Development of an Oral Formulation of Azetirelin, a New Thyrotropin-Releasing Hormone (TRH) Analogue, Using *n*-Lauryl- β -D-maltopyranoside as an Absorption Enhancer

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The effects of formulation factors on the enhancement of colonic absorption of azetirelin by *n*-lauryl- β -D-maltopyranoside (LM) were studied in rats. Coadministration of LM with a small volume of azetirelin solution to the proximal colon increased the AUC of the drug by 8.7-fold. There were no significant differences in the LM-induced absorption profiles of azetirelin between unligated and ligated colon. The addition of a viscous polymer to the drug solution, which delayed the *in vitro* release of both azetirelin and LM, reduced the promoting effects of LM. These results suggest that the action of LM is not affected by sample spreading in the colonic lumen, whereas a rapid release of both azetirelin and LM from the formulation is necessary to maximize the efficacy of LM. Utilizing the balloon sonde method, the effects of LM were also confirmed in the colonic loop of dogs. Based on these results, an enteric capsule formulation of azetirelin containing LM and citric acid (CA), a potential inhibitor of the bacterial degradation of azetirelin in the distal intestine, was prepared and its performance was evaluated in fasted dogs. The bioavailability of azetirelin after the oral administration of this enteric capsule with LM and CA was 43.5% compared with a bioavailability of 14.9% in capsules without LM and CA. Therefore, the delivery of azetirelin and LM to the lower intestine, together with a rapid release of capsule contents, are feasible for the improved peroral bioavailability of azetirelin.

Key words azetirelin; *n*-lauryl- β -D-maltopyranoside; absorption enhancer; TRH analogue; colon; oral bioavailability

Azetirelin, (*N* ^{α} -[[(*S*)-4-oxo-2-azetidiny]carbonyl]-L-histidyl-L-prolineamide) is a novel thyrotropin-releasing hormone (TRH) analogue which shows relative selectivity for action on the central nervous system (CNS) and less thyrotropin (TSH)-releasing activity than TRH.^{1–3)} The poor oral bioavailability of azetirelin (<2% in rats) was previously shown to be due to low intestinal permeability resulting from the high hydrophilicity of the drug.⁴⁾ In a previous report, the effects of various absorption enhancers on the intestinal absorption of azetirelin were evaluated in rats using both an *in vitro* Ussing chamber and *in situ* closed loop methods.⁵⁾ *n*-Lauryl- β -D-maltopyranoside (LM) was selected as the most effective and least harmful absorption enhancer. As LM was much more potent in the colon than in the jejunum, the colon was considered to be a suitable site of action.

It is known that formulation factors such as disposition and delivery rate can influence the efficacy of absorption enhancers. Van Hoogdalem *et al.* reported that the absorption enhancing action of salicylate in rat rectum was more significant when delivered as a bolus than as an infusion.⁶⁾ Moore *et al.* found that sodium salicylate in mineral oil specifically enhanced the absorption of recombinant methionyl human growth hormone from the ileum and colon.⁷⁾ They speculated that mineral oil worked to delay the release of the enhancer and thus prolonged the absorption enhancement period. In light of these reports, the design of an appropriate oral delivery system seems to be important for a successful peroral formulation with absorption enhancers.

In this study, therefore, the effect of formulation factors on the increased colonic absorption of azetirelin due to LM was investigated in rats. In addition, the absorption promoting action of LM was examined in dogs utilizing the balloon sonde

method. Finally, an enteric capsule formulation of azetirelin containing LM was developed, and its *in vivo* performance was evaluated in dogs.

MATERIALS AND METHODS

Animals Male Wistar rats, weighing 230–280 g, and beagle dogs, weighing 10.5–12.5 kg, were not fed for about 24 h before the experiments but were allowed free access to water.

Materials Azetirelin was synthesized in the Central Research Laboratories of Yamanouchi Pharmaceutical Co., Ltd. LM was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Citric acid (CA) was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Hydroxypropyl-methylcellulose (HPMC 60SH4000) and hydroxypropyl-methylcellulose acetate succinate type H (HPMC AS-H) enteric capsules #0 were obtained from Shin-etsu Chemical Co. (Tokyo, Japan). All other chemicals and solvents were of reagent grade and were used without further purification.

