Metabolism of Azetirelin, a New Thyrotropin-releasing Hormone (TRH) Analogue, by Intestinal Microorganisms

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Purpose. We evaluated the effect of luminal bacterial metabolism on intestinal absorption of azetirelin in rats. *In vitro* characteristics of bacterial metabolism of azetirelin were also investigated with the goal of overcoming the low stability of the peptidic drug against luminal microorganisms.

Methods. Plasma azetirelin levels after oral administration to antibiotic-pretreated rats was examined. *In vitro* incubation experiments with bacterial suspensions were also performed to clarify the location of azetirelin breakdown activity as well as the effects of oxygen, pH, and various protease inhibitors on drug metabolism.

Results. Plasma azetirelin levels were sustained after oral administration to antibiotic-treated rats. Incubation with rat luminal contents demonstrated that azetirelin was metabolized by anaerobic bacteria, which are predominant in the distal intestine. Fecal suspensions from rats, dogs, and humans showed comparable metabolic activity. Azetirelin breakdown in the bacterial suspension was pH-dependent and was inhibited in the presence of bacitracin or puromycin.

Conclusions. Bacterial metabolism influences the degree of absorption of azetirelin in the distal intestine. Control of the luminal pH environment may be a practical method for improving the stability of azetirelin against intestinal microorganisms.

KEY WORDS: azetirelin; TRH analogue; intestinal microorganism; metabolism; intestinal absorption.

INTRODUCTION

Azetirelin, (Na-[((S))-4-oxo-2-azetidinyl)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). In a previous report, we demonstrated that the oral bioavailability of azetirelin was poor in rats (<2%), as is also the case of TRH (2). The main reason for this poor oral bioavailability of azetirelin is low intestinal permeability due to the high hydrophilicity of the drug. In addition to this factor, however, degradation of azetirelin was observed on incubation with a suspension of rat cecal contents. Moreover, the results of an *in vivo* excretion study with radiolabeled azetirelin demonstrated presystemic metabolism of the drug (2). These results suggest that bacterial metabolism in the gastrointestinal (GI) tract also influences the oral bioavailability of this peptidic drug.

It is known that various types of microorganisms are distributed throughout the GI tract, and that most are found in the distal intestine where they mediate metabolic reactions such as reduction and hydrolysis (3). A number of studies have investigated the metabolism of orally administered drugs by intestinal microorganisms, some in connection with the low oral bioavailability of the drugs (4,5). However, despite recent interest in the site-specific delivery of peptide drugs to the large intestine (6,7), little is known about the metabolism of orally administered peptide drugs by intestinal microorganisms (8).

The aim of the present study was to examine the effect of bacterial metabolism on the intestinal absorption of azetirelin, an analogue of TRH, following oral administration. *In vitro* incubation experiments were also performed to clarify in detail the characteristics of the metabolism of the drug by microorganisms. Finally, the effect of pH and the addition of protease inhibitors were evaluated in an effort to overcome the luminal bacterial metabolism of azetirelin.

MATERIALS AND METHODS

Materials

Azetirelin was synthesized in the Central Research Laboratories of Yamanouchi Pharmaceutical Co., Ltd., Bacitracin, puromycin, and aprotinin were purchased from Sigma Chemical Co., (St. Louis, MO, USA.). All other chemicals and solvents were of reagent grade and were used without further purification.

Animals

Male Wistar rats (Japan SLC, Inc., Hamamatsu, Japan) weighing 250–330 g, were used. All animal experiments complied with the "Principles of Laboratory Animal Care" (NIH publication #85-23, revised 1985).

