Absorption Mechanism of 1,3-Bis(2-ethoxycarbonylchromon-5-yloxy)-2-((S)-lysyloxy)propane Dihydrochloride (N-556), a Prodrug for the Oral Delivery of Disodium Cromoglycate

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To clarify the absorption mechanism of 1,3-bis(2-ethoxycarbonylchromon-5-yloxy)-2-((S)-lysyloxy)propane dihydrochloride (N-556), a prodrug for the oral delivery of disodium cromoglycate (DSCG), a study was made using rats. N-556 gave the highest plasma level of DSCG following its injection into the loop at the upper part of the small intestine. N-556 was stable in acidic washings of gastric contents, but rapidly hydrolyzed to M1 with twin ethyl residues on DSCG in the washings of the small intestinal contents. N-556 and M1 were hydrolyzed to DSCG via M2 having a mono ethyl residue in the homogenate of the small intestinal mucosa. The oral absorption of M1 following its administration in 50% (v/v) propylene glycol solution was essentially the same as that of N-556. That of M1 administered in aqueous suspension was low. After the oral administration of N-556, a small amount of M2 and a trace of M3 having L-lysyl residue were detected in the portal plasma, but no hydrolytic intermediate except DSCG could be found in the general plasma. The major absorption mechanism of N-556 may thus be concluded as follows: N-556 given orally is transferred to the small intestine in essentially intact form. N-556 is then rapidly diffused to an aqueous layer on the surface of the mucosal membrane and hydrolyzed to M1. The resultant M1 is transported to the mucosal membrane and hydrolyzed to DSCG via M2. DSCG generated in the mucosal membrane is used for general circulation through the portal blood and liver. A small amount of M2 in the mucosal membrane is transported to the portal blood and hydrolyzed to DSCG in the portal blood and/or liver to make DSCG available for the general circulation.

Keywords 1,3-bis(2-ethoxycarbonylchromon-5-yloxy)-2-((S)-lysyloxy)propane dihydrochloride (N-556); disodium cromoglycate; prodrug; absorption mechanism; hydrolysis

Introduction

Disodium cromoglycate (DSCG) is effective for treating allergic diseases such as asthma and allergic rhinitis. It is restricted to topical use as an insufflated powder or aqueous aerosol owing to its poor absorption from the gastrointestinal tract.¹⁻⁴⁾

To improve the oral absorption of DSCG, prodrugs were synthesized and 1,3-bis(2-ethoxycarbonylchromon-5-yloxy)-2-((S)-lysyloxy)propane dihydrochloride (N-556) was selected as suitable for the oral delivery of DSCG.⁵⁾ N-556 is a water-soluble prodrug and the poor lipophilicity of DSCG is improved by introducing the ethyl residues into twin carboxyl groups of DSCG and the resultant decrease in water solubility is overcome by introducing the L-lysyl residue into a hydroxy group.⁶⁾ N-556 gave a high plasma concentration of DSCG in experimental animals⁷⁾ and showed good anti-allergic effects on experimental allergy models in rats.⁸⁾ However, the manner in which N-556 is hydrolyzed to DSCG in the absorption process is not clear.

The absorption mechanism of N-556 in rats is discussed in the following.

Materials and Methods

Materials The structures of the compounds in this study are shown in Fig. 1.

N-556, M1, M2 and M3 were synthesized in our laboratory. DSCG (Fisons Pharmaceuticals Inc., Loughborough, U.K.) was obtained commercially. All other materials were of reagent grade.

Animals Seven or eight-week-old male albino Wistar rats weighing 190-230 g (Japan SLC Inc., Hamamatsu, Japan) were used. They were fasted overnight but had free access to water.

Absorption Site The rats were anesthetized with an intraperitoneal

injection of 50 mg/kg of pentobarbital and the bile duct was ligated following abdominal incision. N-556 was dissolved in saline and administered at 25 mg/kg into the loop at the stomach or each loop of about 10 cm long at the upper, middle or lower intestine. The loop was replaced after the administration of N-556 and about 0.6 ml blood samples were taken from the jugular vein with a heparinized syringe at specified times up to 2 h. The blood samples were centrifuged for 10 min at 3000 rpm to obtain the plasma. The plasma concentration of DSCG was determined by high performance liquid chromatography (HPLC).

