawn immediately and mixed with 100 μl of ice-cold acetonitrile to arrest the reaction. Samples were taken subsequently at predetermined time points up to 24 h, depending on the rate of hydrolysis of a particular ester, and mixed with 2 vol. ice-cold acetonitrile. After centrifugation at 10000 rpm for 15 min, the supernatant was transferred to another tube and stored at −20°C until HPLC analysis.

The % ester remaining in the incubation medium was plotted vs time in a semilogarithmic fashion. Linear regression of the line generates first-order degradation rate constant (k_{obs}, min^{-1}). The second-order rate constant for binding and catalysis (V_{max}/K_m), which serves as an indicator of the catalytic efficiency at subsaturating substrate concentrations, can then be calculated using the following equation:

$$V_{max}/K_m = (\text{volume} \cdot k_{obs})/\text{protein}$$

where volume is the total volume of incubation medium (ml). 'Protein' denotes total protein content in terms of mg. The resulting V_{max}/K_m bears the unit of μmol min^{-1} mM^{-1} mg $protein^{-1}$.

2.2.4. Analytical procedures

The concentrations of acyclovir remaining in the incubation medium were determined by a modified HPLC method reported by Land and Bye (1981). The mobile phase was composed of 2% acetonitrile (v/v) in a 10 mM ammonium acetate buffer (pH 5.0) containing 1.0 mM sodium heptanesulfonate at a flow rate of 1 ml/min. For adequate retention of acyclovir ester prodrugs, the mobile phase composition has been modified to contain 13, 22, 22, 28, 28, 35, 38, 42, and 26% acetonitrile (v/v) in the pH 5.0 ammonium acetate buffer with 0.1 mM sodium heptanesulfonate for propionate, isobutyrate, butyrate, pivalate, valerate, hexanoate, heptanoate, octanoate, and benzoate ester, respectively. The flow rate was all at 2 ml/min.

The HPLC system was a computer-controlled gradient system composed of two Rabbit HP pumps, a Rheodyne injector, a variable-wavelength ultraviolet/visible detector (Knauer, Germany), and a 3390A Hewlett-Packard integrator (Hewlett-Packard Co., Avondale, PA). Samples (20 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. The integrated peak areas were used for quantitative purpose.

The concentration of total proteins in a homogenate was measured using the method of Lowry et al. (1951), with bovine serum albumin as the standard.

3. Results

3.1. Determination of Michaelis-Menten kinetic parameters

The enzymatic degradation kinetic parameters of hexanoate and propionate ester prodrugs in rat nasal homogenate were examined and the Lineweaver-Burk plots are depicted in Fig. 1. Due to the limited solubility of these two substrates (Shao et al., 1994), concentration ranges cannot exceed 0.5 and 5 mM for hexanoate and propionate, respectively. Both plots, nevertheless, exhibited good linearity in their respective concentration ranges. The V_{max}, K_m, and V_{max}/K_m values were calculated to be 3.7 nmol min^{-1} mg $protein^{-1}$, 7.92 mM, and 0.47 nmol min^{-1} mg

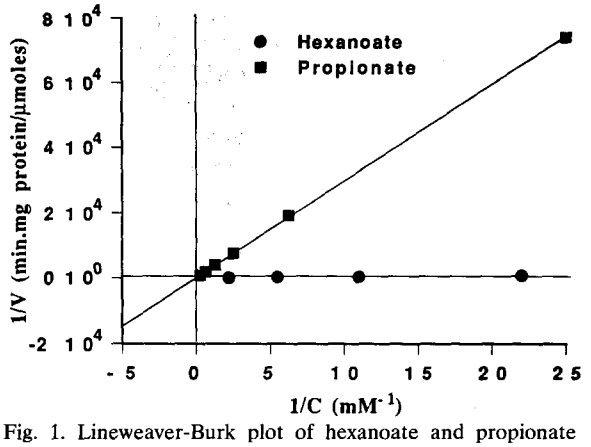


Fig. 1. Lineweaver-Burk plot of hexanoate and propionate ester biodegradation by rat nasal carboxylesterase. Values represent means of three parallel determinations.