Oral Administration to Antibiotic-Pretreated Rats

Rats were pretreated orally with a mixture of 100 mg neomycin sulfate, 50 mg tetracycline hydrochloride, and 50 mg bacitracin sulfate twice daily for 2 days (9). Food was withdrawn during the experimental period but free access to water was given. After pre-treatment, an azetirelin solution was administered orally at a dose of 10 mg/kg (n = 4). Dosing volume was 4 ml/kg. At designated time intervals after administration, the rats were lightly anesthetized with ether and blood was collected from the jugular vein. The blood samples were centrifuged for 20 min at 3000 rpm to obtain plasma (0.25 ml). After extraction with methanol, the plasma concentration of azetirelin was determined by radioimmunoassay as previously described (2). Peak plasma concentration (Cmax) and the time to peak plasma concentration (Tmax) were obtained from the plasma concentration-time curves. Area under the plasma concentration-time curve (AUC) was calculated by the trapezoidal rule

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up to 8 h. Mean residence time (MRT) and mean absorption time (MAT) were calculated by means of moment analysis. Bioavailability (F) was calculated as the ratio of the AUC after oral administration to that after intravenous administration.

Preparation of Bacterial Suspension

Bacterial suspension was prepared as described by Kiriyama et al. (10). Briefly, fresh cecal contents from non-fasted rats were suspended in twice their volume of bicarbonate buffer ($NaHCO_3$, 9.240 g; $Na_2HPO_4 \cdot 12H_2O$, 7.125 g; NaCl, 0.470 g; KCl, 0.450 g; $CaCl_2 \cdot 2H_2O$, 0.073 g; $MgCl_2 \cdot 6H_2O$, 0.087 g/L) whose pH was adjusted to 7.0 by bubbling with CO_2 gas prior to use. The suspension was then filtered through four layers of gauze. Supernatant and the bacterial pellet were obtained by centrifuging the suspension at 3,000 rpm for 5 min. The resultant pellet was washed twice and resuspended in the same volume of bicarbonate buffer solution. In some experiments, the pellet suspension was bubbled with oxygen for 15 min to achieve aerobic conditions. To investigate the effect of pH on metabolic activity, the buffer solution was adjusted to adequate pH by the addition of phosphoric acid or sodium hydroxide before preparation of the pellet suspension. Suspensions of luminal contents from other segments of the rat GI tract were prepared as described above. Fecal suspensions from rats, dogs, and humans were also prepared by the same procedure.

Preparation of Gastrointestinal Tissue Homogenates

Portion of the rat GI tract was rinsed with phosphate buffered saline (PBS) and collected. A 33% homogenate was prepared by homogenizing the whole tissue in ice-cold PBS using a Polytron homogenizer (Kinematica GmbH). The homogenate was centrifuged at 5000 rpm for 10 min, and the resultant supernatant was stored at −80°C until use.

In Vitro Stability Experiments

Stability studies were conducted by incubating 900 μl of the bacterial suspension with 100 μl of azetirelin solution in sealed test tubes under a CO_2 atmosphere at 37°C. The final concentration of the drug in the incubation mixture was 0.1 mM. At predetermined times, 50 μl samples were withdrawn and 150 μl of acetonitrile was added to terminate the metabolic reaction. Fifty microliters of 30 μg/ml L-phenylephrine hydrochloride solution, the internal standard, was then added. After centrifugation, 10 μl of the supernatant was analyzed by HPLC as previously described (2). In some experiments, the suspension was pre-incubated with various protease inhibitors at 37°C for 15 min before the incubation studies. Stability studies in tissue homogenates were conducted by the same procedure except that incubation was carried out under aerobic conditions.

Statistical Significance

Results were expressed as the mean ± S.E. of at least 3 experiments. Statistical analyses were performed using Student's t-test.

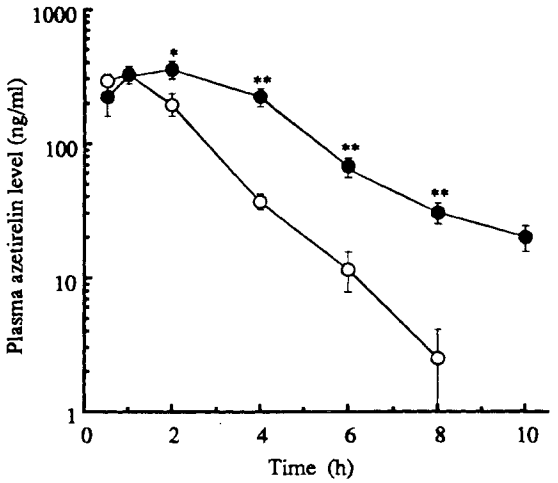

Fig. 1. Mean plasma levels of azetirelin following oral administration to antibiotic-pretreated rats (●) or control rats (○) at a dose of 10 mg/kg. Each point represents the mean ± S.E. of 4 or 5 animals. Significantly different from the control: *$p < 0.05$, **$p < 0.01$.