Hydrolysis in Vitro The rats were bled to death under ether anesthesia, and the stomachs and small intestines were removed. The gastric contents were flushed out with 5 ml of chilled saline and small intestinal contents were flushed out with 10 ml of chilled phosphate buffer (0.1 M, $\text{KH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$, pH 7.0). The washings were centrifuged at 12000 rpm for 15 min at 5°C and the supernatant was used as the washing of the gastric or small intestinal contents. The pH of the washings of the gastric contents was about 3.0.

The small intestine, with the luminal contents flushed out, was everted and the mucosa was scraped off with the edge of a microscope slide glass. The mucosa was homogenized in 3.3 volumes of phosphate buffer (0.1 M, pH 7.0) using an Ultra Disperser (LK-21, Yamato Scientific Co., Ltd., Tokyo, Japan). The homogenate was centrifuged at 12000 rpm for 15 min at 5°C and the supernatant served as the homogenate of the small intestinal mucosa.

Fig. 1. Salt Free Chemical Structure of N-556 and Related Compounds

Hydrolysis in the washings of the gastrointestinal contents and homogenate of the small intestinal mucosa was conducted as follows: N-556 or M1 was dissolved in *N,N*-dimethylacetamide at a concentration of 10 mM. Ten μ l of the solution were mixed with 1.0 ml of the washings of the gastrointestinal contents or homogenate of the small intestinal mucosa. The mixture was incubated at 37°C. At specified times following the start of incubation, hydrolytic reaction was terminated by adding 2.0 ml of 0.1 N ethanolic HCl:CH₃CN (1:1, v/v) to the reaction mixture. After centrifugation at 12000 rpm for 5 min, the concentrations of N-556, M1, M2, M3 and DSCG in the supernatant were determined by HPLC.

Absorption of M1 The rats were anesthetized with ether and the bile duct was ligated after abdominal incision. N-556 and M1 were administered at a dose equivalent to 5 mg/kg of M1 in solution or as an aqueous suspension after the rats recovered from anesthesia. A solution of M1 was prepared by first dissolving it in propylene glycol at about 60°C, and then diluting with an equal volume of warm distilled water. An aqueous suspension of M1 was prepared with distilled water. A dose solution of N-556 was prepared by dissolving it in 50% (v/v) propylene glycol solution or distilled water.

Plasma samples were collected by the same method as for the absorption site and the plasma concentration of DSCG was determined by HPLC. The area under the plasma concentration of DSCG *versus* time curve (*AUC*) was calculated from the trapezoidal rule.

Analysis of Portal and General Plasma An aqueous solution of N-556 was administered to the rats at 50 mg/kg using a stomach tube. Portal and general blood were taken with a heparinized syringe from the portal and jugular veins after abdominal incision under ether anesthesia at specified times following administration. The blood samples were immediately transferred to an iced centrifuge tube and centrifuged at 3000 rpm for 5 min at 5°C.

The concentrations of N-556, M1, M2, M3 and DSCG in the portal and general plasma were determined by HPLC.

HPLC Assay The concentrations of N-556, DSCG and other compounds were determined by HPLC. HPLC apparatus in this study was the same as previously used.⁶⁾

1) Absorption Site and Absorption of M1: The plasma concentration of DSCG was determined as described previously.⁶⁾

2) Hydrolysis *in Vitro*: The concentrations of N-556, M1, M2, M3 and DSCG were determined. A reverse phase packed column ODS-M (5 μ m, 4.6 mm i.d. $\times$ 150 mm, Shimadzu Techno-Research Inc., Kyoto, Japan) was used as the analysis column for N-556, M1 and M2. A mixture of 0.02 M KH₂PO₄-H₃PO₄ (pH 2.5):CH₃CN:CH₃OH (40:30:30, v/v) containing 3 mM sodium 1-octanesulfonate and 2 mM sodium 1-nonanesulfonate was used as the mobile phase for N-556 and M1, and the eluate was monitored at 275 nm at a flow rate of 1.0 ml/min. For M2, a mixture of 0.02 M KH₂PO₄:CH₃CN:CH₃OH (60:15:25, v/v) served as the mobile phase. The eluate was monitored at 254 nm at a flow rate of 0.9 ml/min.