protein^{-1} mM^{-1} for propionate and 70.8 nmol min^{-1} mg protein^{-1}, 2.14 mM, and 33.08 nmol min^{-1} mg protein^{-1} mM^{-1} for hexanoate. The differences in magnitude of these parameters indicate that: (a) hexanoate can be cleaved by nasal carboxylesterase at a much faster maximum velocity; (b) stronger hexanoate-enzyme binding capability as demonstrated by a smaller K_m; and (c) a 70-fold higher first-order rate constant can be obtained for hexanoate at subsaturating concentrations compared to propionate. These observations are in good agreement with previously known characteristics of liver carboxylesterases in which substrate lipophilicity plays an important role in determining the extent and rate of substrate biodegradation (Krisch, 1971).

3.2. Enzymatic hydrolysis of acyclovir esters by rat nasal homogenate

The initial substrate concentration of all prodrugs was kept constant at 0.1 mM, at a subsaturating level, and the respective degradation profile was determined as shown in Fig. 2. A total protein content of 93.3 μg was found to be adequate for valerate, hexanoate, heptanoate, and octanoate ester hydrolysis studies while a 5-fold increment was necessary for pivalate, propionate, isobutyrate, benzoate, and butyrate esters. Decreases in acyclovir ester peaks are accompanied by corresponding increases in acyclovir peak. Acyclovir, on the other hand, was found to be completely stable in the rat nasal homogenate during the course of study. Further, the hydrolysis of the nine ester prodrugs appears to follow apparent first-order kinetics, as expected. The observed first-order hydrolytic rate constants of acyclovir prodrugs, normalized by protein content, were again plotted as a function of carboxylic carbon chain length as shown in Fig. 3. Apparently, ascending side chain length and lipophilicity led to facilitated cleavage of the ester bond in the order of octanoate > heptanoate

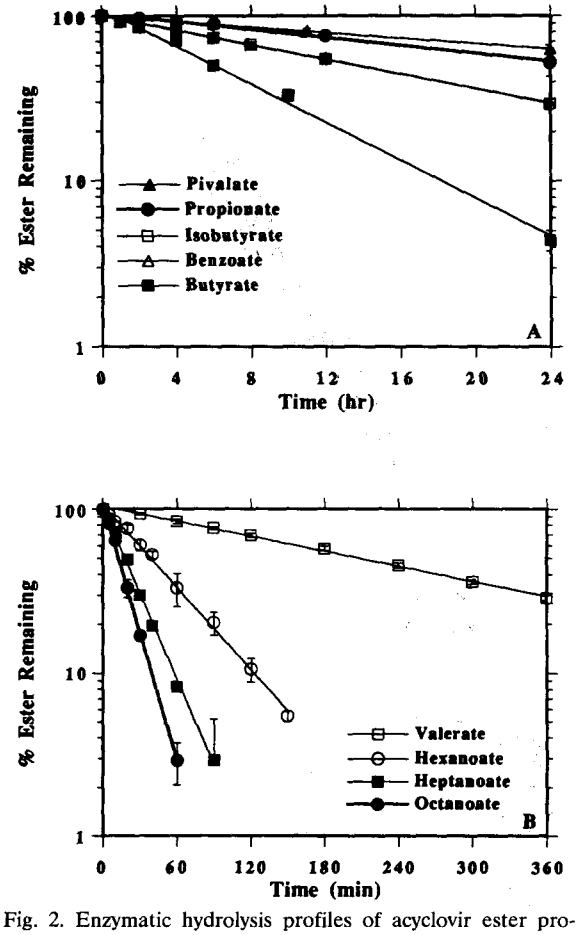


Fig. 2. Enzymatic hydrolysis profiles of acyclovir ester prodrugs in rat nasal homogenate. Initial drug concentration was 0.1 mM. Total protein content was 466.5 μg in A and 93.3 μg in B in a total volume of 0.5 ml. Values represent means ± SD of three measurements.

