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Naringin Exhibits in vivo Prokinetic Activity via Activation of Ghrelin Receptor in Gastrointestinal Motility Dysfunction Rats

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Key Words
Poncirus fructus · Naringin · Ghrelin receptor · Intestinal motility · Prokinetic activity

Abstract
Background and Purpose: Poncirus fructus (PF), also known as the dried immature fruit of *Poncirus trifoliata* (L.) Raf., has long been used as a cure for the treatment of various gastrointestinal disorders in eastern Asia. Recently, it was reported that naringin, a flavonoid constituent of the PF extract, causes the activation of ghrelin receptor in vitro. Although the ghrelin receptor is involved in the enhancement of intestinal motility, there are no studies as yet involving in vivo action of naringin. Therefore, the purpose of the present study is to investigate whether naringin exhibits a prokinetic effect in vivo. ***Methods:*** We measured the intestinal transit rate in rats with gastrointestinal motility dysfunction (GMD) and performed a pharmacokinetic analysis of naringin to investigate the effect of naringin on prokinetic activity in vivo. ***Results:*** The results of this study show that the aqueous extract of PF and its constituent naringin have a strong prokinetic activity in GMD rats via activation of the ghrelin receptor. Surprisingly, pharmacokinetic analysis revealed that naringin has low bioavailability (11%), implying that the prokinetic effect of naringin was largely due to the local activation of ghrelin receptor in the intestine rather than a systemic effect after absorption. Indeed, it turned out that intravenous administration of naringin led to a lower prokinetic effect than when administrated orally to rats, indicating that naringin prefers to act on the intestinal wall rather than getting absorbed into the systemic circuit. This local mode of action might be advantageous for preventing possible systemic side effects since naringin is not well absorbed into the system circuit. ***Conclusions:*** Naringin exhibits an in vivo prokinetic activity by a preferable local activation of ghrelin receptor. Moreover, we propose that naringin could play a role as a leading compound for the development of ghrelin receptor-based prokinetic agents.
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Introduction

Poncirus fructus (PF), the dried immature fruit of *Poncirus trifoliata* (L.) Raf., has been broadly used as a traditional medicine as a cure for various gastrointestinal diseases, especially in eastern Asia. In particular, the aqueous extract of PF (PF-W) is well known as a remedy for the treatment of digestive dysfunctions such as dyspepsia,

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constipation and diarrhea. Indeed, many studies have proved that PF-W enhances gastrointestinal motor function by accelerating the transit of intestinal contents without leading to gastric emptying in human subjects and rodents [1]. It also improved the intestinal transit rate (ITR) in rodents with experimental digestive dysfunctions [2]. Recently, it was revealed that naringin, a chemical component in PF-W, can cause the activation of ghrelin receptor in vitro [3]. Although the ghrelin receptor is well known for its role in gastrointestinal regulation, the effect of naringin on in vivo prokinetic action remains largely unknown.

The ghrelin receptor is a G protein-coupled receptor that induces an increase in intracellular calcium when its endogenous ligand ghrelin, a hunger-stimulating hormone produced mainly from the stomach and pancreas, binds to it. The administration of ghrelin in rodents has been proved to improve gastric emptying, intestinal motility and symptoms of dyspepsia [4–6]. Ghrelin also activates cholinergic excitable neurons, which regulate gastrointestinal motility via the enteric nervous system [7]. Therefore, ghrelin and its receptor are currently being regarded as potential therapeutic targets against various gastrointestinal motility disorders [8].

Naringin belongs to the flavonoid family frequently found in grapefruits, and is responsible for its bitter taste. Various pharmacological benefits of naringin such as antioxidant, anticancer, antibacterial and anti-inflammatory effects have been identified [9–12]. In particular, naringin exerts an anti-ulcer effect on gastric lesions induced by ethanol [13]. Moreover, treatment with naringin prevented an increase of plasma lipid levels such as cholesterol and triglyceride in rats that displayed high fat or disturbed lipid metabolism [14, 15]. Still, there is no clear report about the effect of naringin in terms of intestinal motility. Therefore, the present study investigated possible effects of naringin on in vivo intestinal motility, as well as pharmacokinetic profiles to better understand its action.

