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Uptake of lamivudine by rat renal brush border membrane vesicles

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Abstract

Uptake of lamivudine, a nucleoside analogue antiviral agent, by brush border membrane vesicles (BBMV) prepared from rat renal cortex was investigated. Initial uptake of lamivudine by BBMV was stimulated in the presence of an outward pH gradient. Determination of the kinetic parameters of the initial uptake yielded apparent K_m and V_{max} values of 2.28 mM and 1.56 nmol (mg protein)$^{-1}$ (20 s)$^{-1}$, respectively. The pH-driven uptake of lamivudine was inhibited by organic cations such as trimethoprim and cimetidine. The inhibitory effect of trimethoprim on lamivudine uptake was competitive, with an apparent K_i of 27.6 μM. The uptake of lamivudine was also inhibited by nitrobenzylthioinosine, a representative inhibitor of nucleoside transport, and by other nucleoside analogues, such as azidothymidine and dideoxycytidine, that are excreted by renal tubular secretion. These findings suggest that efflux of lamivudine at the brush border membrane of renal tubular epithelium is mediated by an H^+/lamivudine antiport system, which may correspond to the H^+/organic cation antiport system, and that this system is also involved in the renal secretion of other nucleoside analogues.

Introduction

Lamivudine ((–)-2′-deoxy-3′-thiacytidine, Figure 1) is a cytosine dideoxynucleoside analogue invented by BioChem Pharma Inc., Canada and developed by Glaxo-SmithKline Research and Development, UK, which is used clinically as a very effective agent against human immunodeficiency virus (HIV) and human hepatitis B virus (HBV) (Doong et al 1991; Chang et al 1992; Coates et al 1992; Hart et al 1992). Previous studies in humans and rats have indicated that lamivudine is well absorbed after oral administration, quickly distributed in the whole body, undergoes minimal metabolism and is predominantly and rapidly excreted into urine (Takubo et al 1997; Tsuno-o et al 1997; Johnson et al 1999).

Renal clearance of lamivudine was investigated further because it is the predominant route of elimination of this drug. Studies using isolated perfused rat kidney and rat renal clearance models showed that tubular secretion, in addition to glomerular filtration, is a major component of the renal clearance of lamivudine, and that tubular secretion may be significantly inhibited by co-administered cationic drugs (Sweeney et al 1995; Takubo et al 2000a). Furthermore, the effect of lamivudine on the uptake of organic cations by rat renal brush-border and basolateral membrane vesicles was examined (Takubo et al 2000b). As a result, it was determined that lamivudine is a very weak inhibitor of organic cation transport by the renal tubular epithelium and would not be expected to affect tubular

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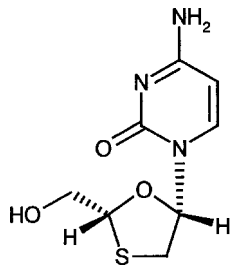

Figure 1 The structure of lamivudine.

secretion of co-administered cationic drugs. However, the uptake characteristics of lamivudine itself and the contribution of the cation transporter to the in-vivo renal excretion of lamivudine have so far not been investigated.

There have been previous studies of the renal tubular secretion of nucleosides and nucleoside analogues. Nelson et al (1988) demonstrated that deoxyadenosine and 5′-deoxy-5-fluorouridine were excreted by tubular secretion that was decreased by dipyridamole and nitrobenzylthioinosine, known inhibitors of nucleoside transport, whereas adenosine underwent net renal reabsorption that was not prevented by the inhibitors. Azidothymidine and dideoxycytidine, which, like lamivudine, are 2′,3′-dideoxynucleoside analogues, were reported to be excreted into urine by tubular secretion (Patel et al 1989; Ibrahim & Boudinot 1991).

