Effect of N-(3-Aminopropionyl)-L-Histidinato Zinc (Z-103) on Healing and Hydrocortisone-Induced Relapse of Acetic Acid Ulcers in Rats with Limited Food-Intake-Time

Mikio ITO, Takao TANAKA and Yoshio SUZUKI

Department of Pharmacology, Faculty of Pharmacy, Meijo University, Nagoya 468, Japan

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Abstract—In the healing test of acetic acid ulcers in rats with limited food-intake-time, Z-103 given, p.o., at doses of 3 and 10 mg/kg, twice a day, for 14 consecutive days from the day after acetic acid injection not only reduced the size and depth of the ulcers, but also promoted the regeneration of the defective mucosa. In the hydrocortisone-induced relapse test of acetic acid ulcers in rats with limited food-intake-time, Z-103 given, p.o., twice a day, at doses of 3 and 10 mg/kg for 20 consecutive days from the 40th day after the acid injection strongly prevented the exfoliation of the regenerated mucosa. Cimetidine (100 mg/kg × 2/day, p.o.), like Z-103, showed a marked relapse-preventive action in addition to the healing-promoting action. However, it was more effective on the healing. Gefarnate (300 mg/kg × 2/day, p.o.) markedly reduced the size and depth of the ulcers and strongly prevented the steroid-induced relapse, but showed no apparent effect on the regeneration of the defective mucosa. These results suggest that Z-103 may be a new therapeutic agent sharing both healing-promoting and relapse-preventive actions on gastric ulcers.

N-(3-Aminopropionyl)-L-histidinato zinc (Z-103) is chemically a chelate compound that consists of L-carnosine and zinc (Fig. 1). It is well-known that L-carnosine containing two amino acids, L-histidine and β-alanine, in its structure exists in mammalian skeletal muscles and possesses wound healing-promoting and immunoregulator actions. In addition to the above pharmacological actions, this compound has been reported to be markedly effective against chronic gastric ulcers such as clamping-cortisone ulcers and acetic acid ulcers in rats (1). On the other hand, zinc deficiency in humans causes epithelial cell abnormalities, impaired sexual development and a variety of reduced psychologic functions, while the deficient state can be improved by supplemental zinc therapy (2, 3). Since ancient times, zinc oxide ointment has been widely used for incisional wound healing. In regards to the anti-ulcer effect of zinc, zinc sulfate has been reported to protect against various kinds of acute gastric lesions in rats (4–7). From the above findings, Z-103 is expected to share the pharmacological actions of L-carnosine and zinc. It has been already reported that Z-103 has potent protective actions against several acute experimental gastric and duodenal lesions in rats (8). However, it is still unclear whether or not this compound has beneficial effects on chronic peptic ulcers.
Therefore, the aim of the present study was to clarify the effects of Z-103 on healing and hydrocortisone-induced relapse of acetic acid ulcers in rats with limited food-intake-time (9, 10).

Materials and Methods

Animals: Male Sprague-Dawley strain SPF rats (Shizuoka Laboratory Animal Center, Shizuoka), weighing approx. 180 g, were used in the experiment. These animals were housed in an air-conditioned room at 23±1 °C and allowed free access to commercial food pellets only between 10:00-11:00 a.m. and 6:00-7:00 p.m., every day from 3 days prior to ulcer induction. However, tap water was always supplied ad libitum.

Drugs: Drugs used were Z-103 (Zeria Pharmaceutical Co., Ltd., Tokyo), ZnCl₂ and L-carnosine (Wako Pure Chemical Industries, Ltd., Osaka), gefarnate (Gefanyl, Sumitomo Pharmaceutical Co., Ltd., Osaka) and cimetidine (Sigma Chemical Co., St. Louis, MO). Z-103, ZnCl₂, L-carnosine and gefarnate were suspended in 0.5% methylcellulose, while cimetidine was suspended in 0.5% gum arabic.