Sample Preparation **Drug Solution:** A drug solution was prepared by dissolving azetirelin in pH 6.5 isotonic phosphate buffer solution.⁴⁾ An appropriate concentration of LM was added when needed. The dosing volume was 150 μ l for rats and 10 ml for dogs.

Gel Formulations: The drug solution was gelled by adding 5, 7.5 and 10% (w/v) of HPMC 60SH4000. The *in vitro* dissolution of azetirelin and LM from the gel formulations was studied using JP XIII dissolution test apparatus No. 2 (Toyama Industry Co., Ltd.) at 37 $\pm$ 0.5°C with a paddle speed of 50 rpm. Five-hundred milliliters of purified water was used as the test solution. One gram of sample on a glass

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dish was placed at the bottom of the vessel. Aliquots of test solution were periodically withdrawn. The concentrations of azetirelin and LM in the solution were assayed by HPLC⁴⁾ and the anthrone method,⁸⁾ respectively.

Enteric Capsule Formulation of Azetirelin with LM: A size #0 HPMC AS-H enteric capsule was filled with a powder mixture consisting of 15 mg of azetirelin, the same amount of LM, and 120 mg of lactose monohydrate. A #4 gelatin capsule filled with 100 mg of citric acid was put in the center of the enteric capsule. The enteric capsule was sealed with HPMC AS-H polymer dissolved in a methylene chloride methanol mixed solvent (1:1) and dried at room temperature. The *in vitro* dissolution of azetirelin from the enteric capsule was tested using JP XIII dissolution test apparatus No. 2 at $37 \pm 0.5^\circ\text{C}$ and a paddle speed of 100 rpm. The dissolution medium (500 ml) was pH 1.2 for the first 1 h, pH 6.5 for the next 1 h, and thereafter pH 7.2. Aliquots of sample were withdrawn periodically and analyzed as previously described.

Animal Experiments Intracolonic Administration to Rats: Rats were anesthetized by an intraperitoneal injection of sodium pentobarbital (32 mg/kg body weight). A small midline abdominal incision of about 1.5 cm was made, the colon was withdrawn, and a silicone tube (i.d. 3 mm, o.d. 5 mm, Natsume Seisakusyo Co., Japan) was then inserted into the proximal end of the colon. The colon was irrigated with 10 ml of saline and returned to the abdominal cavity. The drug solution, containing 0.5 mg/kg azetirelin and 2.5 mM LM, was loaded into a 1 ml syringe and administered into the proximal colon through the silicone tube. Gel formulations were similarly loaded into syringes and were extruded into the colon. At designated times after administration, blood samples were collected from the jugular vein and were analyzed by radioimmunoassay as previously described.⁴⁾ In some experiments, the colon was ligated with a silk suture 1.5 cm from the end of the inserted tube prior to drug administration. To investigate the spreading of the formulations in the colonic lumen, the drug solution and gel formulations were colored with trypan blue before administration. At a pre-determined time after administration, the colon was gently removed and excised to examine it visually.

Colonic Loop Experiment in Dogs: Two dogs were anesthetized by the intravenous administration of sodium pentobarbital (25 mg/kg) followed by subcutaneous maintenance injections every 2 h. A balloon sonde (Create Medic Co., Japan) was lubricated with vaseline and inserted 40 cm into the colon from the anus of the dogs. Air was then introduced to swell the balloon, thus forming a 15 cm long loop in the colon. Ten milliliters of the drug solution containing azetirelin (1 mg/kg) and 2.5 mM LM was introduced into the loop through the sonde. Periodically, 5 ml of blood was taken from the foreleg and analyzed as previously described.

