Comparison of the Antidiarrheal Effects of Zaldaride Maleate and Its Optical Isomers in Rats

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Zaldaride maleate (ZAL), a calmodulin inhibitor, that ameliorates secretory diarrhea in rodents, has a racemic structure. In this study, we compared the antidiarrheal and antisecretory effects of ZAL and its optical isomers, \( R(\pm) \)-isomer and \( S(\pm) \)-isomer, in rats. In Ussing chamber experiments, the inhibitory activity of ZAL on acetylcholine-induced ion transport in the rat colonic mucosa was equipotent for both optical isomers, with \( IC_{50} \) values of approximately 3–4 \( \mu \)mol/L. In castor-oil-induced diarrhea, ZAL and its \( S(\pm) \)-isomer inhibited the incidence of diarrhea, whereas the \( R(\pm) \)-isomer had no effect. In 16,16-dimethyl prostaglandin \( E_2 \)-induced diarrhea, ZAL, the \( S(\pm) \)-isomer and the \( R(\pm) \)-isomer significantly ameliorated diarrhea at doses of 30, 10 and 30 mg/kg (p.o.), respectively; the \( ED_{50} \) values were 25, 10 and above 30 mg/kg (p.o.), respectively. The pharmacokinetic parameters after administration of 30 mg/kg (p.o.) of each compound were as follows: ZAL (\( C_{max} \): 378 ng/ml, \( AUC_{0-\infty} \): 1650 ng·h/ml); \( S(\pm) \)-isomer (\( C_{max} \): 565 ng/ml, \( AUC_{0-\infty} \): 2230 ng·h/ml) and \( R(\pm) \)-isomer (\( C_{max} \): 271 ng/ml, \( AUC_{0-\infty} \): 613 ng·h/ml) (mean, N=4). In conclusion, despite the fact that the antisecretory actions of ZAL and its optical isomers are the same, the antidiarrheal actions of ZAL and its \( S(\pm) \)-isomer are more potent than that of the \( R(\pm) \)-isomer. The antidiarrheal effects of ZAL and its optical isomers may be related to plasma levels.

Key words zaldaride maleate; diarrhea; castor oil; prostaglandin \( E_2 \); calmodulin; optical isomer

Zaldaride maleate (ZAL), 1,3-dihydro-1-[1-[4-methyl-4H,6H-pyrrrol][1,2-\( \alpha \)][4,1-benz-oxazepine-4-yl]methyl][4-piperyn][2H-benzimidazol]-2-one-maleate, has a highly selective and potent inhibitory action on calmodulin (CaM),\(^3\) a Ca\(^{2+}\)-binding protein, and possesses a racemic structure (Fig. 1). CaM is localized in the intestinal tract in rats\(^2\) and regulates intestinal ion transport.\(^3\) The activity of CaM is positively correlated with diarrhea.\(^4\) It has been reported that ZAL ameliorates secretory diarrhea without compromising gastrointestinal propulsive motility in rodents, and that it has few central nervous system effects at the doses studied.\(^5,6\) The antidiarrheal actions after oral administration as a clinical route of the optical isomers of ZAL, an \( R(\pm) \)-isomer and an \( S(\pm) \)-isomer, have not been determined. It is necessary for the development of ZAL as a clinically useful drug to determine whether the activities after oral administration of these three compounds are the same. In this study, we examined the antisecretory actions of ZAL and its optical isomers in vitro using the Ussing chamber, and the antidiarrheal action of these three compounds in vivo using two different rat models of secretory diarrhea.

MATERIAL AND METHODS

Experimental Animals Male Sprague-Dawley rats (Charles River, Atsugi, Japan) weighing 160–180 g were used in the present study. They were housed in a controlled environment at a temperature of 22–24 °C and a humidity of 50–60% with light from 7:00 a.m. to 7:00 p.m. Commercial rat chow and water were provided at libitum prior to the studies. The studies were conducted in compliance with the Guiding Principles for the Care and Use of Laboratory Animals approved by the Japanese Pharmacological Society, and the experimental protocols were approved by the Ethical Committee of the Pharmaceutical Research Institute, Kyowa Hakko Kogyo Co., Ltd.