Induction of acetic acid ulcers in rats with limited food-intake-time (9) and evaluation of healing-promoting action of test drugs: Gastric ulcers were induced in rats by the injection of 20% acetic acid in a volume of 0.05 ml into the serosal layer in the glandular part of the stomach in accordance with the method described by Takagi et al. (11). Test drugs were given orally, twice a day (Z-103, ZnCl₂, L-carnosine and gefarnate, 9:30 a.m. and 5:30 p.m.; cimetidine, 11:30 a.m. and 7:30 p.m.) in a volume of 0.5 ml per 100 g of body weight for 14 consecutive days from day (the 1st day) after the acid injection. Control animals were given only the vehicle instead of test drugs. On the 15th day, the animals were sacrificed by rapid decapitation. The stomachs were removed, filled with 5 ml of 10% formalin and allowed to stand for 5 min. Then, the stomachs were cut open along the greater curvature. The longitudinal and abscissal lengths of the upper-opened part of the ulcer were measured with a micrometer which was set on a stereoscopic microscope, and the product of both lengths (mm²) was expressed in terms of the ulcer index. After measuring the ulcer size, the stomach tissue was again immersed in 10% formalin for 24 hr. The formalin-fixed tissue was then cut so that a little of the normal tissue surrounding the ulcer remained. Thereafter, the central part of ulcer was cut vertically against the serosa along the long diameter. These tissues, cut in half, were embedded in paraffin and cut into 2–3 μm thick sections. The sections were stained with hematoxylin and eosin (HE). Histological measurements were performed under light micrography of HE-stained preparations as shown in Fig. 2a. The following ulcerated regions were as follows: A: Defect of mucosa, B: Extent of ruptured muscularis mucosa, C: Height of marginal mucosa, BT: Thickness of the ulcer base, DA: Defective area in the ulcerated region, R₁ + R₂: Area of regenerated mucosa. On the basis of the above measured values, the following indices were calculated: the index for the decrease in exposed ulcer floor=$\frac{B-A}{B} \times 100$ and the index for mucosal regeneration=$\frac{R₁ + R₂}{C \times B} \times 100$. Drug evaluation was by comparing the ulcer index, defective area in the ulcerated region, the thickness of the ulcer base, the index for the decrease in the exposed ulcer floor and the index for the mucosal regeneration of each test drug with those of the respective control.

Induction of hydrocortisone-induced relapse of acetic acid ulcers in rats with limited food-intake-time (10) and evaluation of the preventive action of test drugs: Acetic acid ulcers were induced in rats as described above. The relapsed ulcers were induced in these animals by daily i.m. injection of 20 mg/kg of hydrocortisone acetate from the 40th to the 59th day after the acid injection. Each test drug was given orally, twice a day, as the ulcer-healing described above was done, from the 40th to the 59th days. Control animals were given only the vehicle. On the 60th day, the animals were sacrificed, and the preventive effects of test drugs on the relapsed ulcers were evaluated by histological measurements as shown in Fig. 2b. The ulcer index, the defective area in the ulcerated region and the thickness of the ulcer base were measured for evaluating the effects of test drugs, as the
ulcer healing was examined. In addition, the following indices were calculated for the evaluation: the index for exposure of ulcer floor = \( \frac{A}{B} \times 100 \) and the index for regenerated mucosa = \( \frac{R_1 + R_2}{B \times C} \times 100 \). The latter index was calculated by the same formula as the index for mucosal regeneration. Because in the case of the relapsed ulcer, the ulcer floor that had been completely covered with regenerated mucosa is once again exposed due to the loss of the regenerated mucosa, we called this the index for regenerated mucosa.

**Statistical analysis:** Results obtained were expressed as the mean±S.E. The data were analyzed by one-way analysis of ANOVA or Kruskal-Wallis. Student’s t-test or a two-tailed Mann-Whitney U-test was then used to determine the difference between groups.

**Results**

1. Ulcer healing

   **Effects of Z-103, gefarnate and cimetidine:** Z-103 at doses of 3 and 10 mg/kg×2/day, p.o., decreased the ulcer index by 54% and 65%, respectively, although it was ineffective at a dose of 1 mg/kg×2/day, p.o. (Fig. 3A). Cimetidine at a dose of 100 mg/kg×2/day, p.o., and gefarnate at 300 mg/kg×2/day, p.o., also decreased the index by 61% and 57%, respectively.

   Z-103 at doses of 3 and 10 mg/kg×2/day, p.o., decreased the defective area of the ulcerated region by 58% and 64%, respectively (Fig. 3B). Cimetidine (100 mg/kg×2/day, p.o.) and gefarnate (300 mg/kg×2/day, p.o.) also decreased the defective area by 70% and 67%, respectively.

   The index for the decrease in the exposed ulcer base is designated as 100, when the ulcer floor is completely covered with the regenerated mucosa. Only Z-103 at a dose of 10 mg/kg×2/day, p.o., and cimetidine (100 mg/kg×2/day, p.o.) were significantly effective in increasing the index, showing 57% and 72% increases, respectively (Fig. 3C). However, Z-103 at the doses of 1 and 3 mg/kg×2/day, p.o., and gefarnate (100 mg/kg×2/day, p.o.) showed no apparent effects on this index.

   The index for the mucosal regeneration is designated as 100 when the ulcer floor is completely covered with the regenerated mucosa.
mucosa equal to the marginal mucosa of the ulcer in height. Z-103 at the doses of 3 and 10 mg/kg x 2/day, p.o., increased the index for the mucosal regeneration by 40% and 60%, respectively (Fig. 3D). Cimetidine (100 mg/kg x 2/day, p.o.) produced a 90% increase in the index. However, no significant difference was seen between Z-103 at both doses and cimetidine. In contrast to both drugs, gefarnate (300 mg/kg x 2/day, p.o.) showed no significant effects on this index.