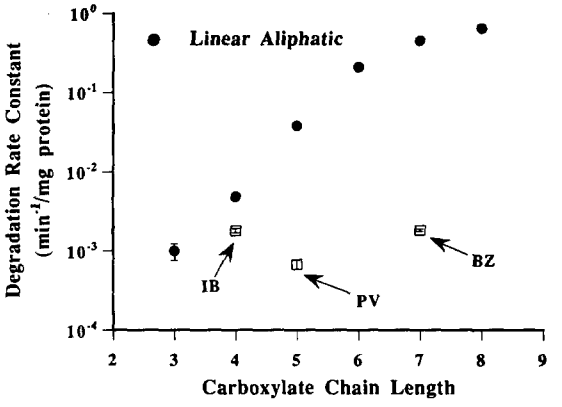


Fig. 3. Relationship between first-order rate constants and acyl side chain length. IB, isobutyrate; PV, pivalate; BZ, benzoate.

> hexanoate > valerate > butyrate > propionate, a phenomenon established for many types of ester prodrugs previously (Hofstee, 1952). 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 retarded hydrolysis. Extended branching of the side chain configuration in pivalate resulted in a further decreased degradation rate constant despite its higher lipophilicity, compared to that of isobutyrate. This is attributed to the steric hindrance of the substituent, thus inhibiting its binding to the active center of the enzyme. Substitution by an aromatic moiety, on the other hand, appears to result in a comparable biodegradation pattern relative to isobutyrate, although exhibiting a slightly improved lipophilicity. Therefore, aromatic derivatization may not offer much significant protection against nasal carboxylesterase-mediated ester hydrolysis. The main controlling factors are the apolar and steric nature of the caboxylic substituent.

3.3. Second-order rate constant for binding and catalysis (V_{max}/K_m)

The V_{max}/K_m values for all nine prodrugs were then calculated from the first-order degradation rate constants and are listed in Table 1. Similarly, V_{max}/K_m values from ester cleavage in rat plasma were also obtained using previously reported first-order rate constants (Shao et al., 1994) and from additional plasma hydrolysis experiments. The 1-octanol/phosphate buffer (pH 7.4) partition coefficients of the prodrugs at 37°C are also compiled in Table 1. Ascending from C3 to C6 in the linear carboxylic side chain resulted in similarly increased V_{max}/K_m values in both nasal homogenate and plasma. Further lengthening of the linear side chain lead to clear differ-

Table 1
Second-order rate constants for binding and catalysis (V_{max}/K_m) of rat nasal carboxylesterases and plasma ester hydrolases using acyclovir esters as substrates

Substrate	PC [a]	V_{max}/K_m [b]		Ratio (nasal/plasma)
		nasal homogenate	plasma	
Propionate	0.14	0.499 ± 0.120 [c]	0.126	4.0
Butyrate	0.83	2.414 ± 0.114	0.445	5.4
Valerate	2.35	18.76 ± 0.32	0.706	26.6
Hexanoate	8.58	103.4 ± 3.7	3.166	32.6
Heptanoate	26.12	225.5 ± 2.4	1.107	203.7
Octanoate	125.6	323.9 ± 9.8	0.795	407.4
Isobutyrate	0.88	0.911 ± 0.051	0.262	3.5
Pivalate	2.01	0.338 ± 0.046	0.108	3.1
Benzoate	1.80	0.918 ± 0.032	0.024	38.2

[a] Partition coefficient in 1-octanol/isotonic phosphate buffer, pH 7.4.
[b] V_{max}/K_m expressed in μmol min^{-1} mg protein^{-1} mM^{-1} ($\times 10^{-3}$).
[c] Means ± SD of three determinations.

