# Renin Inhibitor: Transport Mechanism in Rat Small Intestinal Brush-Border Membrane Vesicles

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The transport characteristics of the renin inhibitor ((3S,4S)-4-[N-morpholinoacetyl-(1-naphthyl)-L-alanyl-N-methyl-(4-thiazolyl)-L-alanyl]amino-3-hydroxy-5-cyclohexyl-1-(4-pyridyl)-1-pentanone; $CH_3$-18) in rat small intestinal brush-border membrane vesicles (BBMV) were examined by a rapid filtration technique. The uptake of $CH_3$-18 was markedly stimulated by an inwardly directed $H^+$ gradient (pH 7.5 inside, pH 5.5 outside) and showed an uphill transport. It was competitively inhibited by tripeptides and tetrapeptides, but not by amino acids or dipeptides. A countertransport effect on the uptake of $CH_3$-18 was observed in the vesicle preloaded with a tripeptide. Effects of the fragments of several renin inhibitors were evaluated by their inhibitory and countertransport effects on the uptake of $CH_3$-18. The morpholino group at the N-terminal was found to be important for the uptake of $CH_3$-18.

KEY WORDS: renin inhibitor; rat intestinal brush-border membrane vesicle; BBMV; proton coupled uphill transport; peptide carrier system.

## INTRODUCTION

Common problems in developing renin inhibitors are low solubility, insufficient oral absorption, and fast hepatic clearance (1–5). We improved the low solubility by using β-cyclodextrin and found that some renin inhibitors with the same substituents were transported well from the intestinal loop into mesenteric venous blood (6). However, they were subject to fast hepatic clearance and their AUCs were low (6). We tried to protect the amido bond in one renin inhibitor ($CH_3$-18) to overcome the hepatic first pass effect and found that the AUC was dramatically evaluated by N-methylation (6). Although some factors lowering the bioavailability of some renin inhibitors were overcome (6), it is still unknown why a saturation phenomenon occurs on the transport process of well-absorbed renin inhibitors from the intestinal loop into mesenteric venous blood. Some renin inhibitors have been suggested to be absorbed by the intestinal peptide transport system (7 and 8), but the transport system is still unclear. Furthermore, the structure of the transporter is unknown. Therefore, it is difficult to determine the structural requirements of orally active renin inhibitors for intestinal transport. Herein, we report the transport mechanism of $CH_3$-18 by using rat intestinal brush-border membrane vesicles (BBMV) and show structural modifications that enhance transport.

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## EXPERIMENTAL SECTION

### Materials

Renin inhibitor ((3S,4S)-4-[N-morpholinoacetyl-(1-naphthyl)-L-alanyl-N-methyl-(4-thiazolyl)-L-alanyl]amino-3-hydroxy-5-cyclohexyl-1-(4-pyridyl)-1-pentanone; $CH_3$-18), the fragments of renin inhibitors, and ceftibuten were synthesized at Shionogi Research Laboratories (Osaka, Japan). Cefaclor and Cephalexin were supplied from Eli Lilly (Indianapolis, Ind.). Cyclacillin was purchased from Takeda Chemical Industries (Osaka, Japan). All other peptides used were obtained from Sigma Chemical Co. (St. Louis, Mo.). All other chemicals were of the highest grade available commercially.

### Animals

Experiments were conducted using male Sprague-Dawley rats (Japan Clea Inc., Osaka, Japan) weighing 240–300 g. Animals were fasted for about 20 hrs prior to the experiment but allowed free access to water.

### Analytical Procedures

The content of the compound $CH_3$-18 was measured by high-performance liquid chromatography (HPLC). The diluted sample was injected onto a column (4.6 × 150 mm) packed with Nucleosil $5C_{18}$ (Chemco Scientific Co., Ltd., Osaka, Japan). The eluent was a mixture of 0.1% trifluoroacetic acid solution and acetonitrile (2:1). The sample was analyzed by UV detection at the range of 230 nm with a photodiode array UV–VIS detector (SPD-M6A, Shimadzu, Kyoto, Japan).

The protein concentration was measured by the Bio-Rad protein assay kit (Bio-Rad, Richmond, Ca.) with bovine γ-globulin as a standard.