Materials and Methods

Sample Preparation

PF-W was prepared as described in a previous report [16]. Briefly, a sample of PF was boiled in distilled water at 100°C for 2 h. The aqueous extract was then filtered and concentrated in vacuo, and powder form was generated by lysophilization. Naringin, domperidone, and Evans blue were purchased from Sigma Aldrich (St. Louis, Mo., USA), and Cortistatin-8 (CST-8) was purchased from Tocris (Bristol, UK).

Animals

Adult male Sprague-Dawley rats (230–300 g) were used for this experiment. The rats were habituated with free access to food and water under a 12:12-hour light:dark cycle at 20–23°C and at 50–60% humidity. All animal experiments were performed in accordance with the ethical guidelines of the Committee on Laboratory Animals at the Seoul National University.

Induction of Gastrointestinal Motility Dysfunction Rats

Sprague-Dawley rats were fasted for 16–20 h with free access to water. The rats were anesthetized with ether, and their abdominal wall was shaved and then sterilized. The peritoneum was then opened. A part of the small intestine was taken out of the abdominal cavity and gently massaged 3 times with fingers starting from the pylorus to the ileocecal valve, and then the abdominal cavity was closed with sutures. The entire procedure was conducted within 10 min. After the operation, the rats were kept in individual cages and allowed to recover for 30 min.

Measurement of ITR

PF-W or naringin was orally and/or intravenously administered in the gastrointestinal motility dysfunction (GMD) rats. At 30 min after the administration of PF-W or naringin, 0.5% (w/v) Evans blue was orally administered to the GMD rats at a dose of 0.1 ml/rat. The rats were sacrificed after 30 min and the entire intestine was rapidly removed. The ITR was calculated by means of the following equation:

$$\mathrm{ITR}\,(\%) = \frac{\text{Distance of Evans blue transport}}{\text{Length of small intestine}} \times 100 \qquad (1)$$

Quantification of Naringin in the Plasma Using High-Performance Liquid Chromatography

The concentration of naringin in the plasma was determined by high-performance liquid chromatography (HPLC) with slight modification of previous methods [17]. Briefly, the plasma samples were loaded into Auto sampler HPLC system (Waters 717 plus, Milford, Mass., USA) that consisted of a 515 HPLC dual pump and a 2487 Dual λ absorbance detector. The C18 analytical column (Gemini NX, 150 × 4.6 mm, 5 μm; Phenomenex, Torrance, Calif., USA) was used for the analysis. All chromatographic separations were conducted in gradient conditions using the mobile phase with pump A (0.1 mol/l ammonium acetate 90%, acetonitrile 10%) and pump B (0.1 mol/l ammonium acetate 10%, acetonitrile 90%). Gradient conditions linearly changed from pump A to pump B as 88:12 at 0 min up to 60:40 at 7 min. The ratio then reverted to its earlier value of 88:12 and maintained this for an additional 2 min. The flow rate was 1 ml/min and UV light at a wavelength of 284 nm was used. To obtain the standard curve, naringin was serially diluted to a concentration of 0.2, 0.5, 1, 5, 10 and 25 μg/ml in rat plasma. Antipyrine (50 μg/ml) was used as an internal standard. The r^2 value was around 0.999 (data not shown), and 0.2 μg/ml was considered as a limitation of detection. The validation data of the analysis are given in table 1.