It is well known that many transport systems contribute to the influx or efflux of various compounds, such as organic anions and cations, sugars and dipeptides, through the epithelial membranes of organs and tissues. As for the absorption of nucleosides across the gastrointestinal tract, it has been suggested that sodium-dependent concentrative nucleoside transport systems and equilibrative nucleoside transport systems (facilitated diffusion) mediate their influx at the brush border membrane and efflux at the basolateral membrane, respectively, of the jejunal epithelium, resulting in efficient transport of nucleosides from the lumen to the blood (Chandrasena et al 1997). Sodium-dependent transport systems are also reported to facilitate reabsorption of nucleosides by the renal tubular epithelium (Griffi th & Jarvis 1996), but renal tubular secretion of nucleosides has not yet been definitively characterised.

As mentioned above, lamivudine is a nucleoside analogue that is extensively excreted by renal tubular secretion, and this secretion appears to be related to the transport of organic cations by the renal tubular epithelium. In this study, uptake of lamivudine by brush-border membrane vesicles (BBMV) prepared from rat renal cortex was investigated to clarify the mechanism of efflux of lamivudine at the brush-border membrane of renal tubular epithelium.

Materials and Methods

Materials

Lamivudine and its ^{3}H-labelled form ([^{3}H]lamivudine) were supplied by GlaxoSmithKline Research and Development, UK. Ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA) and magnesium chloride were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan), and tris-hydroxymethyl-aminomethane (Tris), N-2-hydroxyethyl-piperazine-N′-2-ethanesulphonic acid (HEPES) and 2-N-morpholino-ethanesulphonic acid (MES) from Wako Pure Chemical Industries, Ltd (Osaka, Japan). Other chemicals were obtained from Sigma-Aldrich Co. (MO).

Preparation of vesicles

BBMV were prepared by a modification of the magnesium/EGTA precipitation method described previously (Daniel et al 1991; Hori et al 1993). Briefly, renal cortex was sliced from the kidneys of five 7-week-old male Wistar rats and homogenised for 1 min in buffer A (12 mM Tris, 5 mM EGTA, 300 mM mannitol (pH 7.1); 35 mL) using a homogenizer (ULTRA-TURRAX T25; IKA-Labortechnik (Staufen, Germany)). The homogenate was diluted with same volume of distilled water, and $MgCl_2$ solution (1 M; 700 μL) was added and the mixture was left to stand for 15 min. The homogenate was centrifuged at 1900 *g* for 15 min and the supernatant was centrifuged again at 24000 *g* for 30 min. The resulting pellet was homogenised in buffer B (6 mM Tris, 2.5 mM EGTA, 150 mM mannitol (pH 7.1); 30 mL) and left to stand for 15 min after addition of $MgCl_2$ solution (1 M; 300 μL). The homogenate was centrifuged at 1900 *g* for 15 min and the supernatant was divided equally into two tubes and centrifuged again at 24000 *g* for 30 min. Each resulting pellet was suspended in buffer C (20 mM MES/Tris, 100 mM mannitol, 100 mM KCl (pH 5.5); 3 mL) or buffer D (20 mM HEPES/Tris, 100 mM mannitol, 100 mM KCl (pH 7.0); 3 mL) and left to stand for 30 min. Each suspension was diluted with the same buffer and centrifuged at 24000 *g* for 30 min. Each resulting pellet (BBMV) was resuspended in buffer C or D (1.5 mL) and used in this study. As compared with the activity of alkaline phosphatase (ALP) in the

initial homogenate of renal cortex, the activities of ALP in the BBMV preparations were enriched 7.4–11.4-fold, indicating that BBMV had been separated successfully.

