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The Inhibitory Effects of Korean Red Ginseng Saponins on 5-HT_{3A} Receptor Channel Activity Are Coupled to Anti-Nausea and Anti-Vomiting Action

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Abstract : We performed *in vitro* and *in vivo* studies to know whether the inhibitory effects of ginsenosides on 5-HT_{3A} receptor channel acctivity are coupled to anti-nausea and anti-vomiting action. *In vitro* study, we investigated the effect of compound K (CK) and M4, which are ginsenoside metabolites, on human 5-HT_{3A} receptor channel activity expressed in *Xenopus* oocytes using two-electrode voltage clamp technique. Treatment of CK or M4 themselves had no effect in both oocytes injected with H_2O and 5-HT_{3A} receptor cRNA. In oocytes injected with 5-HT_{3A} receptor cRNA, M4 treatment inhibited more potently 5-HT-induced inward peak current ($I_{5\text{-}HT}$) than CK with dose-dependent and reversible manner. The half-inhibitory concentrations (IC_{50}) of CK and M4 were 36.9 ± 10.1 and 7.3 ± 2.2 μM, respectively. The inhibition of $I_{5\text{-}HT}$ by M4 was non-competitive and voltage-independent. These results indicate that M4 might regulate 5-HT_{3A} receptors. *In vivo* experiments, injection of cisplatin (7.5 mg/kg, i.v.) induced both nausea and vomiting with 1 h latency. These episodes reached to peak after 2 h and persisted for 4 h. Pre-treatment of GTS (500 mg/kg, p.o.) significantly reduced cisplatin-induced nausea and vomiting by 51 ± 8.4 and 48.8 ± 6.4% during 4 h compared to GTS-untreated group, respectively. These results show the possibility that *in vitro* inhibition of 5-HT_{3A} receptor channel activity by ginsenosides might be coupled to *in vivo* anti-emetic activity.

Key words : *Panax* ginseng; ginsenoside metabolites; 5-HT_{3A} receptor; cisplatin; emesis

INTRODUCTION

Ginseng, the root of *Panax ginseng* C.A. Meyer, is well known as a tonic for restoring and promoting human health. In traditional medicine, ginseng alone has been used for the alleviation of symptoms such as anorexia, dyspepsia, vomiting and pain[1]. The main molecular components responsible for the actions of ginseng are ginsenosides, which are also known as ginseng saponins. Ginsenosides have a four-ring, steroid-like structure with sugar moieties attached, and about 30 different forms have been isolated and identified from the root of *Panax* ginseng. They are classified into protopanaxadiol or protopanaxatriol ginsenosides according to the position of sugar moieties at carbon-3 or-6 (Fig. 1)[2].

Ginsenosides regulate several types of ligand-gated ion channel activity. In cells expressing nicotinic acetylcholine receptors, such as bovine chromaffin cells, protopanaxatriol (PT) rather than proptopanaxadiol (PD) ginsenosides, especially ginsenoside Rf and Rg_2, more potently inhibit acetylcholine-stimulated Na^+ influx[3]. More directly, Choi *et al.* (2002) and Sala *et al.* (2002) also showed that PT ginsenosides more potently inhibits acetylcholine-induced inward current in *Xenopus* oocytes expressing several subtypes of neuronal muscle-type nicotinic acetylcholine receptors[4, 5].

Recent reports showed that ginsenosides administered via oral route might pass into large intestine without decomposition by either gastric juice or digestive enzymes[6-9]. By only intestinal microorganisms, some of PD ginsenosides are metabolized into compound K (CK) and some of PT ginsenosides are metabolized into M4. These ginsenoside metabolites are absorbed into the blood. Therefore, ginsenosides might play a role as pro-drugs and ginsenoside metabolites might mediate the various actions of ginsenosides[6-9].

5-Hydroxytryptamine type 3 (5-HT_3) receptor is one of the superfamily of ligand-gated ion channel receptors that share the structural similarity with other ligand-gated ion channels like nicotinic acetylcholine receptors[10]. This