In the case of M3, a Develosil NH₂ packed column (5 μ m, 4.6 mm i.d. $\times$ 150 mm, Nomura Chemical Co., Seto, Japan) was used as the analysis column and a mixture of 0.11 M KH₂PO₄-H₃PO₄ (pH 3.0):CH₃CN (1:2, v/v), as the mobile phase. The eluate was monitored at 240 nm at a flow rate of 1.2 ml/min.

The concentration of DSCG was determined under the same conditions as in the previous paper.⁷⁾

3) Analysis of Portal and General Plasma: Pretreatment of plasma was conducted prior to its injection into the HPLC column. For the analysis of N-556, M1, M2 and M3, 0.5 ml of plasma were taken into a centrifuge tube followed by the addition of 1.0 ml of CH₃CN. The mixture was stirred vigorously and centrifuged at 12000 rpm for 5 min at 5°C. One ml of the supernatant was dried under a N₂ gas stream. The residues thus obtained were dissolved in 0.2 ml of a mixture of 0.02 M KH₂PO₄:CH₃CN:CH₃OH (4:3:3, v/v) and 20 or 30 μ l of the solution were injected into the HPLC column. HPLC conditions were the same as for hydrolysis *in vitro*.

The plasma concentration of DSCG was determined as in the previous paper.⁷⁾

The detection limits of the plasma concentration were 40 ng/ml for N-556 and M1, 25 ng/ml for M2, 30 ng/ml for M3 and 5 ng/ml for DSCG.

Results and Discussion

We reported previously the hydrolysis of an amino acid moiety to be essential to a good oral absorption of water-soluble prodrugs of DSCG.⁶⁾ In this paper, examination was made of the absorption mechanism of N-556 possessing

Fig. 2. Plasma Levels of DSCG after Administration of N-556 into the Gastrointestinal Loop in Rats

N-556 was given into each loop at a dose of 25 mg/kg. Each point and vertical bar represents the mean $\pm$ S.E. of 3 rats. ○, stomach; ●, small intestine (upper); ▲, small intestine (lower).

twin ethyl residues and an L-lysyl residue.

For determination of the absorption site, the plasma concentration of DSCG was measured following the administration of N-556 into the loop at different parts of the gastrointestinal. Absorption from each loop at the gastrointestinal is shown in Fig. 2.

The highest concentration of DSCG in the plasma was observed when N-556 was administered into the loop at the upper part of the small intestine. The plasma concentration of DSCG was moderate after the administration of N-556 into the loop at the middle and lower parts of the small intestine. However, it was quite low for the stomach part. The absorption rate of N-556 from the upper part of the small intestine was greater than that from the middle or lower part of the small intestine. The difference of the absorption rate from each part of the small intestine may be attributed to the hydrolysis rate of an L-lysyl residue of N-556 in each loop, since the hydrolysis rate of an amino acid moiety plays an important role for the oral absorption of water-soluble prodrugs of DSCG.⁶⁾ N-556 is thus shown to be mainly absorbed from the upper part of the small intestine including the duodenum.

The *in vitro* hydrolysis of N-556 and M1 in washings of the gastrointestinal contents and homogenate of the small intestinal mucosa was subsequently conducted. These results are shown in Figs. 3 and 4.

N-556 was stable in the acidic washings of gastric contents. It was rapidly hydrolyzed to M1 still with twin ethyl residues in the washings of small intestinal contents; M1 was stable. The digestive enzymes, trypsin or α -chymotrypsin, may possibly participate in the hydrolysis of the L-lysyl residue of N-556 in the washings of small intestinal contents, as discussed previously.⁶⁾ In the homogenate of the small intestinal mucosa, N-556 and M1 were hydrolyzed to DSCG *via* M2 which had a mono ethyl residue.