Pharmacokinetic Analysis

Pharmacokinetic parameters were determined using the noncompartmental analysis (WinNonlin version 3.1; Pharsight, Mountain View, Calif., USA): clearance (CL), volume of distribution at the steady state (V_{ss}), mean residence time (MRT), maxi-

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Table 1. Intra- and interday validation of HPLC analysis for determining naringin concentration in plasma samples

	Theoretical concentration			
	LLOQ 0.2	LQC 1	MQC 5	HQC 25
Intraday accuracy and precision				
Mean estimated concentration, μg/ml	0.2	1	4.9	25.1
Precision, CV, %	4.1	7.6	5.3	0.2
Accuracy, %	103.7	102	99.6	100.2
Interday accuracy and precision				
Mean estimated concentration, μg/ml	0.2	1	4.9	25.1
Precision, CV, %	13.4	9.3	4.3	0.4
Accuracy, %	100.2	101.7	98.1	100.2

LLOQ = Lower limit of quantification (μg/ml); LQC = low quality control (μg/ml); MQC = medium quality control (μg/ml); HQC = high quality control (μg/ml); CV = coefficient of variation.

mum plasma concentration (C_{max}), and time to reach a C_{max} (T_{max}). The total area under the plasma concentration-time curve from time zero to time infinity (AUC) was calculated using the trapezoidal method. $AUC_{t-\infty}$ from the last measured time to time infinity was calculated from the concentration at last measured time (C_t) divided by velocity constant at the terminal phase (k). Therefore, the total $AUC_{t-\infty}$ was calculated by the summation of $AUC_{(0-t)}$ and $AUC_{(t-a)}$ (see table 2).

The bioavailability of naringin was calculated from the compensated equation 1 because of the dose difference between oral (25 mg/kg/ml) and intravenous (5 mg/kg/ml) injection (5-fold).

$$F = \frac{PO\ AUC_{0-\infty}}{5 \times IV\ AUC_{0-\infty}}, \quad (2)$$

where PO $AUC_{0-\infty}$ indicates AUC from orally administered rats, and IV $AUC_{0-\infty}$ refers to AUC from intravenously administered rats.

Table 2. Summary of pharmacokinetic parameters of naringin after intravenous (IV; 5 mg/kg) and oral (PO; 50 mg/kg) administration in rats

Parameter	IV (n = 4)	PO (n = 4)
C_{max}, μg/ml		2.7±1.8
T_{max}, min		22.5±8.7
AUC_{inf}, min·μg/ml	411.9±91.5	227.2±55.2
MRT_{inf}, min	67.1±29.7	164.2±135.8
CL, ml/min/kg	12.6±2.7	
V_{ss}, ml/kg	798.1±251.8	
Bioavailability, %	11.0	

Results

PF-W and Its Constituent Naringin Exert Prokinetic Activity in GMD Rats via Activation of Ghrelin Receptor

First, it was necessary to investigate whether the prokinetic activity of PF-W is mediated via the action of the ghrelin receptor. The ITR of GMD rats resulting from PF-W administration measured by equation 1 was used to validate the prokinetic activity. As shown in figure 1, oral administration of PF-W (500 mg/kg) greatly enhanced ITR (33.6 ± 1.9%, n = 4) compared to sham-operated control rats (21.6 ± 2.6%, n = 4). Remarkably, when CST-8 (100 μmol/animal) – a ghrelin receptor antagonist – was pretreated before PF-W administration, ITR was significantly inhibited (27.4 ± 1.3%, n = 4; fig. 1a). As expected, none of the vehicles (DMSO-containing saline) affected ITR in GMD rats (19.8 ± 1.9%, n = 4). Furthermore, the administration of CST-8 alone did not have any effect on ITR (22.2 ± 3.0%, n = 4). Therefore, these data strongly suggest that the increase in ITR as a result of PF-W administration could be due to the activation of the ghrelin receptor, because the pretreatment of a ghrelin receptor antagonist (CST-8) inhibited the prokinetic action of PF-W.

