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An aqueous extract of *Poncirus fructus* activates the prokinetic activity of 5-HT receptor subtype 4 without hERG interaction

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ABSTRACT

Aim of the study: Poncirus fructus (PF) – also known as the dried, immature fruit of *Poncirus trifoliata* (L.) Raf. (Rutaceae) – is a natural substance that has long been used for various gastrointestinal disorders in eastern Asia. An aqueous extract of PF (PF-W) has particularly potent gastroprokinetic effects, but its molecular mechanism was not well understood. Identification of the underlying prokinetic mechanism of PF-W was pursued in the present study.

Materials and methods: Changes in *in vitro* cAMP levels and *in vivo* intestinal transit rate (ITR) caused by PF-W were measured after pretreatment with GR125487, an antagonist for serotonin receptor subtype 4 (5-HT4R). An [^{3}H] astemizole binding assay and electrophysiology experiments were performed to determine if PF-W has any interaction with the human *ether-à-go-go* related gene (hERG) potassium channel.

Results: PF-W induced an increase in intracellular cAMP in 5-HT4R-expressing HEK293T cells, indicating that PF-W does activate 5-HT4R. Moreover, pretreatment with GR125487 successfully blocked the increase, suggesting that the response was 5-HT4R-specific. More importantly, pretreatment of GR125487 in rats inhibited the elevation of ITR by PF-W, indicating that the prokinetic effect of PF-W was indeed exerted via 5-HT4R. On the other hand, both [^{3}H]-astemizole binding assay and electrophysiological experiments revealed that PF-W did not interfere at all with the hERG channel.

Conclusion: It was found that PF-W exerts its prokinetic activity through a 5-HT4R-mediated pathway, with no interaction with hERG channels. Therefore, PF-W is a good candidate that might be developed as a prokinetic agent with fewer expected cardiac side effects.

1. Introduction

The dried, immature fruit of *Poncirus trifoliata* (L.) Raf. (Rutaceae), also known as *Poncirus fructus* (PF), has been widely used as a traditional medicine for diverse gastrointestinal (GI) disorders, especially in eastern Asia (Lee et al., 1996, 2005a; Yi et al., 2004). PF extracts are a common ingredient in over-the-counter drugs for gastrointestinal disorders in Korea. The popularity of PF for the treatment of GI disorders recently was found to be due mostly to its remarkable prokinetic activity. An earlier study reported that an aqueous extract of PF (PF-W) had strong prokinetic activity in normal subjects, accelerating the transit of intestinal contents without affecting gastric emptying (Lee et al., 2005b). Moreover, PF-W showed a dramatic prokinetic effect in rats with experimental GI motility dysfunction (Lee et al., 2005a). A recent report indicated that PF-W may also be useful as a weight-suppression agent because of its notable prokinetic activity (Shim et al., 2009).

Although the prokinetic effect of PF-W seems obvious, it is still unclear how this extract exerts its prokinetic activity at the molecular level. Therefore, the present study aimed to identify the molecular underpinnings of the prokinetic activity of PF-W.

Among the pathways related to intestinal motility, serotonin (5-hydroxytryptamine, 5-HT) is known as a mediator that influences the GI tract. Generally, 5-HT plays a role as a neurotransmitter in the central nervous system, but the majority (95%) of 5-HT is found in the GI tract (Kim and Camilleri, 2000). 5-HT interacts with seven different 5-HT receptor subtypes, but only five that are involved in intestinal motility are found in the gut (De Maeyer et al., 2008).

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Among these, 5-HT receptor subtype 4 (5-HT4R) is of interest due to its strong association with potent prokinetic activity (Gershon and Tack, 2007). Basically, 5-HT4R is a member of the G_s family, which stimulates adenyl cyclase when activated (De Maeyer et al., 2008). Adenyl cyclase then catalyzes the conversion of adenosine triphosphate (ATP) into cyclic adenosine monophosphate (cAMP). Thus, activation of 5-HT4R results in the increase of intracellular cAMP level. Stimulated 5-HT4R in the enteronervous system enhances the release of acetylcholine, which in turn strengthens the synaptic transmission, leading to the excitation of smooth muscles in the myenteric plexus, so it is regarded as *prokinetic* (Gershon and Tack, 2007). It therefore seems logical to investigate whether the prokinetic action of PF-W has any relationship with 5-HT4R.

However, it should also be noted that some 5-HT4R agonists, such as cisapride, are notorious for their critical cardiac adverse effects, because they block the human *ether-à-go-go* related gene (hERG) potassium channel (Mohammad et al., 1997). Since that report, it has become a regular practice in the field of drug development to screen out any compound that might interact with hERG, for fear of cardiac side effects. If PF-W activates 5-HT4R in a manner similar to cisapride, it is plausible that it may block hERG as well.

Therefore, in the present study, through both *in vitro* and *in vivo* experiments, we examined whether the prokinetic activity of PF-W is linked to a 5-HT4R-mediated system. The possibility of interaction between PF-W and hERG was also verified.