RESULTS

Effect of Antibiotic Treatment on Oral Absorption of Azetirelin

Feces of rats were collected after 2 days of treatment with antibiotics to examine azetirelin metabolic activity. The degradation rate constant of azetirelin in the fecal suspension of normal rats was 10.2×10^{-3} min^{-1}. In contrast, that in the fecal suspension of antibiotic-treated rats was negligible. These results indicated that metabolic activity of intestinal microorganisms was almost completely suppressed by antibiotic pretreatment. Fig. 1 shows mean plasma concentration-time curves of azetirelin following single oral administration to rats at a dose of 10 mg/kg. The duration of plasma azetirelin levels in antibiotic-treated rats was longer than that in the normal rats, and a significantly higher plasma concentration was maintained over 10 h after administration. Table I summarizes pharmacokinetic parameters. Significant increases in Tmax, MRT and MAT were observed after antibiotic treatment. Cmax was not changed, whereas F in the antibiotic-treated rats was almost double that of the control. These results suggest that the intestinal absorption of azetirelin is influenced by suppression of bacterial metabolism in the GI tract.

Table I. Pharmacokinetic Parameters of Azetirelin Following Oral Administration to Antibiotic-Treated and Control Rats

Parameter		Control	Antibiotic-treated
Cmax	(ng/ml)	349.8 ± 27.3	376.4 ± 51.8
Tmax	(h)	1.0 ± 0.3	2.7 ± 0.5*
AUC_{0-8h}	(ng·h/ml)	792.0 ± 72.7	1542.5 ± 197.2*
F	(%)	1.6	3.1
MRT	(h)	1.71 ± 0.09	2.81 ± 0.10***
MAT	(h)	1.29	2.44

Note: Each value represents the mean ± S.E. of 4–5 animals. * $p < 0.05$, *** $p < 0.001$ compared with the control.

Table II. Rate Constants of Azetirelin Degradation in Luminal Contents and Tissue Homogenates of the Gastrointestinal Tract of Rats

Segment	Rate constant ($\times 10^{-3}$ min^{-1})	
	Luminal content	Tissue
Stomach	0.03 ± 0.02	0.11 ± 0.06
Proximal small intestine	<0.01	0.21 ± 0.15
Distal small intestine	2.01 ± 0.28	0.13 ± 0.07
Cecum	10.51 ± 0.48	—
Colon	9.77 ± 0.39	<0.01

Note: Each value represents the mean ± S.E. of 3 experiments.

Stability of Azetirelin in Bacterial Suspension

Table II shows the rate constants of azetirelin degradation in luminal contents and tissue homogenates of the rat GI tract. Azetirelin appears to be stable in tissue homogenates prepared from any region of the GI tract. In contrast, the drug was metabolized in the luminal contents of the cecum and colon. Slight metabolic activity was also found in contents of the distal small intestine, but no appreciable activity was observed in the stomach or proximal small intestine.

Fig. 2 shows the effect of various conditions on the degradation rate constants of azetirelin in rat cecal contents. To investigate the location of the azetirelin breakdown activity, a suspension of cecal contents was centrifuged to obtain a supernatant and a bacterial cell pellet. As shown in Fig. 2, degradation of azetirelin occurred only in the resuspended pellet, and no metabolic activity was found in the cell-free supernatant. Also shown is the effect of aerobic conditions on the degradation rate constant of azetirelin. After bubbling oxygen through the bacterial suspension for 15 min, a significant decrease in the degradation rate constant was observed, indicating that the metabolic activity of cecal contents was oxygen-sensitive.