### Transport Studies with Brush-Border Membrane Vesicles

The brush-border membrane was isolated from the rat small intestine according to the calcium precipitation method of Kessler et al. (9). The uptake studies were carried out at 30°C by the same rapid filtration technique as described by our colleagues (10 and 11). The freshly isolated vesicles were resuspended with 10 mM Hepes buffer (pH 7.5) containing 100 mM mannitol, 100 mM KCl and made up to a final concentration of 10–15 mg protein/ml. The vesicle suspension (20 μl) was added to 200 μl of a reaction mixture containing 0.7 mM $CH_3$-18, 100 mM mannitol, 100 mM NaCl, and 10 mM Mes (pH 5.5) to start the transport. The reaction was stopped by the addition of 1 ml of ice-cold stop solution, containing 100 mM mannitol, 100 mM KCl, 10 mM Mes (pH 5.5), and 1.5% (w/v) β-cyclodextrin to avoid the adsorption of renin inhibitor to Millipore filter (DAWP, 0.65 μm, 2.5 cm diameter). Then, the mixture was immediately filtered by the filter, followed by washing with 5 ml of ice-cold stop solution. $CH_3$-18 trapped on the filter was dissolved with 200 μl

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of the HPLC eluent and measured by HPLC. More than 95% of $CH_3$-18 trapped on the filter was extracted.

To test the functional integrity of the brush-border membrane vesicles, the $H^+$ gradient-dependent ceftibuten uptake was examined (10). A typical overshoot uptake of ceftibuten was observed in the presence of an inwardly directed $H^+$ gradient (pH 5.5 outside and pH 7.5 inside) and the peak uptake value was three times higher than that at equilibrium. The enrichment of the marker enzymes in the brush-border membrane vesicles was almost the same as reported previously by our colleagues (10).

The inhibitory effect of various compounds on the renin inhibitor uptake was examined by their addition to the reaction solution at a final concentration of 10 mM. The reaction was stopped at 30 sec.

The countertransport effect was studied as follows. Brush-border membrane vesicles were suspended with pH 5.5 buffer solution to a protein concentration of about 20 mg protein/ml. Before the uptake experiment, a portion of the vesicle suspension (10 μl) was preloaded with 10 μl of pH 5.5 buffer solution containing various compounds (20 mM) for 5 min at 30°C, then the uptake experiment was carried out as described above. The reaction was stopped at 30 sec.

## RESULTS AND DISCUSSION

### Effect of a Transmembrane $H^+$ Gradient on the Uptake of $CH_3$-18

The time course of the uptake for $CH_3$-18 (Table I), which was absorbed well from the intestine (6), was examined in the presence and absence of an $H^+$ gradient (the extravesicular pH 5.5 and 7.5, respectively). $CH_3$-18 was taken up rapidly with an inwardly directed $H^+$ gradient (pH 5.5 outside and pH 7.5 inside) compared with that without the $H^+$ gradient (pH 7.5 outside and pH 7.5 inside) while each approached the same equilibrium value (Fig. 1). In addition, an overshoot phenomenon was observed at 60 sec under the $H^+$ gradient. Figure 2 shows the effect of an $Na^+$ gradient on the uptake of $CH_3$-18. An inwardly directed $Na^+$ gradient did not significantly stimulate the initial uptake for 60 sec under either extravesicular pH condition.

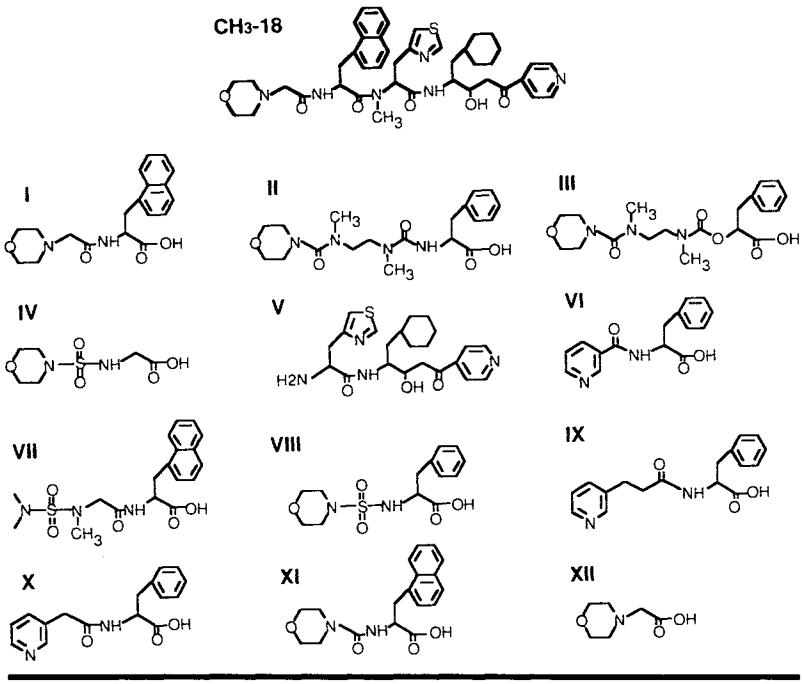
### Inhibitory Effect of Amino Acids and Oligopeptides