The thickness of the ulcer base, unlike other parameters, was not affected by any test drugs (data not shown).

**Effects of ZnCl₂ and L-carnosine:** When ZnCl₂ and L-carnosine were given p.o., twice a day, at 4.7 and 7.7 mg/kg, respectively, doses which are contained in 10 mg of Z-103, both compounds neither alone nor in combination were effective in promoting the ulcer healing (Fig. 4A, B, C and D). In addition, both compounds alone were ineffective even at a ten-fold dose.

2. **Ulcer relapse**

On the 40th (Fig. 5a) and 60th days (Fig. 5b) after acetic acid injection, the ulcer base in hydrocortisone-untreated rats was completely covered with regenerated mucosa. However, on the 60th day, in the steroid-treated rats, the ulcer base was exposed due to the loss of regenerated mucosa (Fig. 5c). Thus, the apparently relapsed ulcers were caused by 20 days treatment with hydrocortisone (20 mg/kg/day, i.m.).
Effects of Z-103, gefarnate and cimetidine: Z-103 at doses of 3 and 10 mg/kg x 2/day, p.o., decreased the ulcer index by 64% and 60%, respectively (Fig. 6A). Cimetidine (100 mg/kg x 2/day, p.o.) and gefarnate (300 mg/kg x 2/day, p.o.) also induced an approx. 60% decrease in the ulcer index.

Z-103 at 3 and 10 mg/kg x 2/day, p.o., and gefarnate (300 mg/kg x 2/day, p.o.) decreased the defective area in the ulcerated region by 62%, 54% and 61%, respectively (Fig. 6B). Cimetidine (100 mg/kg x 2/day, p.o.) decreased the defective area by 43% only.

The index for the exposure of the ulcer was decreased approx. 55% by Z-103 at doses of 3 and 10 mg/kg x 2/day p.o., and gefarnate (300 mg/kg x 2/day, p.o.) (Fig. 6C). However, the decrease in the index by cimetidine (100 mg/kg x 2/day, p.o.) was only 36%.

Z-103 at 3 and 10 mg/kg x 2/day, p.o., increased the index for the regenerated mucosa by 124% and 119%, respectively (Fig. 6D). Cimetidine (100 mg/kg x 2/day, p.o.) also increased the index by 92%. The increasing action of gefarnate (300 mg/kg x 2/day, p.o.) was 157%, being more potent than that of cimetidine (100 mg/kg x 2/day, p.o.). However, there was no significant difference between Z-103 at both doses and gefarnate.

The thickness of the ulcer base was little affected by any test drugs (data not shown).
Discussion

Acetic acid ulcers in rats, which closely resemble chronic gastric ulcers in humans (11), have been widely used to evaluate the effects of anti-ulcer agents on ulcer healing. However, histamine H₂-receptor blocking agents (i.e., cimetidine), which markedly promote the healing of human peptic ulcers, are not so effective against ulcers in experimental animal models, compared to human peptic ulcers (12, 13). Our previous studies indicated that the healing of acetic acid ulcers in rats was markedly delayed by the food-intake in a fixed period twice a day (9:30–10:30 a.m. and 6:00–7:00 p.m.) (9) and cimetidine markedly promoted the healing of acetic acid ulcers in rats with limited food-intake-time (14). Therefore, in the first experiment, we examined the healing-promoting effect of Z-103 in comparison with cimetidine and gefarnate by using a model of acetic acid ulcers in rats with limited food-intake-time. It has been considered that the preventive action of Z-103 on acute gastric lesions in rats is mainly due to its local action, because the compound possesses the ability to adhere to the gastric mucosa, especially to the ulcerated part. The gastric content may disturb the local action of this agent on the gastric mucosa. For this reason, Z-103 was given twice a day 30 min prior to the beginning of food-intake in the morning and evening. For a comparison, gefarnate was also given prior to food-intake. However, only cimetidine was given after food-intake because of its strong anti-secretory action. In this experiment, cimetidine and gefarnate were given p.o. twice a day at a dose of 100 and 300 mg/kg, respectively, which had been proven to accelerate the healing of acetic acid ulcers in rats (14, 15).