N-556 was hydrolyzed to M1 in the washings of small intestinal contents and thus the permeability of M1 to the gastrointestinal was assessed. For this purpose, it was administered orally in solution or as an aqueous suspension and the plasma concentration of DSCG was determined.

Fig. 3. Hydrolysis of N-556 in the Washings of the Gastric (a) and Small Intestinal Contents (b)

Each point represents the mean of 3 experiments. ●, N-556; ▲, M1; ■, M2.

Fig. 4. Hydrolysis of N-556 (a) and M1 (b) in the Homogenate of the Small Intestinal Mucosa

Each point represents the mean of 3 experiments. ●, N-556; ▲, M1; ■, M2; ○, DSCG.

Fig. 5. AUCs after Oral Administration of M1 and N-556 in Solution or Suspension in Rats

M1 and N-556 were administered at a dose equivalent to 5 mg/kg of M1. Each column and bar represents the mean $\pm$ S.E. of 3 rats.

In this experiment, the bile duct of rats was ligated, since a portion of DSCG that reaches the liver through the portal vein is excreted in the bile without being transferred to the general circulation.⁷⁾ Oral absorption of N-556 and M1 in solution or suspension following their oral administration is shown in Fig. 5.

AUC after the oral administration of M1 in 50% propylene glycol solution was similar to that after oral administration of N-556 in aqueous or 50% propylene

TABLE I. Portal Plasma Levels of N-556, M1, M2, M3 and DSCG in Rats after Oral Administration of N-556 at a Dose of 50 mg/kg

Assayed compound	Plasma level (ng/ml)				
	0.08 h	0.25 h	0.5 h	1.0 h	2.0 h
N-556	— ^{a)}	—	—	—	—
M1	—	—	—	—	—
M2	114.2 $\pm$ 9.8 ^{b)}	84.6 $\pm$ 34.3	77.1 $\pm$ 17.1	—	—
M3	—	39.9 $\pm$ 22.6	—	—	—
DSCG	819.3 $\pm$ 77.4	1575.1 $\pm$ 150.7	1081.8 $\pm$ 206.5	430.4 $\pm$ 121.2	253.8 $\pm$ 62.2

a) Not detected. b) Each value represents the mean $\pm$ S.E. of 3 animals.

TABLE II. General Plasma Levels of N-556, M1, M2, M3 and DSCG in Rats after Oral Administration of N-556 at a Dose of 50 mg/kg

Assayed compound	Plasma level (ng/ml)				
	0.08 h	0.25 h	0.5 h	1.0 h	2.0 h
N-556	— ^{a)}	—	—	—	—
M1	—	—	—	—	—
M2	—	—	—	—	—
M3	—	—	—	—	—
DSCG	295.2 $\pm$ 23.6 ^{b)}	383.6 $\pm$ 58.7	511.6 $\pm$ 23.4	193.3 $\pm$ 87.0	42.9 $\pm$ 4.8

a) Not detected. b) Each value represents the mean $\pm$ S.E. of 3 animals.

glycol solution. AUC after the oral administration of M1 in aqueous suspension was very low.

Low water solubility of M1 thus appears quite likely to

Fig. 6. Proposed Absorption Mechanism of N-556

be a rate-limiting factor for oral absorption, although it has suitable lipophilicity for permeation of the membrane.⁶⁾ According to a model proposed by Ho and Higuchi *et al.*,⁹⁻¹¹⁾ an aqueous layer on the surface of the membrane plays an important role for the oral absorption of drugs whose partition coefficients are sufficient for the membrane permeation. Poor absorption following the administration of M1 in suspension may thus be the result of its low concentration in the aqueous layer. That M1 is diffused to the aqueous layer in high concentration may be the reason for the increased oral absorption following the administration of M1 in solution. On the other hand, N-556 may easily diffuse to the aqueous layer and continuously provide M1 by liberating an L-lysyl residue. M1 generated in the aqueous layer may be immediately transported to the mucosal membrane. This may explain the improved oral absorption of N-556.