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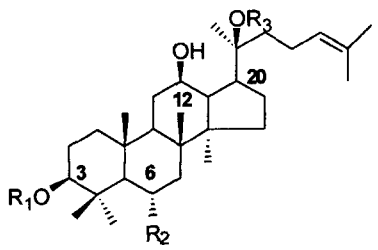
receptor mediates the rapid and transient excitatory synaptic transmission as well as nicotinic acetylcholine receptors. 5-HT_3 receptor is present in both the central and peripheral nervous systems and is involved in physiological and pathological processes that mediate nausea, vomiting and pain perception.[11] In this study, we investigated *in vitro* and *in vivo* studies whether the inhibitory effects ginsenosides on 5-HT_{3A} receptor channel acctivity are also coupled to anti-nausea and anti-vomiting action. *In vitro* study, we investigated the effect of CK and M4, which are ginsenoside metabolites, on human 5-HT_{3A} receptor channel activity expressed in *Xenopus* oocytes using two-electrode voltage clamp technique. *In vivo* experiments, we have investigated the effect of GTS on cisplatin-induced anti-emetic or anti-nausea using cats.[12] We found that M4 inhibited more potently 5-HT-induced inward peak current ($I_{5\text{-}HT}$) than CK with dose-dependent and reversible manner and pre-treatment of GTS antagonized cisplatin-induced retching and vomiting with time-dependent manner.

MATERIALS AND METHODS

Materials

All purified ginsenosides and other ginseng related compounds were obtained from the Korea Ginseng and Tobacco Research Institute (Korea). Fig. 1 shows the structures of the representative ginsenoside CK and M4. Ginsenoside metabolites used in this study were dissolved in dimethyl sulfoxide (DMSO) and were diluted with bath medium before use. Final DMSO concentration was less than 0.01%. Other chemical agents were obtained from Sigma (St. Louis, MO).



Ginsenosides metabolites	R1	R2	R3
CK	-H	-H	-Glc
M4	-H	-OH	-H

Fig. 1. Chemical structure of ginsenoside metabolites. The structure shown is common to ginsenoside metabolites. Side chains for CK are R_1 and R_2, -H and R_3, -Glc. Side chains for M4 are R_1 and R_3, -H and R_2 -OH. Glc is glucopyranoside.

Oocyte preparation

Xenopus laevis care and handling were in accordance with the guide for the *Care and Use of Laboratory Animals* published by NIH, USA. Frogs underwent surgery only twice, separated by at least 3 weeks. To isolate oocytes, frogs were anesthetized with an aerated solution of 3-amino benzoic acid ethyl ester. Oocytes were separated by treatment with collagenase, by gentle shaking for 2 h in $CaCl_2$-free medium containing 82.5 NaCl, 2 mM KCl, 1 mM $MgCl_2$, 5 mM HEPES, 2.5 mM sodium pyruvate, 100 units penicillin/ml, and 100 μg streptomycin/ml. Only stage 5 or 6 oocytes were collected and maintained at 18°C with continuous gentle shaking in ND96 (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, and 5 mM HEPES, pH 7.5) supplemented with 0.5 mM theophylline and 50 μg gentamycin/ml. All solutions were changed every day. All experiments were performed within 2-4 d following isolation of the oocytes.[4]

cRNA preparation of 5-HT_{3A} receptor and microinjection

Recombinant plasmid (Guthrie Research Institute, Sayre, PA) containing human 5-HT_{3A} cDNA insert was linearized by digestion with appropriate restriction enzymes. The cRNAs from linearized templates were obtained by using an *in vitro* transcription kit (mMessage mMachine; Ambion, Austin, TX) with a T7 polymerase. The RNA was dissolved in RNase-free water at 1 μg/μl, divided into aliquots, and stored at -70°C until used. Oocytes were injected with H_2O or human 5-HT_{3A} receptor cRNAs (5-10 ng) by using a Nanoject Automatic Oocyte Injector (Drummond Scientific, Broomall, PA). The injection pipette was pulled from glass capillary tubing used for recording electrodes and the tip was broken to ~20 μm-OD[4].

Oocyte recording

A single oocyte was placed in a small Plexiglas net chamber (0.5 ml) and was constantly superfused with ND96 medium in the absence or presence of 5-HT or ginsenoside metabolites during recording. The microelectrodes were filled with 3 M KCl and had a resistance of 0.2-0.7 MΩ. Two-electrode voltage-clamp recordings were performed at room temperature with Oocyte Clamp (OC-725C, Warner Instrument) with Digidata 1200A. For most of the electrophysiological experiments, the oocytes

were clamped at a holding potential of -80 mV. For current-voltage relationship, voltage ramps were applied from -100 to +60 mV for 300-ms. In different membrane holding potential experiments, oocytes were clamped at the indicated holding potentials[4].