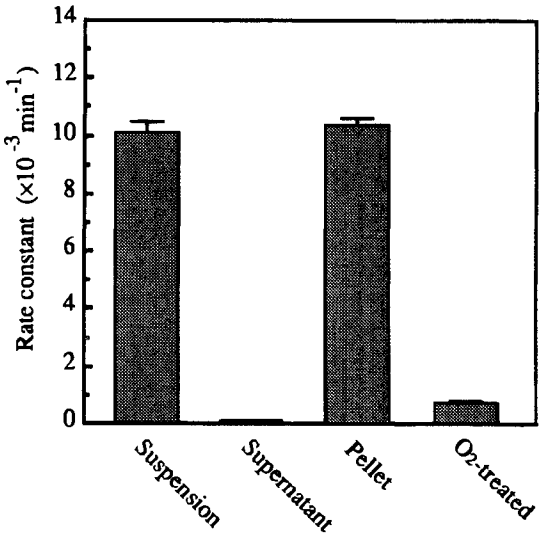

Fig. 2. Effect of various conditions on rate constants for the degradation of azetirelin in rat cecal contents. The suspensions were prepared by suspending cecal contents in two volumes of bicarbonate buffer solution. Supernatant and pellet were obtained by centrifuging the suspension. Oxygen treatment was carried out as described in the text. Results are expressed as the mean ± S.E. of 3 experiments.

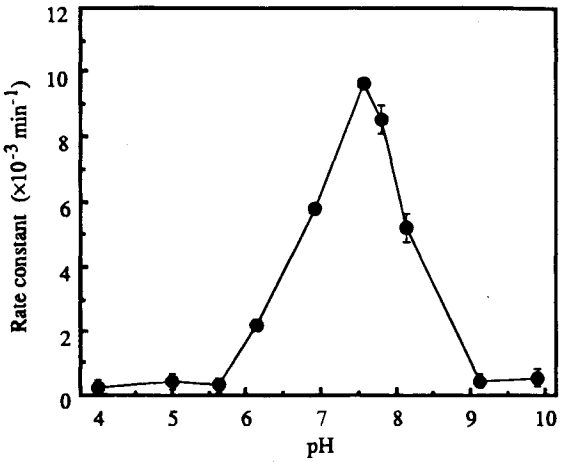

Fig. 3. Effect of pH on the degradation rate constant of azetirelin in a rat cecal suspension. Each point represents the mean ± S.E. of 3 experiments.

Species differences in the metabolic activity of a fecal suspension was also investigated. The degradation rate constants of azetirelin in a 33% fecal suspension of rats, dogs, and humans were 10.2×10^{-3}, 8.2×10^{-3}, and 9.7×10^{-3} min^{-1}, respectively. These results indicate that feces of the three species have comparable metabolic activity against azetirelin, although those of rats and humans were slightly greater than that of dogs.

Effect of pH and Protease Inhibitors on Bacterial Metabolism of Azetirelin

The bacterial pellet obtained by centrifugation was resuspended in a pH-adjusted buffer solution and used for the incubation experiments. Fig. 3 shows the effect of pH on the degradation of azetirelin in bacterial suspension. The metabolic activity of the suspension was pH-dependent, with the first-order rate constant reaching a maximum value at pH 7.4 of 9.6×10^{-3} min^{-1}. No degradation occurred below pH 5.5 or above pH 9.

Table III shows the effect of various protease inhibitors on the half lives of metabolism of azetirelin in bacterial suspension. The half lives were calculated from the first order rate constants. Degradation of azetirelin was not affected in the

Table III. Effects of Various Protease Inhibitors on the Degradation of Azetirelin in Rat Cecal Contents

Inhibitor	Concentration	Half life[a] (min)	Ratio
Control	—	78.7 ± 1.0	1.00
Na-GC	5 mM	86.9 ± 6.6	1.10
EDTA	5 mM	79.6 ± 8.3	1.01
Aprotinin	0.1%	78.2 ± 3.6	0.99
Bacitracin	1 mM	100.6 ± 5.6*	1.28
	2 mM	129.9 ± 3.0***	1.65
	5 mM	172.3 ± 5.5***	2.19
Puromycin	5 mM	107.5 ± 1.0***	1.37

* $p < 0.05$, *** $p < 0.001$ compared with the control.
[a] Each value represents the mean ± S.E. of 3 experiments.

presence of sodium glycocholate, EDTA, or aprotinin, whereas the addition of bacitracin and puromycin significantly inhibited the breakdown of the drug in the cecal contents. The inhibitory effect of bacitracin was more potent than that of puromycin and was concentration-dependent.