Measurement of [^{3}H]lamivudine uptake

The uptake of [^{3}H]lamivudine into BBMV was measured by a rapid filtration technique (Murer & Gmaj 1986). Briefly, a portion of the BBMV suspension (9–12 mg protein mL^{-1}; 40 μL) was placed in a silanised glass tube and incubated at 25°C for 20 min. Uptake was then initiated by adding 4 volumes of buffer D containing [^{3}H]lamivudine with or without other compounds and the solution was incubated at 25°C until uptake was terminated by adding ice-cold stop solution (20 mM HEPES/Tris, 100 mM mannitol, 0.1 mM $MgCl_2$ (pH 7.0)). The reaction mixture was poured immediately onto a pre-wetted filter (0.45 μm pore size) and the filter was washed with ice-cold buffer E (20 mM HEPES/Tris, 100 mM mannitol (pH 7.0)). The filter was placed in a glass vial and solubilized in scintillation cocktail (10 mL) to measure radioactivity. In order to correct for non-specific adsorption of [^{3}H]lamivudine onto the BBMV, a sample of each vesicle suspension was filtered immediately after addition of [^{3}H]lamivudine and the radioactivity present on the filter was determined. The control radioactivity on the filter was determined each time an experiment was performed, and subtracted from the radioactivity detected when estimating [^{3}H]lamivudine uptake. Uptake of lamivudine (pmol (mg protein)$^{-1}$) was evaluated from the amount of [^{3}H]lamivudine (pmol) calculated from the specific radioactivity, and the total protein content (mg protein) of the BBMV suspension.

Analytical methods

The protein content of the BBMV preparations was determined with a commercial kit (BCA Protein Assay Kit; Pierce, IL). Radioactivity on the filters was measured with a liquid scintillation counter (Wallac 1410; Wallac Berthold Japan Co., Ltd, Tokyo, Japan). The activity of ALP was determined with a commercial kit (Alkaline phospha-B-test Wako; Wako Pure Chemical Industries, Ltd, Osaka, Japan).

Statistical analysis

Data are expressed as means±s.d. of results from separate experiments. Kinetic parameters of lamivudine uptake by BBMV were estimated by the equation $v = V_{max}[S]/(K_m+[S])+\alpha[S]$, where v is the initial uptake of [^{3}H]lamivudine (20 s), [S] is the concentration of [^{3}H]-lamivudine, V_{max} is the maximum velocity, K_m is the affinity constant and α is the non-specific uptake constant. These parameters were estimated simultaneously according to the non-linear regression as expressed in the above equation. Non-linear regression analysis was carried out using the WinNonlin program (Pharsight Corporation, CA). The differences between data for the effect of various compounds on lamivudine uptake were analysed using a one-way analysis of variance followed by a multiple range test (Dunnett test). Statistical significance was assumed when the corresponding P value was lower than 0.05. Effect of trimethoprim on lamivudine uptake by BBMV was estimated using the Dixon plot, which was prepared using reciprocals of uptake rates versus concentrations of trimethoprim. The resulting line at each concentration of [^{3}H]lamivudine was analysed by linear regression using the Excel program (Microsoft, WA). The value of K_i was estimated from the intersections of the lines and expressed as means± s.d. of trimethoprim concentration.

Results

Uptake of lamivudine by BBMV

Uptake of lamivudine by BBMV was determined for 5 min after addition of [^{3}H]lamivudine to the BBMV suspension (Figure 2). Uptake of lamivudine increased gradually in the absence of a pH gradient (pH_{in} = 7.0,

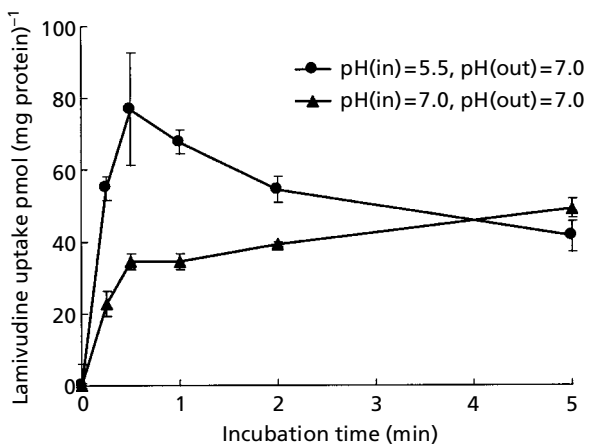

Figure 2 Effect of H^+ gradient on uptake of [^{3}H]lamivudine by rat renal BBMVs. The BBMVs were incubated at 25°C with 50 μM [^{3}H]lamivudine. Data are presented as the mean±s.d. of three determinations.