Animals

The experiments were performed on male cats (2.0-3.0 kg) bred at Japan SLC, Inc. Prior to the experiments, they were housed communally at 22 ± 2°C and had free access to food and water. On the day of the experiment, each cat was presented with approximately 200 g of commercially available tinned cat food (Whiskas Effen Foods, Australia) 30 min before being transferred to the laboratory.

Administration of drugs

To facilitate the intravenous administration of drugs, the animals were lightly anaesthetized with 5% halothane (carrier: N_2 80%, O_2 20%) and a temporary cannula was inserted into the cephalic vein. Cisplatin (7.5 mg/kg) was infused intravenously over a 4 min period (time=0 min) followed immediately oral injection by GTS (500 mg/kg)[13]. Control group was injected by vehicle through oral route. The cannula was removed and the animals transferred to individual observation cages for the assessment of emesis. All animals were conscious within 1-5 min of discontinuation of the anaesthetic. Cat food (Feline DietR 5003, PMIR Feeds, USA) and water was available ad libitum. Cisplatin (Sigma, USA) was formulated in saline (0.9% w/v). GTS was administered orally in a volume of 500 mg/kg (p.o). After 1 h cisplatin was administered in a volume of 7.5 mg/kg (i.v.)[12,14].

Measurement of emesis

Animal behavior was recorded remotely and analyzed at the end of the experiment. Emesis was characterized by rhythmic abdominal contractions that were either associated with the oral expulsion of solid or liquid material from the gastrointestinal tract (i.e., vomiting) or not associated with the passage of material (i.e. nausea movements). Episodes of nausea and/or vomiting were considered separately, when the animal changed its location in the observation cage or when the interval between nausea and/or vomits exceeded 5s[12].

Data analysis

To obtain the concentration-response curve for 5-HT-induced current in the presence of CK or M4, the observed peak amplitudes were normalized and plotted and then fitted to the Hill equation below using Origin software (Northampton, MA). $y/y_{max} = [A]^n/([A]^n + [IC_{50}]^n)$, where y, % inhibition at given concentration of CK or M4, y_{max}, maximal % inhibition, IC_{50} is the concentration of CK or M4 producing half-maximum inhibition of the control response to 5-HT, and [A] is the concentration of CK or M4. n is the interaction coefficient. All values are presented as means ± S.E.M. The differences between means of control and CK or M4 treatment data were analyzed using unpaired Students *t* test. A value of $P<0.05$ was considered statistically significant. In each animal, the

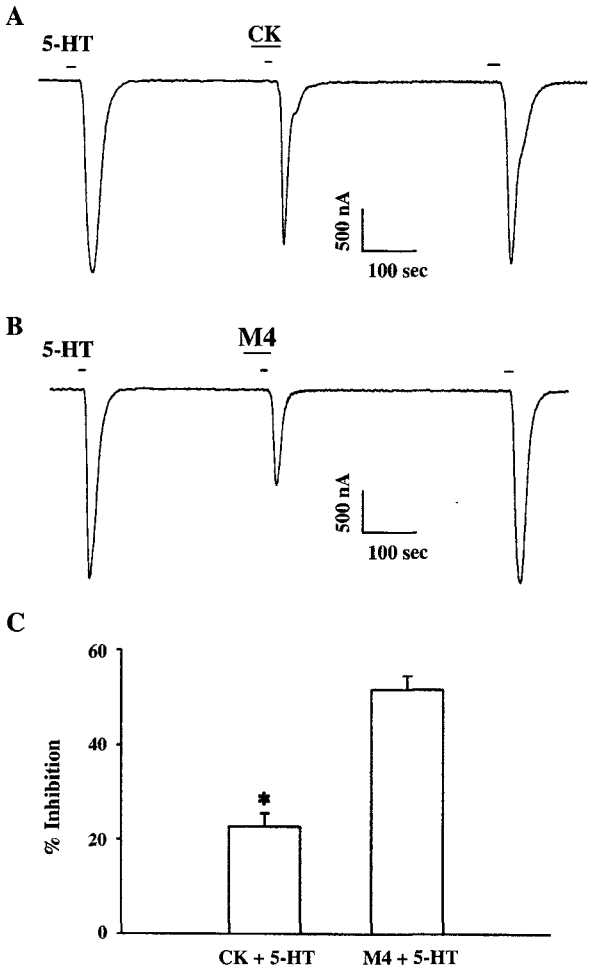


Fig. 2. Effect of ginsenoside metabolites, CK or M4, on $I_{5\text{-}HT}$ in oocytes expressing the 5-HT_{3A} receptor. **A.** 5-HT (10 μM) induced a reversible inward current ($I_{5\text{-}HT}$). The cells were pretreated with CK for 30 s before 5-HT was applied. **B.** The cells were pretreated with M4 for 30s before 5-HT was applied. **C.** Summary of CK- or M4-induced inhibition of $I_{5\text{-}HT}$. Holding potential was -80 mV. Tracings are representative of results with eighteen separate oocytes from three different frogs. *$P < 0.01$ compared with M4 + 5-HT.