Materials The reagents used in the present study were ZAL, \( R(\pm) \)-isomer and ZAL \( S(\pm) \)-isomer (Novartis Consumer Health, Inc., Nyon, Switzerland), castor oil (Kanto Chemical Co., Inc., Osaka, Japan), 16,16-dimethyl prostaglandin \( E_2 \) (dmpGE2) (Sigma Chemical Co., Inc., St. Louis, MO, U.S.A.) and acetylsalicylic acid (Ach) (Wako Pure Chemical Industries Co., Ltd., Osaka, Japan). All other reagents were analytical grade. ZAL and its optical isomers were dissolved in dimethyl sulfoxide or were suspended in distilled water containing 0.2% (w/v) methyl cellulose 400. dmpGE2 was diluted in saline solution. The reagents or the vehicle was administered orally or intraperitoneally to rats at a volume of 5 ml/kg or was applied to a perfusion solution at a volume of 1 ml/l.

Methods Castor-Oil-Induced Diarrhea The current study was carried out according to a slightly modification of a previously described method.\(^6\) Rats were fasted for 18–20 h prior to the experiments but had free access to water. On the day of the experiments, they were screened to exclude animals with preexisting diarrhea and were acclimated in individual cages for 1–2 h. ZAL, one of the two ZAL optical

![Fig. 1. Chemical Structure of Zaldaride Maleate](#)
isomers or the vehicle was administered orally 1 h before oral administration of castor-oil (10 ml/kg). After 1 h, the individual cages for each rat were inspected for the presence or absence of diarrhea. Unformed wet stools and watery stools were regarded as diarrhea.

**dmPGE₂-Induced Diarrhea** These experiments were performed as previously described⁶; non-fasted rats were used. On the day of the experiments, they were screened to exclude animals with preexisting diarrhea and were acclimated in individual cages for 3 h. ZAL, one of the two of ZAL optical isomers or the vehicle was administered orally 1 h before intraperitoneal administration of dmPGE₂ (500 µg/kg). After 1 h, each cage was inspected for evacuated stools that were scored according to an arbitrary scoring criteria as follows: hard stool or no defecation, score 0; ordinary stool, score 1; wet but formed stool, score 2; unformed stool, score 3; severely watery stool, score 4. The average of these scores is defined as the fecal output index. The evacuated stools were also dried and weighed.

**Ussing Chamber Experiments** Non-fasted rats were sacrificed by cervical dislocation, and the distal colon was carefully removed without stretching. Mucosal preparations were obtained by stripping away the serosa and smooth muscle of the distal colon. The mucosal preparations were mounted in Ussing chambers (surface area 0.693 cm²) and bathed on each side with 10 ml of Krebs–Henseleit solution. The composition of this perfusion solution was (mmol/l): NaCl 119.0; KCl 4.7; MgSO₄·7H₂O 1.2; KH₂PO₄ 1.2; CaCl₂·2H₂O 1.8; NaHCO₃ 24.9; glucose 11.1 and ascorbic acid 0.05 (pH 7.4). This solution was warmed at 37°C and gassed with carbogen (5% CO₂, 95% O₂).

The change in the short-circuit current (∆Isc), an indicator of chloride ion secretion, was measured continuously as the response to adding ACh. ACh (1 mmol/l) was added to the serosal side of the preparation because the maximum response was obtained in this manner. After a 30-min equilibration period, ZAL, one of the two of ZAL optical isomers or vehicle was added to the bath on the serosal side of the preparation, and 10 min later ACh (100 µmol/l) was applied to the serosal side of the preparation. The results were calculated according to the following formula:

\[
\text{Change in Isc} \text{(%)} = \left( \frac{\text{response to ACh after adding the test compounds or the vehicle (Isc)}}{\text{maximum response to ACh (Isc)}} \right) \times 100
\]

**Plasma Concentration** Rats were fasted for 18—24 h but had free access to water prior to these experiments. ZAL and its optical isomers were administered orally to rats at a dose of 30 mg/kg. After 0.5, 1, 2, 3, 4, 6, 8, 12, 24, 30, and 48 h, the animals were anaesthetized lightly with diethyl ether and plasma samples were collected from the abdominal veins.

The plasma concentrations of the compounds were determined as follows. After adding an internal standard solution to the sample plasma, 2 mol/l of NaOH was added and mixed thoroughly. Disopropyl ether was then added and mixed for 5 min. After centrifuging, the organic layer was transferred and evaporated under nitrogen gas. The residue was dissolved and HPLC was performed with fluorescence detection (Ex. 280 nm, Em. 310 nm). Plasma pharmacokinetics of ZAL and its optical isomers were characterized according to 3 parameters: the area under the plasma concentration time curve (AUC), the half-life (T½) and the maximum plasma concentration (Cmax). The AUC was calculated according to the trapezoidal rule.