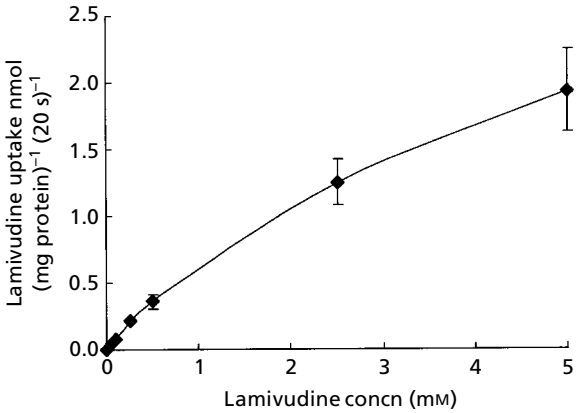

Figure 3 Concentration dependence of [^{3}H]lamivudine uptake by rat renal BBMVs. The BBMVs (pH(in) = 5.5, pH(out) = 7.0) were incubated at 25°C for 20 s with [^{3}H]lamivudine. Data are presented as the mean±s.d. of three determinations.

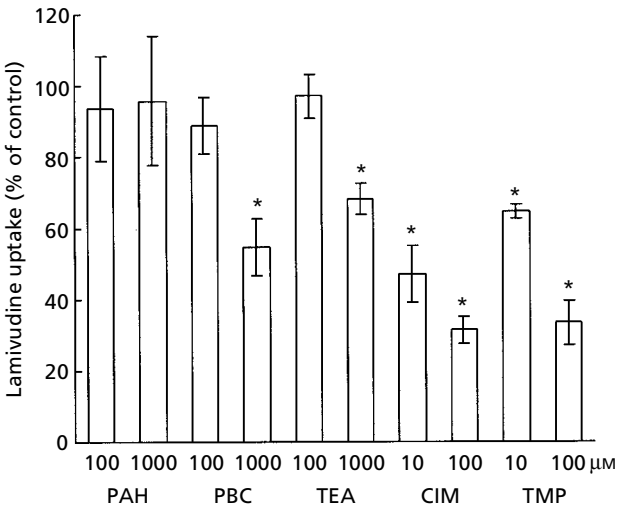

Figure 4 Effect of organic anions and cations on [^{3}H]lamivudine uptake by rat renal BBMVs. The BBMVs (pH(in) = 5.5, pH(out) = 7.0) were incubated at 25°C for 30 s with 100 μM [^{3}H]lamivudine. Data are presented as the mean±s.d. of three preparations. *$P < 0.05$ compared with control. PAH: p-aminohippuric acid; PBC: probenecid; TEA: tetraethylammonium; CIM: cimetidine; TMP: trimethoprim.

pH_{out} = 7.0), but uptake of lamivudine was stimulated against its concentration gradient in the presence of an outward pH gradient (pH_{in} = 5.5, pH_{out} = 7.0), and reached a peak after 30 s. The maximum uptake accounted for 77.0±15.7 pmol (mg protein)$^{-1}$ and was about twice that in the absence of a pH gradient. Uptake of lamivudine after 5 min reached a plateau of approximately 50 pmol (mg protein)$^{-1}$ under both conditions tested.

The pH-driven uptake of lamivudine by BBMV after 20 s was determined at several concentrations of [^{3}H]-lamivudine (50–5000 μM) (Figure 3). The uptake of lamivudine was concentration dependent and exhibited saturation at higher concentrations of lamivudine. The non-linear regression fitting showed that K_m was 2.28±0.387 mM, V_{max} was 1.56±0.254 nmol (mg protein)$^{-1}$ (20 s)$^{-1}$ and α was 0.172±0.0245 nmol (mg protein)$^{-1}$ (20 s)$^{-1}$ (mM)$^{-1}$.

Effect of various compounds on lamivudine uptake

The effect of organic anions or cations on the uptake of lamivudine by BBMV 30 s after addition of [^{3}H]-lamivudine in the presence of an outward pH gradient (pH_{in} = 5.5, pH_{out} = 7.0) is summarised in Figure 4. The uptake of lamivudine was strongly inhibited by trimethoprim (TMP) and cimetidine (CIM), being decreased to about 30% of control values with 100 μM of each inhibitor. Tetraethylammonium (TEA) and probenecid (PBC) at concentrations of 1 mM also reduced the uptake of lamivudine to 68% and 55% of control values, respectively. No significant effect of p-aminohippuric acid (PAH) on the uptake of lamivudine by BBMV was observed.