latency to nausea or vomiting following the administration of the respective emetogen and the total number episodes of nausea and/or vomiting were calculated for the duration of the experiment. Latency data is expressed as the mean time (min) of only the animals that retched or vomited; all other data is expressed as the mean ± S.E.M.

RESULTS AND DISCUSSION

As shown in Fig. 2, the addition of 10 μM 5-HT to the bathing solution induces a large inward current ($I_{5\text{-}HT}$) in oocytes injected with 5-HT_{3A} receptor cRNA. $I_{5\text{-}HT}$ was blocked by the selective 5-HT_{3A} receptor antagonist, 0.5 μM MDL-72222, and in H_2O-injected control oocytes neither 5-HT nor CK and M4 induced any inward current. CK or M4 (100 μM each) themselves had no effect in oocytes expressing the 5-HT_{3A} receptor at a holding potential of -80 mV (data not shown). Pretreatment for 1 min with CK before 5-HT application caused slight inhibition of $I_{5\text{-}HT}$ in oocytes expressing the 5-HT_{3A} receptor. However, pretreatment for 1 min with M4 caused substantial, reversible inhibition (Figs. 2A and 2B, n = 18 with three different frogs). Thus, after 30 s pretreatment with CK or M4, $I_{5\text{-}HT}$ was inhibited by 22.7 ± 2.8 and 51.7 ± 3.0%, respectively (Fig. 2C, $^*P < 0.01$ compared with M4 + 5-HT). Inhibition by CK and M4 following 30s pretreatment was dose-dependent (Fig. 3A). IC_{50} was 36.9 ± 9.6 μM for M4 (n = 10-12, with three different frogs for each point) (Fig. 3A).

To clarify the mechanism by which M4 inhibits $I_{5\text{-}HT}$, we analyzed the effect of 100 μM M4 on $I_{5\text{-}HT}$ evoked by different 5-HT concentrations (Fig. 3B). Following pretreatment with M4 for 30s the presence of increasing concentrations of 5-HT did not cause any substantial rightward shift of the dose-response curve, although pretreatment with M4 significantly inhibited $I_{5\text{-}HT}$ at 5-HT concentration of 10, 30, and 100 μM (Fig. 3B, $^*P < 0.01$).

In experiments on the current-voltage relationship, the membrane potential was held at -80 mV and a voltage ramp was applied from -100 to +60 mV for 300 ms. In the absence of 5-HT, the inward current at 100 mV was < 0.01 μA and the outward current at +60 mV was about 0.1 μA. The addition of 5-HT to the bathing medium mainly induced an inward current at negative voltages and an outward current at positive voltages. Pretreatment with CK or M4 before the application of 5-HT reduced both inward and outward currents. The reversal potential was near 0 mV both with 5-HT alone and with 5-HT plus CK or M4. This indicates that 5-HT induces a cation