Figure 3 shows the effects of amino acids and oligopeptides (10 mM) on the uptake of $CH_3$-18 in the presence of an $H^+$ gradient. Amino acids and dipeptides, except for His-Gly, had little effect on the uptake of $CH_3$-18. However, tripeptides (Ala-Ala-Ala and Glu-Ala-Ala) and tetrapeptides (Ala-Ala-Ala-Ala and Gly-Gly-Gly-Gly) inhibited the uptake of $CH_3$-18. The order of the inhibitory effect was roughly estimated to be tripeptide > tetrapeptide > dipeptide, amino acid. These findings suggested that the transport system of oligopeptides larger than a dipeptide played an important role in the transport of the renin inhibitor like $CH_3$-18.

### Inhibitory Effect of Amino β-Lactams Antibiotics

Figure 3 shows the effect of amino β-lactam antibiotics on the uptake of $CH_3$-18. All antibiotics except for ceftibuten inhibited the uptake of $CH_3$-18. Cyclacillin showed the strongest inhibition on the uptake of $CH_3$-18, as well as on the uptake of ceftibuten (11). Therefore, ceftibuten was expected to show a marked inhibition on the uptake of $CH_3$-18, however, it did not inhibit the uptake of $CH_3$-18 at all (Fig. 3). The two compounds inhibited by the third compound do not always show mutual inhibition in the transport studies with BBMV. It is difficult to account for this discrepancy at present.

Table I. Molecular Structures of Renin Inhibitor and the Constituents



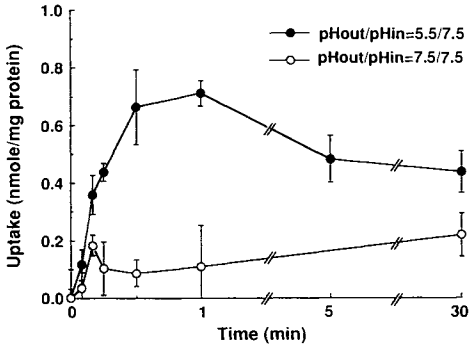



**Figure 1** Effect of an inwardly directed $H^+$ gradient on the uptake of $CH_3$-18 by intestinal BBMV. Membrane vesicles were suspended in either Mes buffer (pH 5.5; ●) or Hepes buffer (pH 7.5; ○). Data represent the mean ± S.E. (n = 4–6).

### Inhibitory Effect of Renin inhibitor Fragments

Figure 4 shows the effects of renin inhibitor fragments on the uptake of $CH_3$-18. Some fragments are part of the renin inhibitors that are well-absorbed from the intestine as reported previously (6). Table I shows the molecular structures of the fragments. I and XII are the N-terminal fragments of well-absorbed renin inhibitors, VIII is the N-terminal fragment of poorly-absorbed renin inhibitors, and V is the C-terminal fragment of well-absorbed renin inhibitors (6). Some fragments inhibited the uptake of $CH_3$-18. Especially, I, VI, XI, and XII inhibited the uptake strongly. I and XII were the fragments of well-absorbed renin inhibitors. The common features of the fragments, which inhibit the uptake strongly, were the presence of the morpholino group in the N-terminal fragments such as I, XI, and XII. However, the effect of inhibition was decreased when a sulfonyl group was adjacent to the morpholino group such as IV and VIII. Bulky substituents such as the sulfonyl group may interfere with the access of morpholino group to the binding site of the carrier protein. The C-terminal fragment of the renin inhibitor such as V also inhibited the uptake of $CH_3$-18, but the effect was not so strong. These findings suggest that the N-terminal substituent is more important than the C-terminal substituent on the transport in the BBMV.

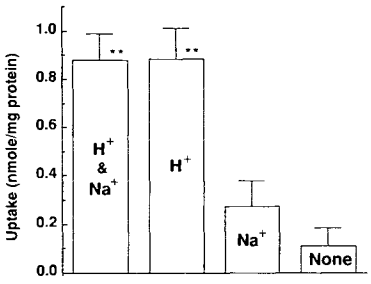



**Figure 2** Effect of an $Na^+$ gradient on the uptake of $CH_3$-18 by intestinal BBMV at different extravesicular pHs 5.5 and 7.5. $H^+$ and $Na^+$ in the columns indicate the presence of $H^+$, $Na^+$, or both gradients. "None" shows the absence of both gradients. Data represent the mean ± S.E. (n = 4–6). Asterisk (**) on the right of the bar shows that the value is statistically significant ($P < 0.01$) compared with the uptake of None.