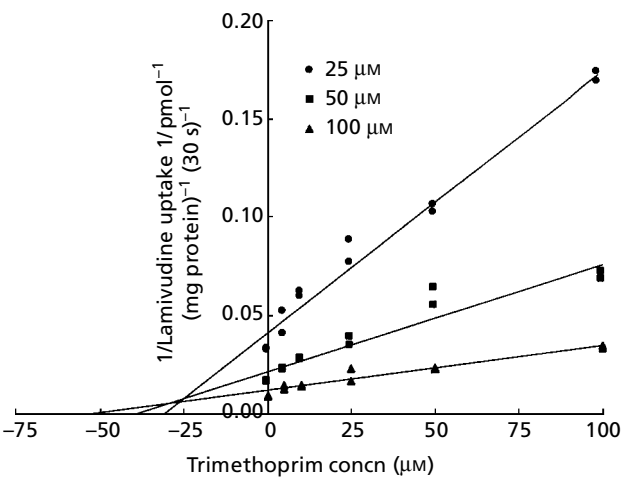

Figure 5 Dixon plot of [^{3}H]lamivudine uptake by rat renal BBMVs in the presence of various concentration of trimethoprim. The BBMVs (pH(in) = 5.5, pH(out) = 7.0) were incubated at 25°C for 30 s with 25–100 μM [^{3}H]lamivudine and 0–100 μM trimethoprim.

The pH-driven uptake of lamivudine by BBMV after 30 s was determined at several concentrations of [^{3}H]-lamivudine (25–100 μM) with trimethoprim (5–100 μM) (Figure 5). Dixon plot analysis indicated that the in-

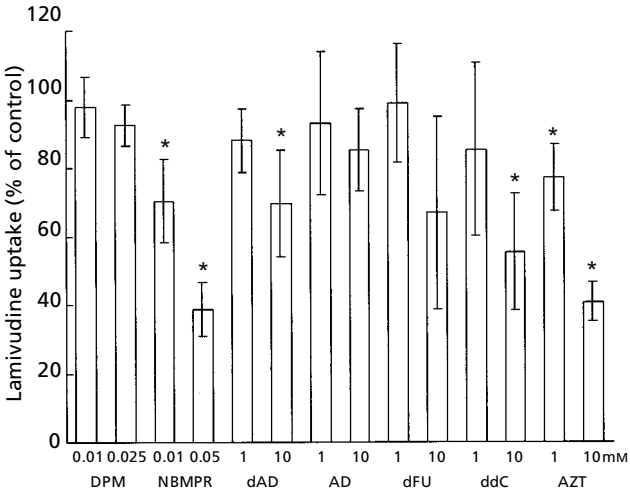

Figure 6 Effect of various nucleosides and inhibitors of nucleoside transport on [^{3}H]lamivudine uptake by rat renal BBMVs. The BBMVs (pH(in) = 5.5, pH(out) = 7.0) were incubated at 25°C for 30 s with 100 μM [^{3}H]lamivudine. Data are presented as the mean±s.d. of three preparations. *$P < 0.05$ compared with control. DPM: dipyridamole; NBMPR: nitrobenzylthioinosine; dAD: deoxyadenosine; AD: adenosine; dFU: deoxyfluorouridine; ddC: dideoxycytidine; AZT: azidothymidine.

hibitory effect of trimethoprim on lamivudine uptake was competitive with an apparent K_i of 27.6±2.87 μM.