Oral Administration of Enteric Capsules to Dogs: Enteric capsules of azetirelin with or without LM and CA were orally administered to 5 dogs which had fasted 24 h. Thirty milliliters of water was given after administration of the capsules. Blood samples were taken from the foreleg up to 8 h and assayed as previously described. The systemic availability of azetirelin ($F\%$) was calculated by comparing the AUC_{0-8h} with that after intravenous administration.

Pharmacokinetic and Statistical Analyses: The peak plas-

Fig. 1. Effect of LM on Azetirelin Absorption Following the Administration of Drug Solution into the Proximal Colon of Rats

●, unligated colon; ○, ligated colon; □, control (drug solution without LM was administered into the unligated colon). Results are expressed as the means $\pm$ S.E. of 4 to 5 animals. Inset: Pharmacokinetics of azetirelin following drug administration.

ma concentration (C_{max}) and the peak plasma concentration time (T_{max}) were obtained from the plasma concentration time curves from individual animals. The area under the plasma concentration-time curve (AUC) was calculated by the trapezoidal rule up to the last sampling point. The oral bioavailability (F) in dogs was calculated by comparing the AUC with that after intravenous administration. Statistical analyses were performed using Student's *t*-test.

RESULTS

Effect of Ligation on LM-Induced Colonic Absorption of Azetirelin Figure 1 shows mean plasma concentration time curves of azetirelin following administration of the drug solution with or without LM into the proximal colon of rats. A marked increase in plasma azetirelin level was observed when 2.5 mM LM was coadministered. The mean AUC of azetirelin with LM was 8.7 times greater than that of the control. Figure 1 also shows the effect of colonic ligation on LM-induced azetirelin absorption enhancement. Although initial absorption in the unligated colon was slightly faster than that in the ligated colon, similar plasma concentration time profiles were obtained in both the ligated and unligated colon.

The disposition of a colored drug solution in the colorectal lumen after administration into the proximal colon was visually determined. The drug solution stayed in the loop when the colon was ligated, whereas it spread through the large intestine and reached the end of the rectum within 30 min when the colon was not ligated. These results indicate that the effects of LM are hardly affected by the disposition or spreading of the drug solution in the colorectal lumen.

Effect of Release Rate on LM-Induced Colonic Absorption of Azetirelin Figure 2 shows the *in vitro* dissolution profiles of azetirelin and LM from the gel formulations. Although the release of LM from each gel was slightly faster than that of azetirelin, the dissolution profiles of these two components were almost the same. The dissolution rate of both azetirelin and LM decreased with an increased amount of polymer in the gels.

Figure 3 shows plasma concentration time profiles of aze-

Fig. 2. *In Vitro* Dissolution Profiles of Azetirelin (Open Symbol) and LM (Closed Symbol) from Gel Formulations Containing (A) 5% (w/v), (B) 7.5% (w/v) and (C) 10% (w/v) HPMC

Results are expressed as the means of 3 experiments.

Table 1. Pharmacokinetics Following Intracolonic Administration of Azetirelin Solution and Gel Formulations with LM in Rats

Parameter	Control ^{a)}	Solution	HPMC gel		
			5%	7.5%	10%
$T_{\max}$ (h)	—	0.3±0.1	0.5±0.2	0.3±0.1	0.3±0
$C_{\max}$ (ng/ml)	—	317.5±53.0	207.3±20.7	134.6±18.2	54.4±12.1
AUC_{0-4h} (ng·h/ml)	52.8±13.2	457.2±86.2	289.0±35.0	153.6±18.1	75.5±9.8
Ratio	1.0	8.7	5.5	2.9	1.4

Results are expressed as the means±S.E. of 4 to 5 animals. a) Drug solution without LM.

Fig. 3. Plasma Concentration Time Profiles of Azetirelin Following the Intracolonic Administration of Drug Solution and Gel Formulations with LM in Rats

●, drug solution with LM; ○, 5% gel; ▲, 7.5% gel; △, 10% gel; □, drug solution without LM. Results are expressed as the means±S.E. of 4 to 5 animals.

etirelin after the administration of gel formulations containing 2.5 mm LM. The enhanced absorption of azetirelin was apparent when compared to the control drug solution. However, the plasma azetirelin levels of these gels were lower than that of the drug solution with LM combination. Table 1 summarizes the pharmacokinetics of solution and gel formulations. It was found that both AUC and $C_{\max}$ decreased with an increased amount of polymer in the gels, and a delay in the *in vitro* release rate resulted in a reduced absorption of azetirelin. The results suggest that a quick release of the components is needed for the maximum promoting action of LM.