2. Materials and methods

2.1. Reagents

PF-W was prepared using the method previously described (Shim et al., 2009). Briefly, a 2-kg sample of PF was boiled in 30 L of distilled water at 100 °C for 2 h. The aqueous extract was then filtered, concentrated *in vacuo*, and a powder was obtained by lyophilization. Evan's blue, theophylline and cisapride were purchased from Sigma (St. Louis, MO). GR125487 sulfamate was purchased from Tocris (Ellisville, MO). [^{3}H] astemizole (20 Ci/mmol, MT-100917) was purchased from Moravek Biochemical (Brea, CA).

2.2. Cloning of the rat 5-HT4R gene

The full-length rat 5-HT4R cDNA (NM_012853) was cloned using the RT-PCR technique. Custom-designed primers corresponding to rat 5-HT4R, which includes a flanking EcoRI restriction enzyme site followed by a Kozak sequence (Forward) and NotI restriction enzyme site (Reverse), were synthesized. The primer sequences appear below:

- Forward: 5′-G/GAATTC/GCCACC/ATGGACAGACTTGATGCTAATGT G-3′
- Reverse: 5′-ATAAGAAT/GCGGCCGC/TTAGGACTGGCTTCTTTTCAA G-3′

A standard RT-PCR was performed against the rat dorsal root ganglion cDNA library with a protocol of 94 °C (30 s) – 60 °C (30 s) – 72 °C (1 min) with 30 cycles. The amplicon was subcloned into multiple cloning sites (EcoRI, NotI) of pcDNA 3.1. It was found that the gene had 100% identity with a GENBANK rat 5-HT4R sequence.

2.3. Transient transfection of rat 5-HT4R into HEK293T cells and drug treatment

HEK293T cells were grown up to 10^7 cells/well in 6-well plates. Transfection of rat 5-HT4R was done using FuGENE® HD Transfection Reagent (Roche, Basel, Switzerland). In the control groups, pcDNA 3.1 mock vector was transfected. Then, 24 h after transfection, the media were removed and the cells were washed 2 times with 1 mL of NBS buffer (1 M NaCl, 1 M KCl, 500 mM $CaCl_2$, 500 mM $MgCl_2$, 500 mM glucose, 1 M HEPES). After washing, 1 mL of 5 mM theophylline was added to inhibit 3′,5′-cyclic-AMP phosphodiesterases (Essayan et al., 1995), followed by incubation for 15 min at 37 °C. Afterward, 1 mL of cisapride or PF-W dissolved in NBS buffer was added and incubated for another 15 min at 37 °C, reaching a final concentration of 1 μM (cisapride) and 100 μg/mL (PF-W). For the 5-HT4R inhibitor study, 10 nM of GR125487 was included from the theophylline treatment onward.

2.4. Measurement of cAMP in the cell

After the drug treatment, the media were quickly removed and the cells were washed 2 times. For cell lysis, 0.1N HCl was added and incubated for 20 min at room temperature. The cells were then scraped, put into microtubes, and centrifuged for 10 min at 1000 × *g*. After centrifugation, supernatants were obtained and cAMP measurement was performed using a cyclic AMP EIA kit (Cayman Chemical, Ann. Arbor, MI) following the manufacturer's protocol. Specifically, cAMP levels were obtained by the acetylation method and normalized by the protein amount in each well, measured by the standard BCA assay method.

2.5. Measurement of rat intestinal transit rate (ITR)

Measurement of the rat intestinal transit rate was done using the same method previously reported (Shim et al., 2009). Briefly, SD rats weighing 200–250 g were fasted for 16–20 h with *ad libitum* access to water. 30 min after oral administration of PF-W, 0.5% (w/v) Evan's blue was administered orally to the rats. After an additional 20 min, the rats were sacrificed and the whole intestine was rapidly removed. The intestinal transit rate (ITR) was calculated using the following equation, as described previously (Lee et al., 2005a):

$$\text{ITR}\ (\%) = \left(\frac{\text{Distance of Evan's blue transport}}{\text{Length of small intestine}}\right) \times 100$$

where the length of the small intestine was determined by measuring the distance from the pylorus to the ileocecal valve. The experiment was approved by the Animal Care and Use Committee of the College of Pharmacy, Seoul National University, and the institutional guidelines for the care and use of laboratory animals were followed throughout the study.

2.6. hERG membrane preparation

The hERG membrane was prepared using the following methods from Finlayson et al., with slight modification (Finlayson et al., 2001). Briefly, hERG stable CHO cell lines, established by LG Life Sciences Ltd., were cultured on a 100 mm Petri dish until they reached 90% confluency. After washing twice with PBS, cells were harvested with trypsin-EDTA and centrifuged for 10 min. The supernatants were then removed, and the pellets were resuspended in a 0.32 M sucrose-containing protease inhibitor cocktail (Cat #539137; Calbiochem, San Diego, CA). After homogenization on ice with a glass/Teflon homogenizer, centrifugation was done and the supernatants were kept on ice. The remnant pellets were resuspended with milli-Q water and taken through the same homogenization process again. The supernatants from this step were combined with those previously obtained and subjected to ultracentrifugation (48,000 × *g*, 20 min, 4 °C). The pellets were then resuspended with 1 mL of membrane buffer (10 mM HEPES, 5 mM KCl, 130 mM NaCl, pH 7.4). The amount of membrane protein was assessed using the standard BCA method, and the aliquots were frozen in liquid