The effect of various compounds related to nucleosides on the pH-driven uptake of lamivudine is summarised in Figure 6. The uptake of lamivudine was strongly inhibited by nitrobenzylthioinosine (NBMPR), being reduced to 39% of control by 50 μM NBMPR. Azidothymidine (AZT), dideoxycytidine (ddC), deoxyadenosine (dAD) and deoxyfluorouridine (dFU) at concentrations of 10 mM also reduced the uptake of lamivudine to about 40–70% of control. No significant effect of adenosine (AD) or dipyridamole (DPM) on the uptake of lamivudine by BBMV was observed.

Discussion

Previously, we demonstrated that lamivudine inhibited the uptake of TEA by BBMV under an outward H^+ gradient and suggested that lamivudine possesses affinity for the H^+/organic cation antiport system (Takubo et al 2000b). In the present study we therefore first examined the relationship between the uptake of lamivudine by BBMV and the H^+ gradient. The time profile of the uptake of lamivudine by BBMV indicated that the uptake of lamivudine increased gradually without an H^+ gradient, but the initial uptake was stimulated by an outward H^+ gradient, and under both conditions the uptake reached the same level. These findings indicate the presence of an H^+/lamivudine antiport system at the renal brush border membrane.

Cationic drugs such as CIM and TMP significantly reduced the pH-driven uptake of lamivudine by BBMV. The uptake of lamivudine was also inhibited by a typical organic cation, TEA, but not by a typical organic anion, PAH. These findings suggest that the H^+/lamivudine antiport system corresponds to the H^+/organic cation antiport system. The effects of an anionic drug, PBC, on the uptake of lamivudine observed in this study are consistent with this conclusion because Hsyu et al (1988) reported that a higher concentration (10 mM) of PBC competitively inhibited the pH-driven uptake of N′-methylnicotinamide, a classical substrate of the organic cation transporter, by BBMV.

Kinetic analysis of the concentration dependence of lamivudine uptake by BBMV under an outward H^+ gradient resulted in values for K_m of 2.28 mM and for V_{max} of 1.56 nmol (mg protein)$^{-1}$ (20 s)$^{-1}$. Maeda et al (1993) calculated K_m and V_{max} values of 0.21 mM and 3.83 nmol (mg protein)$^{-1}$ (10 s)$^{-1}$, respectively, for pH-driven uptake of TEA by rat renal BBMV. Takahashi et al (1993) also reported that uptake of disopyramide, a cationic drug, by rat renal BBMV is mediated by the H^+/organic cation antiport system and estimated a K_m of 0.068 mM and a V_{max} of 1.25 nmol (mg protein)$^{-1}$ (30 s)$^{-1}$. Comparison of these kinetic parameters suggests that the uptake capacity of the H^+ counter-transport system for these compounds is similar, but that the affinity of lamivudine for the antiport system is much lower than that of the other organic cations. The low affinity of lamivudine for the antiport system is also supported by our previous finding that the IC50 value (concentration resulting in 50% inhibition) of lamivudine for the pH-driven uptake of TEA by BBMV is 2.67 mM (Takubo et al 2000b).

An apparent reduction of the renal clearance (CLr) of lamivudine by co-administered TMP has been observed in non-clinical and clinical studies (Moore et al 1996; Takubo et al 2000a), and therefore the effect of TMP on the pH-driven uptake of lamivudine by BBMV was dissected. Dixon plot analysis indicated TMP competitively inhibits the uptake of lamivudine with a K_i value of 27.6 μM. We have already confirmed that TMP reduces the CLr of lamivudine at steady-state plasma concentrations in rats (Takubo et al 2000a), and the ratio of the CLr for tubular secretion in the presence and absence of TMP [(CLr (+TMP)−GFR)/(CLr (control)−GFR)] was calculated to be 0.188. In order to clarify the relative contribution of the H^+/lamivudine

antiport system to the CLr of this drug, prediction of the decrease in the CLr was attempted by using the values of K_m and K_i obtained in this study.