**Statistical Analysis** The data from the Ussing chamber experiments are expressed as the mean±S.E.M. for the experimental group. Statistical significance between the vehicle-treated group and the test compound-treated groups was analyzed using one-way analysis of variance (ANOVA) followed by Dunnett’s test. The data from the castor-oil diarrhea experiments are expressed as the ratio of the number of animals with diarrhea to the total number of animals in each group. Statistical significance between the vehicle-treated group and the test compound-treated groups was analyzed using Fisher’s exact test. The data from the dmPGE₂ diarrhea experiments are expressed as the mean±S.E.M. for the experimental group. Statistical significance between the vehicle-treated group and the test compound-treated groups was analyzed using the Kruskal–Wallis test followed by the Steel test. The ED₅₀ and IC₅₀ values were calculated by the Probit (L) method. A p-value less than 0.05 was regarded as statistically significant and is denoted by an asterisk.

**RESULTS**

**Castor-Oil-Induced Diarrhea** The incidence of diarrhea induced by oral administration of castor-oil was an all or none response. In the vehicle control group, castor oil caused diarrhea in all rats. ZAL at a dose of 30 mg/kg (p.o.) significantly reduced the incidence of diarrhea induced by castor-oil administration (Fig. 2). The S(+) isomer at doses of 20 mg/kg (p.o.) and higher also significantly inhibited castor oil-induced diarrhea. However, at doses up to 30 mg/kg (p.o.), the R(−)-isomer had no effect (Fig. 2).

**dmPGE₂-Induced Diarrhea** At doses of 30 mg/kg (p.o.) and higher, ZAL significantly improved dmPGE₂-induced diarrhea (Fig. 3 upper). The R(−) and S(+) isomers significantly ameliorated dmPGE₂-induced diarrhea at doses of 30 and 10 mg/kg (p.o.), respectively (Fig. 3). The ED₅₀ value for each compound was as follows (mg/kg, p.o.): ZAL 25; S(+) isomer 10; R(−)-isomer >30 (Table 1). At doses of 3 mg/kg (p.o.) and higher, ZAL significantly

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*Fig. 2. Effect of ZAL and Its Optical Isomers on Castor-Oil-Induced Diarrhea in Rats*

Each column represents the ratio of rats with diarrhea to the total number of animals for each group. *+: p<0.05, **: p<0.01; statistically significant vs. the value of the control group. ZAL: zaldaride maleate; S(+): S(+) isomer; R(−): R(−)-isomer.*
reduced the dry weight of the evacuated stools (Fig. 3 lower). At doses of 10 mg/kg (p.o.) and higher, the S(+) isomer also decreased the dry weight (Fig. 3 lower). Only at a dose of 3 mg/kg (p.o.) did the R(−) isomer inhibit the dry weight of the evacuated stools (Fig. 3 lower).

**Ussing Chamber Experiments** At concentrations of 10 μmol/l and higher, ZAL significantly reduced the increase in Isc response induced by ACh in the rat colonic mucosa (IC50=4.0 μmol/l) (Fig. 4, Table 3). At concentrations of 10 μmol/l and higher, the R(−) and S(+) isomers also significantly inhibited the ACh-induced effect (Fig. 4). The IC50 values for the R(−) and S(+) isomers on ACh-induced Isc response were 3.7 and 2.9 μmol/l, respectively (Table 2).

**Plasma Concentrations of Each Compound** Plasma concentration values for ZAL after administration of 30 mg/kg (p.o.) were Cmax. 378 ng/ml (mean of 4 animals) and AUC0−12. 1650 ng·h/ml (mean of 4 animals) (Table 3). The R(−) isomer at a dose of 30 mg/kg (p.o.) rapidly increased in the plasma to Cmax. 271 ng/ml (mean of 4 animals) and AUC0−12. 613 ng·h/ml (mean of 4 animals) (Table 3). At a dose of 30 mg/kg (p.o.), the plasma level of the S(+) isomer reached a peak 1 or 2 h after administration with a Cmax of 565 ng/ml (mean of 4 animals) and AUC0−12. 2230 ng·h/ml (mean of 4 animals) (Table 3).