Analysis of portal and general plasma was conducted to determine the presence of hydrolytic intermediates after the oral administration of N-556. The results are summarized in Tables I and II.

M2 having a mono ethyl residue was identified in the portal plasma up to 30 min following the administration of N-556. The peak plasma concentration of M2 was 114.2 ng/ml at 5 min and decreased to below the detection limit at 60 min. N-556 and M1 could not be found at any time, while M3 was present in trace amounts only at 15 min after the administration. In general plasma, no hydrolytic intermediate except DSCG was noted at any time after the administration of N-556.

That M2 and M3 were present in the portal plasma suggests the possibility of a pathway by which N-556 is directly transported to the mucosal membrane. However, such a pathway would only be a minor route based on the results of the hydrolysis *in vitro*. M2 and M3 in the portal blood may be hydrolyzed to DSCG in the blood and/or liver.

Based on the above, the proposed absorption mechanism of N-556 is shown in Fig. 6.

Orally administered N-556 is transferred to the small

intestine in essentially intact form without hydrolysis in the stomach. N-556 is rapidly diffused to an aqueous layer on the surface of the mucosal membrane and hydrolyzed to lipophilic M1 by digestive enzymes and/or by general acid-base catalysis. M1 thus produced is transported to the mucosal membrane and hydrolyzed enzymatically to DSCG *via* M2. DSCG generated in the membrane is transported to the general circulation through the portal blood and liver. A small amount of M2 is transported to the portal blood and hydrolyzed to DSCG in the portal blood and/or liver, thus making DSCG available for the general circulation. As a minor pathway, N-556 after diffusing to an aqueous layer is transported to the mucosal membrane and hydrolyzed to DSCG *via* M3. DSCG generated in the mucosal membrane is transferred to the general circulation. A trace of M3 is also transported to the portal blood and hydrolyzed to DSCG in the portal blood and/or liver so that DSCG becomes available for the general circulation.

References

- 1) L. S. Schanker, D. J. Tocco, B. B. Brodie and C. A. M. Hogben, *J. Pharmacol. Exp. Ther.*, **123**, 81 (1958).
- 2) C. A. M. Hogben, D. J. Tocco, B. B. Brodie and L. S. Schanker, *J. Pharmacol. Exp. Ther.*, **125**, 275 (1959).
- 3) G. F. Moss, K. M. Jones, J. T. Ritchie and J. S. G. Cox, *Toxicol. Appl. Pharmacol.*, **17**, 691 (1970).
- 4) S. R. Walker, M. E. Evans, A. J. Richards and J. W. Paterson, *J. Pharm. Pharmacol.*, **24**, 525 (1972).
- 5) T. Mori, K. Nishimura, S. Tamaki, S. Nakamura, H. Tsuda and N. Kakeya, *Chem. Pharm. Bull.*, **36**, 338 (1988).
- 6) A. Yoshimi, H. Hashizume, S. Tamaki, H. Tsuda, F. Fukata, K. Nishimura and N. Yata, *J. Pharmacobio-Dyn.*, **15**, 339 (1992).
- 7) A. Yoshimi, H. Hashizume, M. Kitagawa, K. Nishimura and N. Kakeya, *J. Pharmacobio-Dyn.*, **15**, 681 (1992).
- 8) S. Nakamura, H. Yamamura, S. Kohno, K. Ohata, A. Yoshimi, K. Nishimura and N. Kakeya, *Jpn. J. Allergol.*, **39**, 1621 (1990).
- 9) A. Suzuki, W. I. Higuchi and N. F. H. Ho, *J. Pharm. Sci.*, **59**, 644 (1970).
- 10) A. Suzuki, W. I. Higuchi and N. F. H. Ho, *J. Pharm. Sci.*, **59**, 651 (1970).
- 11) N. F. H. Ho, W. I. Higuchi and J. Turi, *J. Pharm. Sci.*, **61**, 192 (1972).