Of the indices and the values measured in the ulcerated region, the ulcer index indicates the size of the ulcers, while the defective area of the ulcerated region is expressed as the defective area of vertical sections of the ulcers, indicating the depth of the ulcers. The thickness of the ulcer base denotes the degree of

Fig. 5. Micrographs of ulcer bases from hydrocortisone-untreated rats on 40th (a) and 60th days (b), and from rats on the 60th day treated with the steroid (20 mg/kg/day, i.m.) for the 40th to the 59th day after acetic acid injection (c).
granulation proliferation beneath the ulcer floor. Moreover, the index for the decrease in the exposed ulcer floor and the index for the mucosal regeneration represent the degree of the regeneration of the defective mucosa. In this experiment, Z-103 (10 mg/kg × 2/day, p.o.), like cimetidine (100 mg/kg × 2/day, p.o.), not only diminished the size and depth of the ulcers, but also remarkably promoted the regeneration of the defective mucosa. On the other hand, gefarnate (300 mg/kg × 2/day, p.o.) showed no apparent effect on the regeneration of the defective mucosa, although it was as effective as Z-103 and cimetidine in decreasing the size and depth of ulcers. The thickness of the ulcer base was little affected by any test drug.

In the second experiment, we examined the preventive effects of Z-103, cimetidine and gefarnate on hydrocortisone-induced relapse of acetic acid ulcers in rats with limited food-intake-time. Human chronic peptic ulcers are characterized by recurrence or relapse. However, there have been very few reports concerning experimental animal models for evaluating the preventive effects of anti-ulcer agents on the recurrence or relapse of peptic ulcers. Although it has been demonstrated that relapsed ulcers can be produced after healing of acetic acid ulcers in rats (16), the time when the relapse is induced and the severity of the relapsed ulcers are variable from rat to rat. Therefore, in the present study, in order to induce definitely relapsed ulcers as
soon as possible after healing, we gave hydrocortisone (20 mg/kg) i.m. daily to the rats under a condition of limited food-intake time for 20 days from the 40th day after the acid injection. Our previous study indicated that the steroid-induced relapse of acetic acid ulcers in rats was remarkably increased by limiting the food-intake-time (12). In the present experiment, all rats treated with the steroid had an apparent relapse of ulcers on the 60th day, although none of the steroid-untreated animals had a relapse. Z-103 (3 and 10 mg/kg×2/day, p.o.) showed a marked preventive effect on the relapse of the ulcers, being as effective as gefarnate (300 mg/kg×2/day, p.o.). Both drugs were slightly more potent in protecting the regenerated mucosa than cimetidine.

It has been reported with respect to the anti-ulcer action of L-carnosine or zinc alone that L-carnosine is effective at over 200 mg/kg/day, s.c., on clamping-cortisone ulcers and acetic acid ulcers in rats (1), while zinc sulfate protects against ethanol-induced gastric necrosis at 20 mg/kg, p.o., expressed as zinc (4). In the present experiment, L-carnosine at 7.7 mg/kg×2/day, p.o., and ZnCl₂ at 4.7 mg/kg (2.3 mg/kg as Zn)×2/day, p.o., which is contained in 10 mg of Z-103, either alone or in combination was ineffective in promoting the healing of acetic acid ulcers. In addition, even at 10 times higher doses, both compounds alone were ineffective on the ulcer healing. This result suggests that the anti-ulcer effect of Z-103 may be due to the action of a chelate compound that consists of L-carnosine and zinc rather than the result of synergism of each action of L-carnosine and zinc.

The mechanisms of the anti-ulcer actions of Z-103 have not been well defined. It has been reported that Z-103 strongly prevents ethanol-induced gastric damages and deep necrosis in vivo (17, 18) and ethanol-induced damages of surface epithelial cells isolated from rat stomachs in vitro (18) without affecting mucosal prostaglandin (PG) E₂ level. This result suggests that Z-103 has a cytoprotective action without the mediation of endogenous PGs. Furthermore, Z-103 has been shown to prevent compound 48/80-induced gastric lesions in rats in vivo and to inhibit compound 48/80-induced release of histamine from isolated rat mast cells in vitro (19). Therefore, in the present study, the preventive effect of Z-103 on the steroid-induced relapse of acetic acid ulcers may be partly due to the PG-independent cytoprotective and membrane-stabilizing actions of this agent.

More recently, we found in the healing of acetic acid ulcers in rats that the serum gastrin level in the food-intake-time limited group was significantly lower than that in the food-intake-time non-limited group (M. Ito et al., unpublished data). Gastrin has been reported to exert a trophic action (i.e., it stimulates the proliferation of gastric mucous cells) in addition to the stimulating action on gastric acid secretion (20). Therefore, we are now investigating the effect of Z-103 on serum gastrin level as one of mechanism of the ulcer-healing promoting action of this agent.

References
8 Ishikawa, M., Seiki, M., Ueki, S., Tanaka, Y., Soeda, M., Hori, Y., Tagashira, E. and Okabe, S.: Effect of a new compound, zinc L-carnosine (Z-