Effect of LM on Azetirelin Absorption in Colons of Dogs To examine the absorption promoting effect of LM in the dog colon, a colonic loop experiment utilizing a balloon sonde was carried out. Figure 4 shows the absorption profiles of azetirelin after administration of the drug solution with or

without 2.5 mm LM into the colonic loop of dogs. A rapid increase in plasma concentration was observed in each dog when LM was added. The AUC increased from 107.9 to 391.4 ng·h/ml in dog 1, and 192.7 to 349.6 ng·h/ml in dog 2. The results indicate that LM promotes absorption in the dog colon, as observed in rats.

***In Vivo* Performance of Azetirelin Enteric Capsules with LM and CA after Peroral Administration to Dogs**

An enteric capsule formulation of azetirelin containing LM was prepared. CA, a pH controlling agent, was also administered with the intention of suppressing bacterial degradation of azetirelin in the distal intestine.⁹⁾ The *in vitro* dissolution profile of azetirelin from the capsule is shown in Fig. 5. Dissolution did not occur at either the pH 1.2 or pH 6.8 medium up to 2 h, whereas a rapid release of azetirelin was demonstrated after changing the medium to a pH 7.2 buffer. The amount of azetirelin released within 30 min was more than 90% of the total amount, suggesting that the capsule released its contents quickly above pH 7.

Figure 6 shows the mean plasma concentration time profiles of azetirelin following oral dosing of the enteric capsules to dogs which had fasted for 24 h. A significant increase in plasma azetirelin level was observed compared with the control enteric capsules without LM and CA. Absorption lag-times of 1 to 5 h were noted in all 5 dogs. As shown in Table 2, bioavailability was improved from 14.9% to 46.1%. These results suggest that the enteric capsules, which dissolve at a relatively high pH level, containing LM and CA significantly improve the oral bioavailability of azetirelin.

DISCUSSION

The present study demonstrated that differences between the disposition of drug solution in ligated and unligated colon did not affect the LM-induced absorption profile of

Fig. 4. Effect of LM on the Absorption of Azetirelin Following the Administration of a Drug Solution into the Colonic Loop of Dogs
■, 2.5 mM LM; □, without LM.

Fig. 5. *In Vitro* Dissolution Profile of Azetirelin from the Enteric Capsule Determined by the JP XIII Paddle Method at a Paddle Rotation Speed of 100 rpm

Results are expressed as the means of 3 experiments.

Fig. 6. Mean Plasma Concentration Time Profile of Azetirelin Following Oral Administration of Enteric Capsules Containing LM and CA to Fasted Dogs

▲, with LM and CA; △, Control (without enhancers). Results are expressed as the means $\pm$ S.E. of 5 animals.

Table 2. Pharmacokinetics after Oral Administration of Enteric Capsules of Azetirelin with or without LM and CA in Dogs

Parameter	Control	with LM and CA
T_{max} (h)	4.6 ± 0.6	4.2 ± 0.3
C_{max} (ng/ml)	141.5 ± 40.4	$402.4 \pm 63.7^{**}$
AUC_{0-8h} (ng·h/ml)	524.3 ± 149.0	$1529.6 \pm 218.9^{**}$
F (%) ^{a)}	14.9	43.5

Results are expressed as the means $\pm$ S.E. of 5 animals. a) Bioavailability. **: $p < 0.01$, compared with the control.

azetirelin. This suggests that factors such as the disposition or distribution of the administered formulation in the colonic lumen have only minor effects on the absorption promoting action of LM. Diffusion of the drug solution in the intestinal lumen may increase the effective absorptive surface area. However, it may also cause dilution of the drug solution by secreted luminal fluid which reduces the effective concentration of both the adjuvant and drug, and thus decreases the effect of the enhancer. The results of this study suggest that these opposing factors counteracted each other and apparently had a negligible effect on the absorption promoting ability of LM.