DISCUSSION

In this study, we examined the effect of antibiotic treatment on plasma azetirelin levels after oral administration to rats. Although the oral bioavailability of azetirelin in antibiotic-treated rats was not remarkably improved, the duration of plasma azetirelin level was longer than that in normal rats. In pilot studies, we confirmed that plasma concentration following intravenous administration was not influenced by antibiotic treatment. These findings suggest that the increase in duration of plasma azetirelin levels by antibiotic treatment may be attributable to changes in presystemic absorption from the GI tract. A possible explanation is that the suppression of luminal bacterial metabolism resulted in the continuous absorption of azetirelin during transit throughout the whole intestine. Together with the results of *in vitro* stability studies in luminal contents, this finding suggests that bacterial metabolism should be closely considered when absorption of azetirelin is targeted at the distal intestinal region.

We previously demontrated that the low oral bioavailability of azetirelin is mainly due to poor intestinal permeability resulting from its high hydrophilicity (2). One practical formulation approach to improving the oral bioavailability of azetirelin is through the use of absorption promoters. However, as many absorption promoters act more intensively in the large rather than in the small intestine (11), the distal intestine may become an important site for absorption of the drug. Consequently, a peroral formulation of azetirelin should both enhance intestinal permeability and suppress bacterial breakdown.

Results of *in vitro* incubation studies with rat luminal contents showed that azetirelin is metabolized by an oxygen-sensitive bacteria localized mainly in the large intestine. This bacterial strain seems to commonly inhabit the gut of laboratory animals and humans, since similar metabolic activity was noted in fresh feces of rats, dogs and humans. Similarities in the composition of the intestinal flora of common laboratory animals and humans have also been demonstrated by Drasar (12). It is known that luminal contents in the lower gut contain about 10^{11} microorganisms per gram, most of which are obligatory anaerobic (3). Macfarlane et al. reported that some of the predominant species such as Bacteroides and Streptococcus could potentially play a major role in proteolysis in the human large intestine (13). It is possible that one of these proteolytic bacteria is involved in the metabolism of azetirelin.

In order to ascertain the contribution of microorganisms to the luminal metabolism of azetirelin, the location of the azetirelin breakdown activity was investigated after separating a cecal suspension by centrifugation. The lack of metabolic activity in the supernatant fluid indicated that azetirelin was metabolized by neither extracellular enzymes of bacteria nor by luminal pancreatic proteases. This result partly agreed with our previous finding that azetirelin was stable in luminal fluid collected from the small intestine in rats (2). We have not succeeded in identifying the metabolites of azetirelin or the types of bacterial enzymes involved in this metabolism; further investigation is required to obtain this information.

Among the protease inhibitors used in the inhibitory experiments, bacitracin and puromycin were found to be effective in reducing azetirelin degradation in a bacterial suspension. The inhibitory effect of bacitracin was more potent. This cyclic polypeptide antibiotic is known to inhibit the post-proline cleaving enzyme (14), the main azetirelin metabolizing enzyme, as well as the enzyme which degrades TRH in the body. We therefore suggest that this peptidase may play a part in the metabolic pathway of azetirelin by luminal microorganisms. This possibility should be further examined.

A second means of suppressing the bacterial metabolism of azetirelin was through control of the luminal pH environment to mild acidic conditions. Metabolic activity of the bacterial suspension showed a pH-dependent profile, with no breakdown noted below pH 5.5. In pilot studies, azetirelin breakdown in bacterial suspension was almost completely suppressed in weakly acidic conditions prepared by the addition of citric acid. It remains to be determined whether the induction of a transient and mild change in pH of the distal intestine would have any adverse effect on microflora composition or human health. However, it is known that intestinal bacteria transiently alter luminal pH through the production of short-chain fatty acids by fermentation of carbohydrates. Thus, the use of organic acids, commonly used as pharmaceutical additives, as pH-controlling agents may represent one practical approach to overcoming the luminal bacterial metabolism of azetirelin.

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