Since our recent work [3] already found that naringin, a constituent of PF-W, is an agonist of ghrelin receptor in vitro, we sought to investigate whether naringin has an in vivo prokinetic activity as well. As shown in figure 1, when 50 mg/kg of naringin was orally administrated to GMD rats, it induced an increase of ITR up to nearly 40% (39.9 ± 4.0%, n = 4), whereas sham-operated rats had an increase of ITR of 21.0 ± 2.6% (n = 4). More importantly,

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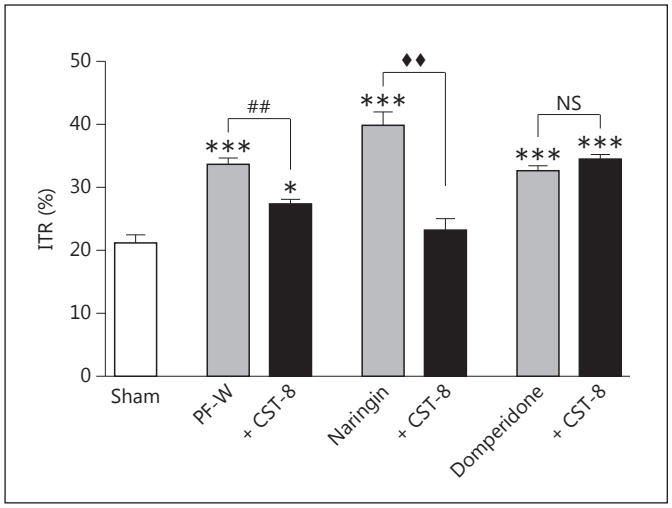


Fig. 1. Prokinetic activity of PF-W and its constituent naringin in GMD rats. The ITR was measured after administration of PF-W, naringin or domperidone in GMD rats. PF-W, naringin and domperidone significantly increased the ITR in GMD rats. This increase was abolished by pretreatment with CST-8 ghrelin receptor antagonist, except for domperidone. * $p < 0.05$ vs. sham; *** $p < 0.001$ vs. sham; ## $p < 0.01$ vs. PF-W; ♦♦ $p < 0.01$ vs. naringin (ANOVA, Tukey's post hoc test). Error bars indicate SEM.

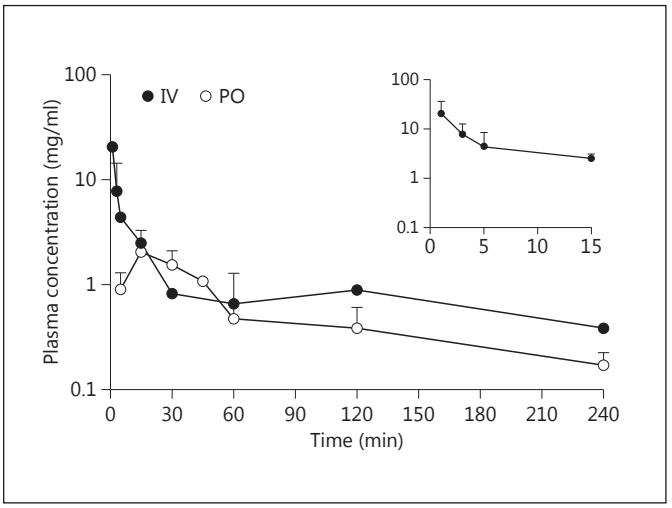


Fig. 2. Pharmacokinetic time-concentration profiles of naringin after intravenous (IV) and oral (PO) administration of naringin into rats. The inset graph represents a magnification of the intravenous pharmacokinetic profile from 5 to 15 min.

pretreatment with CST-8 significantly dissolved the increase of ITR to almost that of sham-operated groups (23.3 ± 3.3%, n = 4). Therefore, it is evident that naringin has indeed an in vivo prokinetic effect via the activation of the ghrelin receptor.

We further questioned whether the inhibitory effect of CST-8 on intestinal motility was due to specific inhibition of the ghrelin receptor. To prove that, domperidone, a prokinetic agent based on a different mechanism (via inhibition of dopamine receptors [18]), was administered to GMD rats. As shown in figure 1, administration of domperidone (20 mg/kg) induced a dramatic enhancement of ITR (32.7 ± 1.5%, n = 4). Because the enhanced motility was related to the dopamine receptor, pretreatment of the ghrelin receptor antagonist CST-8 did not cause any changes in the domperidone-induced ITR enhancement (34.5 ± 1.3%, n = 4). Thus, these data suggest that CST-8 specifically inhibits intestinal motility only when induced by the activation of the ghrelin receptor.