ences in the second-order rate constants such that continual increases were observed in nasal homogenate while gradual decline was noted in plasma. This phenomenon tends to suggest some intrinsic differences in the catalytic capacity of nasal and plasma carboxylesterases in cleaving medium-chain carboxylic esters. The nasal homogenate/plasma ratios of V_{max}/K_m were calculated and listed in Table 1. In the case of the most lipophilic prodrug (octanoate), over 400-fold difference was observed in its degradation in nasal homogenate over that in plasma. Another striking difference appears to exist with the aromatic prodrug. An extremely small second-order rate constant of 0.024 nmol min^{-1} mg $protein^{-1}$ mM^{-1} was found in plasma, being the most enzyme-resist candidate of all nine esters. Its hydrolysis in nasal homogenate, on the other hand, manifested an enhanced rate constant which was faster than propionate and pivalate and equivalent to isobutyrate. As a result, a 38-fold higher second-order rate constant in nasal homogenate can be obtained over that in plasma.

3.4. Site-dependent degradation of hexanoate by the respiratory mucosal homogenates

Hexanoate was used as a model compound to compare the relative efficiency of various segments of the respiratory tract in hydrolyzing ester prodrugs. The kinetic profiles of tracheal, pulmonary parenchymal, and nasal homogenates containing the same amount of total protein (93.3 μg in 0.5 ml) are illustrated in Fig. 4. Decreases in hexanoate concentration in tracheal and pulmonary tissue homogenates similarly follow apparent first-order kinetics. The degradation rate constants were found to be $0.016 \pm 7.89 \times 10^{-3}$, $0.104 \pm 2.15 \times 10^{-3}$, and $0.207 \pm 7.33 \times 10^{-3}$ min^{-1} mg $protein^{-1}$ for hexanoate in tracheal, pulmonary, and nasal tissue homogenates, respectively. Therefore, nasal mucosa appears to possess maximum carboxylesterase activity on the unit protein basis, while the conducting airways contain the least.

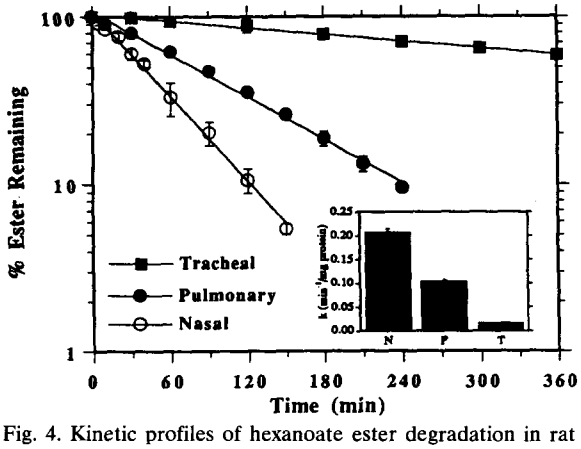


Fig. 4. Kinetic profiles of hexanoate ester degradation in rat tracheal, pulmonary parenchymal, and nasal tissue homogenates. Values denote means ± SD of three determinations. Inset: first-order degradation rate constants.

4. Discussion

Intranasal and respiratory drug delivery are known to provide better bioavailability for a wide range of chemical entities, partly due to the weak nasal enzymatic activity compared to the gastrointestinal tract and partly due to the avoidance of hepatic first-pass metabolism. Nevertheless, the nasal mucosa contains phase I, conjugative phase II and proteolytic enzymes (Sarkar, 1992). High levels of certain enzymes in the respiratory mucosae have been previously documented, with carboxylesterase being a typical example (Bogdanffy, 1990).

Although carboxylesterases are widely distributed in mammalian tissues, much information on the structure and function of this family of enzymes has been derived from liver esterases which are known to reside predominately in the endoplasmic reticulum. Since the early eighties, interest in the area of industrial toxicology prompted extensive work on the histological localization of carboxylesterases in the respiratory tract (Stott and McKenna, 1985; Bogdanffy et al., 1987, 1991; Morris, 1990). Various organic solvents and chemical ingredients containing propylene glycol monomethyl ether acetate, ethyl acrylate, and dibasic esters are widely used in synthetic, paint, and coating industries which are known to cause lesions in the olfactory mucosa

leading to subsequent loss of sensory cells. Carboxylesterase plays an important role by cleaving inhaled esters to their corresponding free acids, which contribute to the observed histological changes following chronic inhalation exposure.