In the rat study, we found that CLr was mainly determined by intrinsic renal clearance and that the concentration of lamivudine in renal cortex was about 40 μM, much lower than the K_m value (Takubo et al 2000a). The ratio [(CLr (+TMP)−GFR)/(CLr (control)−GFR) can therefore be assumed to be determined by $1/(1+Iu_{TMP}/K_i)$, where Iu_{TMP} is the unbound concentration of TMP. The plasma concentration of TMP was maintained at 10.5 μM in the rat study (Takubo et al 2000a). Literature data indicate that the mean ratio of TMP concentration in kidney and plasma (C_{kidney}/C_{plasma}) was about 10 in the elimination phase after rats received intravenous dose of TMP, and that the unbound fraction of TMP, as determined from the extent of its plasma protein binding, is 0.65 (Tu et al 1989; Nouws et al 1991; Gustafsson et al 1999). Based on these values, the ratio of the CLr was predicted to be 0.288, similar to the ratio observed in the rat study in vivo. We therefore suggest that the reduction of the CLr of lamivudine by co-administered TMP is caused by the effect of TMP on the H^+/lamivudine antiport system at the renal brush border membrane.

The effect of various compounds related to nucleosides on the pH-driven uptake of lamivudine by BBMV was also investigated by using NBMPR and DPM, which are representative inhibitors of nucleoside transport, AD, which is a nucleoside reabsorbed at the renal tubule, and dAD, AZT, ddC and dFU, which are a nucleoside (dAD) and nucleoside analogues excreted by renal tubular secretion (Nelson et al 1988; Griffith & Jarvis 1996). The uptake of lamivudine was strongly inhibited by NBMPR and also reduced by dAD, AZT and ddC. However, no significant effect of AD and dFU on the uptake of lamivudine was observed. These findings indicate that certain nucleosides possess an affinity for the H^+/lamivudine antiport system.

There have so far been only a few studies of the transport systems involved in renal secretion of nucleosides. Recently, it was shown that cloned rat kidney organic anion transporter (rOAT1) and cation transporter (rOCT1) expressed in *Xenopus laevis* oocytes stimulate the uptake of nucleoside analogues by the oocyte, suggesting that they participate in their influx at the basolateral membrane of renal tubular epithelium (Chen & Nelson 2000; Wada et al 2000). However, the transporter systems facilitating efflux of nucleosides at the renal brush border membrane have not yet been definitively identified. It is possible that p-glycoprotein (P-gp) expressed at the renal brush border membrane could mediate the efflux of nucleosides (Lieberman et al 1989). However, Leung & Bendayan (1999) showed that accumulation of vinblastine, an established P-gp substrate, by cultured cells expressing P-gp was not affected by dideoxynucleoside analogues, implying that P-gp does not play a significant role in the renal tubular transport of nucleoside analogues.

Our results indicate that lamivudine is secreted by the H^+/organic cation antiport system, and they have been corroborated by the latest report of Leung & Bendayan (2001) describing uptake properties of lamivudine by a continuous renal epithelial cell line. Interestingly, it has been reported that renal secretion of AZT in the rat in vivo is mediated by an organic cation transport system at the renal brush border membrane, and also that AZT is a competitive inhibitor of the pH-driven uptake of organic cations by BBMV (Griffiths et al 1992; Aiba et al 1995). These findings suggest that efflux of AZT at the renal brush border membrane is also mediated by the H^+/organic cation antiport system, and therefore it would be possible some nucleoside analogues excreted by renal secretion also have affinity for the H^+/organic cation antiport system.

In this study we observed strong inhibitory effects of NBMPR on the pH-driven uptake of lamivudine, whereas DPM, without a nucleoside structure, was not inhibitory, indicating that the H^+/organic cation antiport system possesses a higher substrate specificity for nucleoside analogues. NBMPR with its bulky substituent is more hydrophobic than lamivudine, and therefore the higher affinity of NBMPR for the H^+/organic cation antiport system is reasonable in light of the suggestion that higher affinity of substrates for the H^+/organic cation antiport system correlates with increasing hydrophobicity (Wright & Wunz 1998; David et al 1995).

Conclusion

In this study we identified an H^+/lamivudine antiport system, corresponding to the H^+/organic cation antiport system, at the brush border membrane of the renal tubular epithelium. It is likely that renal tubular secretion of other nucleoside analogues is also mediated by the organic cation antiport system.

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