**DISCUSSION**

In the Ussing chamber experiments, the antisecretory actions of ZAL and its optical isomers were equipotent and their IC50 values were approximately 3—4 μmol/l. The IC50 value for the inhibitory action of ZAL on CaM activity is 3.3 μmol/l,13 and the potencies of both optical isomers are almost the same (Towart et al., unpublished observations). In addition, these compounds have a high selectivity for CaM. These findings suggest that the inhibitory actions of ZAL and its optical isomers on ACh-induced intestinal ion secretion are dependent on their activity against CaM.

ACh was used in the Ussing chamber experiments because it is widely accepted as a physiologically important mediator that modulates gastrointestinal ion transport and gastrointestinal propulsive motility. ACh stimulates gastrointestinal ion secretion via an increase in intracellular Ca2+ levels and the stimulation of protein kinase C mediated by muscarinic receptors located in the gastrointestinal mucosa. Moreover, external ACh releases internal ACh from enteric cholinergic
neurons via muscarinic or nicotinic receptors localized on enteric neurons. CaM inhibitors such as trifluoperazine are reported to inhibit ACh release from cholinergic nerve endings.7 These findings suggest that ZAL and its optical isomers inhibit intestinal ion secretion by a reduction in ACh release from cholinergic neurons, and that they partly block intestinal ion secretion elicited by increased Ca$^{2+}$ levels within intestinal epithelial cells.

It has been reported that ZAL ameliorates dmPGE$_2$- and castor-oil-induced diarrhea without reducing gastrointestinal propulsive motility in rodents.5,6 dmPGE$_2$, a stable analogue of prostaglandin E$_2$, stimulates adenylyl cyclase activity and intestinal ion secretion via type 4 of EP receptors.8,9 This compound also inhibits ion absorption in the intestinal mucosa. Adenylyl cyclase is localized in the intestinal tract, and the activity of some isoforms is regulated by CaM.10,11 These findings suggest that ZAL and its optical isomers ameliorate dmPGE$_2$-induced diarrhea by inhibition of adenylyl cyclase activity within the intestinal mucosa. Castor-oil, which contains ricinoleic acid, increases the biosynthesis of PGs,12,13 nitric oxide14–17 and platelet-activating factor18 in the intestinal tract. These compounds stimulate ion secretion through the intestinal mucosa.11,19,20 Nitric oxide production induced by castor-oil is caused by CaM-regulated constitutive nitric oxide synthase activation,21 and the biosynthesis of PGs from arachidonic acid is regulated by CaM.22,23 In addition, platelet-activating factor receptors are controlled by a CaM-dependent phosphorylation and dephosphorylation process.24 Based on these findings, we conclude that ZAL and its S(+)-isomer inhibit the incidence of diarrhea evoked by castor oil by inhibition of arachidonic acid production, CaM-dependent adenylyl cyclase activity25–31 and CaM-regulated constitutive nitric oxide synthase activity.32 It is believed that ZAL inhibits secretory diarrhea without reducing gastrointestinal propulsive motility. However, at the high doses studied, the antidiarheal effect of ZAL may in part be related to inhibition of this motility.

In this study, the order potency for the antidiarheal effects of ZAL and its optical isomers on dmPGE$_2$- and castor-oil-induced diarrhea were as follows: S(+)-isomer $\geq$ ZAL $>$ R(--)-isomer (dmPGE$_2$ diarrhea model); S(+)-isomer $>$ ZAL, R(--)-isomer (castor oil diarrhea model). The order of the ED$_{50}$ values for the effects of these three compounds on dmPGE$_2$-induced diarrhea was S(+)-isomer $>$ ZAL $>$ R(--)-isomer. The rank order of the plasma concentrations of ZAL and its optical isomers after oral administration was as follows: S(+)-isomer $>$ ZAL $>$ R(--)-isomer (C$_{max}$); S(+)-isomer $>$ ZAL $>$ R(--)-isomer (AUC$_{0-12}$). These results suggest that the antidiarheal effects of ZAL and both optical isomers relate to their plasma concentration levels.

In conclusion, despite the fact that the antisecretory actions of ZAL and its optical isomers are the same, the antidiarheal actions of ZAL and its S(+)-isomer are more potent than that of the R(--)-isomer. These actions may be related to plasma levels; however, the plasma concentrations of ZAL and both optical isomers for inhibition of CaM activity or ACh-induced ion secretion were higher than the plasma levels for improved experimental secretory diarrhea. The mechanisms of action of those compounds have not been determined. It is possible that ZAL and its optical isomers act locally rather than systemically in the intestinal tract in rats. Further studies are required to learn whether ZAL and the two isomers act systemically or locally in the intestinal tract.

REFERENCES