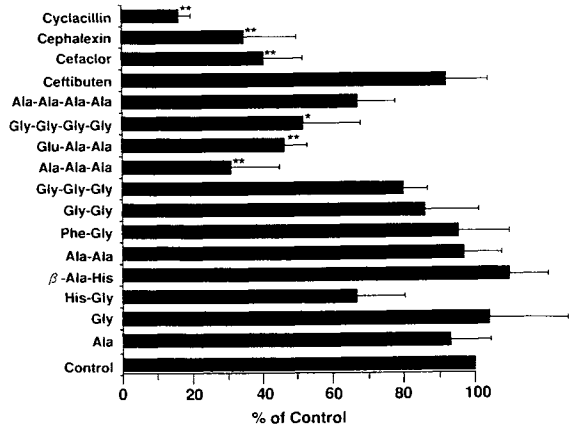



**Figure 3** Inhibitory effects of amino acids, oligopeptides, and amino β-lactam antibiotics on the uptake of $CH_3$-18 by intestinal BBMV under $H^+$ gradient. Control value of the initial uptake of $CH_3$-18 is 0.59 ± 0.05 nmol/mg protein/30 sec. Data represent the mean ± S.E. (n = 4–6). *P < 0.05, **P < 0.01 compared with control.

### Countertransport Effect of Oligopeptides and Fragments of Renin Inhibitors

The countertransport effects of oligopeptide and some fragments of renin inhibitors were studied to confirm that $CH_3$-18 was transported by a carrier mechanism. Representative compounds, which inhibited the uptake of $CH_3$-18 strongly, showed significant countertransport effects except for XII (Fig. 5). XII did not show the countertransport effect at all. This discrepancy between the inhibitory effects and the countertransport effects of XII may be explained as follows. The affinity of XII to the carrier protein is very strong at one binding site of carrier protein and XII can inhibit the uptake of $CH_3$-18 very strongly. However, XII lacks the affinity to carrier protein, which is necessary for being transported by a carrier protein, thus XII can not be transported and does not stimulate countertransport as a result. On the other hand, XII could combine with the carrier protein so strongly that it cannot separate from carrier protein to initiate countertransport. Compounds I and VI and Ala-Ala-Ala increased the uptake of $CH_3$-18 and appeared to have suitable affinity to the carrier protein for renin inhibitor.

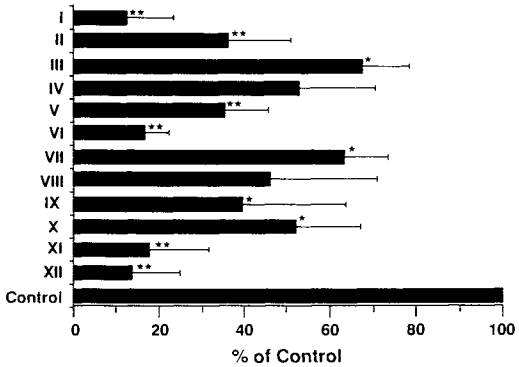



**Figure 4** Inhibitory effects of the fragments on the uptake of $CH_3$-18 by intestinal BBMV under $H^+$ gradient. Control value of the initial uptake of $CH_3$-18 is 0.63 ± 0.04 nmol/mg protein/30 sec. Data represent the mean ± S.E. (n = 4–6). *P < 0.05, **P < 0.01 compared with control.

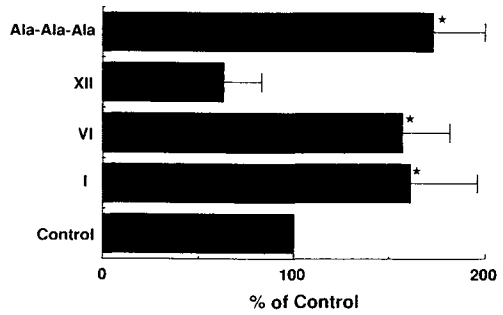



**Figure 5** Countertransport effects of the fragments, I, VI, and XII, and Ala-Ala-Ala on the uptake of $CH_3$-18. Control value of the initial uptake of $CH_3$-18 is 0.14 ± 0.03 nmol/mg protein/30 sec. Data represent the mean ± S.E. (n = 4–5). *P < 0.05 compared with control.

On the basis of these findings, it may be possible to design the molecular structure of a renin inhibitor absorbed better than the present compounds by using peptides and the fragments of the renin inhibitors even though the structure of the carrier protein is unknown.

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