Sutton *et al.* reported that the absorption enhancing effect of palmitoyl L-carnitine on the colonic absorption of cefoxitin, a poorly absorbed antibiotic, was more significant in the ligated colon than in the unligated colon.¹⁰⁾ They speculated that this is due to the spreading and subsequent dilution of the sample solution in the unligated colon. Their finding is not consistent with our results. One explanation may be the hyper-tonic solution used in their experiments, which might accelerate the flux of water from the intestinal wall to the lumen, thus diluting the components. In general, it is known that both movement and fluid secretion in the colon are less extensive than those in the small intestine and, therefore, less likely to produce significant dilution of a drug solution.¹¹⁾

Through the studies with gel formulations showing various dissolution profiles, a correlation was found between the *in vitro* dissolution rate of azetirelin and LM and the extent of absorption of azetirelin, *i.e.*, a delay in dissolution rate reduced the effects of LM. This result indicates that the localized concentration of azetirelin and LM at the absorption site is important for the promoting effect of LM. This suggested a strategy for designing a successful peroral formulation of azetirelin, a formulation which permits the concomitant and concentrated release of both the drug and LM in the lower intestine, an optimal site of promoting action by LM.

The absorption promoting effect of LM was also confirmed in dog colons by experiments utilizing the balloon sonde method. The *AUC* of azetirelin when coadministered with 2.5 mM LM to the colon improved by 1.8- to 3.6-fold in both of two dog. It is worth noting that the effect of LM found in the dog colon was less than that obtained in the colonic loop experiment in rats.⁵⁾ This suggests that differences in sensitivity to the promoting action of LM exist between the colonic membranes of rats and dogs. Further investigation will be required to obtain information on species dif-

ferences in the absorption promoting action of LM.

In this study, an enteric capsule which dissolves at a relatively high pH (above 7) was used as a prototype formulation to deliver azetirelin and LM to the colon. Smith *et al.* reported that the luminal pH of the dog small intestine is below 6.6 both in the upper and middle portions and rises to 7.5 in the ileum.¹²⁾ Therefore, the capsule would not disintegrate in the upper and middle small intestine, whereas it may dissolve and begin to release the components after arrival in the ileum. Consequently, the capsule releases its contents mainly in the lower intestine, which allows for a highly localized concentration of LM and azetirelin in the colon. Citric acid was also included in the capsule with the intention of improving the stability of azetirelin against luminal bacterial enzymes. A weak absorption promoting effect was also expected with this organic acid as demonstrated in our previous study.⁷⁾

Lag times observed in plasma concentration time profiles after the oral administration of the enteric capsules are mainly attributable to variations in the time it took for the capsule to reach the lower intestine after gastric emptying and small intestinal transit. However, a much shorter lag time of 1 h was observed in one dog, which suggests that disintegration of the enteric capsule occurred in the stomach. The gastric pH of dogs in a fasted state is known to vary from the acidic to neutral region.^{13,14)} Since the absorption promoting effect of LM was found to be more significant in the colon than in the small intestine,⁷⁾ this might influence the effect of LM observed in this study. The use of other colon specific delivery systems utilizing a pH-independent release mechanism¹⁵⁻¹⁷⁾ may produce a more effective and reproducible promoting action by LM. This possibility should be investigated in the future.

In conclusion, the effects of formulation factors such as dissolution and disposition on LM-induced azetirelin absorp-

tion profiles were investigated. It was demonstrated that the rapid release of azetirelin as well as LM is necessary to obtain the maximum efficacy of LM. Based on these results, an enteric capsule formulation of azetirelin using LM as an absorption enhancer was developed and its *in vivo* performance was evaluated in fasted dogs. It was shown that the formulation improves the oral bioavailability of azetirelin.

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