Recent biochemical and histological examination further revealed that respiratory carboxylesterase locates selectively in certain cell populations while absent in others. Gaustad et al. (1991) found that over 80% carboxylesterase activity is distributed in the cytosolic fraction, a striking contrast to its microsomal localization in the liver. Histochemical visualization with α-naphthyl butyrate (Bogdanffy et al., 1987) and immunohistochemical staining with a carboxylesterase antibody (Olson et al., 1993) both revealed rich presence of this enzyme in the olfactory epithelium, especially in cells of Bowman's glands and sustentacular cells.

The influence of this naturally existing enzyme, for detoxification purposes, on the nasal delivery of ester prodrugs has not been previously examined in detail. Our current findings using acyclovir prodrugs as model compounds agree well with research efforts of other environmental toxicologists in that a strong enzymatic barrier is present in the upper respiratory mucosa (Stott and McKenna, 1985; Bogdanffy et al., 1991). Determination of Michaelis-Menten parameters indicated a K_m value in the millimolar range. The K_m values in this range have also been reported previously for a variety of ester compounds (Stott and McKenna, 1985). Comparison between V_{max}, K_m, and V_{max}/K_m values of propionate and hexanoate suggests that substrate lipophilicity is an important factor in determining its cleavage efficiency by nasal carboxylesterase.

The specific activity of nasal carboxylesterase, normalized by protein content, appears to be 3–400-fold stronger than ester hydrolase activity in plasma. With medium-chain aliphatic esters (C7, C8), the catalytic affinity of this enzyme reached a remarkably high level. For short-chain (C3, C4) and branched-chain aliphatic esters, the catalytic difference between nasal and plasmic carboxylesterases becomes less pronounced. The aromatic derivative, benzoate, appears to effectively resist degradation by plasma yet being vulnerable to nasal carboxylesterase attack. All these observations tend to emphasize the marked ability of nasal mucosa in handling lipophilic substrates, regardless of their aliphatic or aromatic structures.

Comparison of carboxylesterase activities in different regions of the respiratory tract further revealed that nasal mucosa contains the richest carboxylesterase, with the order being nasal > pulmonary parenchymal (lower alveolar region) > tracheal. The nasal mucosa appears to assume primary responsibility in hydrolyzing various esters aerosolized in the breathing air.

Direct interpretation of any outcome following intranasal or pulmonary prodrug delivery is difficult without knowing the subcellular localization of carboxylesterase. Bogdanffy et al. (1987) observed that even the mucus blanket overlying the respiratory epithelium was stained positively, by histochemical visualization. This enzyme localization pattern certainly causes presystemic breakdown of ester prodrugs even before transcellular partitioning could take place. Our previous study involving incubation of hexanoate ester with phosphate buffer nasal washings indeed indicated moderate hydrolysis (Shao et al., 1994). The relative distribution of carboxylesterase in the plasma membrane of nasal epithelium is not clear at present. Any membrane-bound carboxyleterase will form a second barrier against prodrug diffusion. Due to the rich and even distribution of this enzyme in the cytoplasmic space, most prodrug molecules surviving the mucus and lipid bilayers are likely to be cleaved prior to traversing the basolateral membrane.

If the carboxylesterase activity in the mucus and plasma membrane can be circumvented through proper prodrug design, such an approach may eventually prove to be extremely valuable for both nasal topical and systemic delivery purposes. As anticipated (Sarkar, 1992), the prodrugs could be rapidly absorbed across the nasal epithelial bilayers and cleaved subsequently by the cytosolic carboxylesterase leading to regeneration of the parent compound. The concentration of parent compound in the nasal epithelium, therefore, could reach a desirable level for local effect, or diffusion across basolateral membrane may af-

ford sufficient input to the vascular circulation for systemic response.

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