Overall, it was confirmed that PF-W and its constituent naringin strongly enhanced intestinal motility in GMD rats, and the enhancement was specifically inhibited by pretreatment of the ghrelin receptor antagonist CST-8.

Naringin Is Rarely Absorbed through the Intestinal Membrane

Next, the pharmacokinetic profile of naringin was investigated. The pharmacokinetic profiles of naringin after intravenous and oral administration are illustrated in figure 2, and pharmacokinetic parameters are summarized in table 2. It should be noted that due to limitations in the detection of naringin, the oral dose of naringin (25 mg/kg) was increased 5 times more than that of intravenous administration (5 mg/kg). Surprisingly, it was found that the bioavailability of naringin calculated from equation 2 turned out to be very low (11%), suggesting that naringin is poorly absorbed through the intestinal membranes. Therefore, these data provided us with an insight that the effect of naringin on intestinal motility could be a result of direct action on intestinal membranes, since naringin was found to be poorly absorbed into the systemic circuit.

Oral Administration of Naringin Is More Favorable than Intravenous Route in Terms of Intestinal Motility

Finally, naringin was administrated by different routes (intravenous and oral), and which route of administration is more beneficial in terms of intestinal motility was ascertained. Because of the low bioavailability (around 10%; table 1) of naringin, different doses were chosen be-

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tween intravenous (5 mg/kg) and oral (50 mg/kg) administration to achieve a similar plasma concentration of naringin between the two groups. In the case of GMD rats administered by the intravenous route, naringin slightly increased the ITR (30.2 ± 4.0%, n = 4). However, in orally administered GMD rats, the ITR was found to be dramatically increased (39.9 ± 4.0%, n = 4). Assuming that the plasma concentration of both groups was comparable, it could be determined that oral administration is more satisfactory than intravenous administration in terms of prokinetic action.

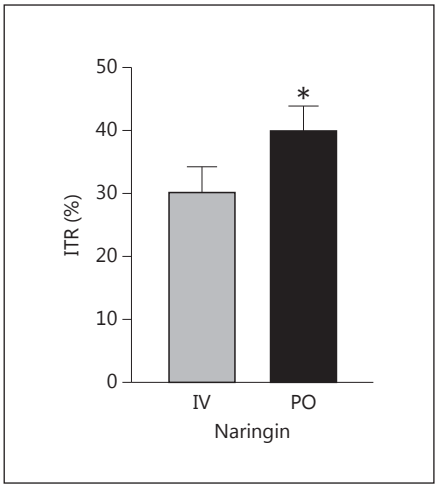


Fig. 3. Comparison of the ITR in orally and intravenously administrated rats. IV = Intravenous; PO = oral. Note that the ITR value of oral administration here is identical to that shown in figure 1. * $p < 0.05$ (Student's t test).

Discussion

The present study clearly shows that the prokinetic activity of PF-W is achieved in a ghrelin receptor-dependent manner. Moreover, the present study revealed that naringin, a constituent of PF-W, exhibits prokinetic activity in vivo for the first time. The present finding is in agreement with our previous work that proved that naringin works as a ghrelin receptor agonist in vitro [3].

The prokinetic effect of PF-W has already been known in both humans and rodents and even in GMD rats [1, 2]. Although the prokinetic activity was evident, the underlying molecular mechanism of this activity was uncertain until the involvement of serotonin receptor subtype 4 (5-HT4R) was reported [19]. Nevertheless, it seemed that the activation of 5-HT4R by PF-W is not quite robust when compared to that by cisapride, a synthetic agonist of 5-HT4R [19]. These unresolved questions became the starting point for investigating the molecule responsible for the prokinetic activity of PF-W, and it turned out that the ghrelin receptor is activated by PF-W and naringin in vitro [3].

Therefore, it appears that PF-W is involved in at least two mechanisms to enhance intestinal motility: the 5-HT4R and the ghrelin receptor. Thus, this dual-action mechanism might be the source of strong prokinetic activity of PF-W. Indeed, this could be estimated from our current data: the increased ITR by PF-W was not completely dissolved by CST-8 (fig. 1; third column from the left; asterisk indicates difference compared to sham), suggesting the involvement of a different prokinetic mechanism (probably a 5-HT4R-mediated one) that cannot be inhibited by ghrelin receptor antagonist CST-8. In contrast, the increased ITR by naringin was almost completely offset by CST-8 (fig. 1; fifth column from the left; note that there is no asterisk), which also supports the dual-action mechanism of PF-W. Although it was found that naringin is the single compound in PF-W that can activate ghrelin receptor, it is still vague as to which single compound in PF-W can activate 5-HT4R. Further studies are required to find the key player other than naringin involved in PFW on intestinal motility.

However, the prokinetic effect by ghrelin receptor activation is not totally new. In fact, there were many reports that showed the prokinetic effects of ghrelin and/or ghrelin receptor. For instance, it was reported that the administration of ghrelin in rodents enhanced gastric contractions dose-dependently, which accelerated gastric emptying of liquid and solid food by increasing antropyloric coordination [4, 20]. The administration of ghrelin in diabetic mice with gastroparesis was found to stimulate a gastric motor effect on gastric emptying [21]. Ghrelin also normalized burn-induced delayed gastric emptying and intestinal transit [22]. Vagotomized rats showed delayed small ITR due to reduced expression of ghrelin receptor in the small intestinal muscle layers [23]. Moreover, the activation of ghrelin receptor improved gastric emptying in a mouse model of dyspepsia [5], and accelerated gastric emptying in a postoperative or lipopolysaccharide-induced septic ileus rat model [24, 25]. However, we believe that the present study is the first report that identified the action of naringin on the ghrelin receptor in vivo.

The pharmacokinetic analysis of naringin in the present study showed some interesting characteristics. Firstly,

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naringin turned out to be a very poorly absorbed compound (bioavailability = 11%). In fact, the low bioavailability of naringin interfered with our pharmacokinetic experiments in the initial setup, and was the reason why a 5 times higher oral dose (25 mg/kg) than that of intravenous administration (5 mg/kg) was required to overcome the detection limits of the HPLC device. We are inclined to think that the low naringin bioavailability is related to its action on P-glycoprotein 1 (P-gp) transporter [26]. In fact, naringin is well known for its capability to enhance the bioavailability of other drugs that are P-gp substrates [27]. Thus, naringin is considered as a P-gp inhibitor [28], probably due to competing with other P-gp substrates. Since P-gp transports its substrates back into the lumen, chances are that naringin might have been pumped back into the lumen, resulting in such a lower bioavailability. Obviously, further investigation is mandatory to conclude its involvement with P-gp. Secondly, despite its low bioavailability, naringin can still be used as a prokinetic agent.

Because the target organ for a prokinetic agent is mostly the small intestine, absorption might not be a critical factor. However, we cannot exclude the possibility that the different doses of naringin between intravenous and oral administration have led to a calculated bioavailability far from the real value. Again, because of limits of detection, the use of different dose regimens was inevitable. Still, considering that ITR from the orally administered group was larger than that of the intravenously administered group (39.9 ± 4.0 vs. 30.2 ± 4.0%; fig. 3), it is difficult to imagine that naringin has a good bioavailability in the end. Moreover, considering that many prokinetic agents suffered from systemic side effects such as heart failure [29, 30], low bioavailability can even be advantageous for prokinetic agents.

Conclusion

The present study showed that naringin in PF-W exerts prokinetic activity via the specific activation of ghrelin receptor in vivo. Although naringin has low bioavailability, it could be rather advantageous because its target site is the small intestine and it has less severe systemic side effects. Therefore, naringin could be developed not only as a ghrelin receptor-based prokinetic agent, but also as a leading compound to develop a novel prokinetic agent.

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