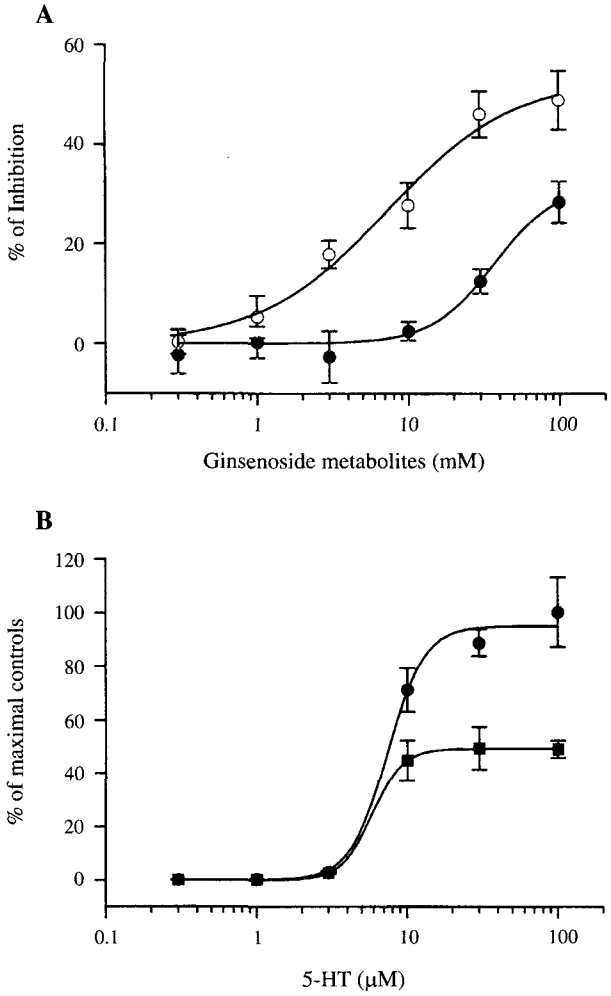


Fig. 3. Dose-dependent effects of CK and M4 on $I_{5\text{-}HT}$. **A.** % Inhibition by CK (●) or M4 (○) of $I_{5\text{-}HT}$ was calculated from the average of the peak inward current elicited by 5-HT and of the peak inward current elicited by 5-HT after pretreatment with CK or M4 for 30s as described in **Materials and Methods**. **B.** Dose-response relationship for 5-HT and 5-HT plus 100 μM M4. $I_{5\text{-}HT}$ was measured with the indicated concentration of 5-HT in the absence (●) or presence (■) of 100 μM M4. Values are means ± S.E.M. (n = 10-12 oocytes).

current[15]. In addition, pretreatment with M4 followed by 5-HT did not affect 5-HT receptor channel behavior because M4 and CK did not alter the reversal potential of the 5-HT_{3A} receptor (Fig. 4A).

The inhibitory effect of M4 on $I_{5\text{-}HT}$ was independent of the membrane holding potential (Fig. 4B). M4 inhibited $I_{5\text{-}HT}$ by 54.1 ± 7.2, 68.3 ± 4.8, 67.1 ± 1.6, and 65.7 ± 3.3% at -80, -60, -40, and -20 mV membrane holding potentials, respectively (n = 10-15, with three different frogs).

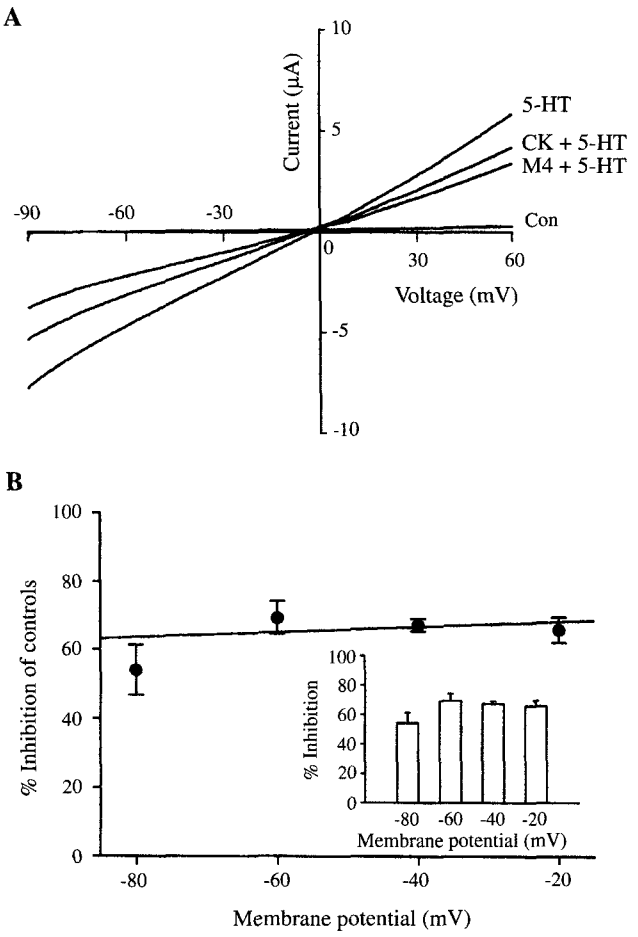


Fig. 4. Voltage-independent inhibition of $I_{5\text{-}HT}$ by M4. **A.** The representative current-voltage relationship was obtained using voltage ramps from -100 and +60 mV of 300-ms duration. Voltage steps were applied before and after application of 10 μM 5-HT in the presence or absence of 100 μM CK or M4. **B.** Summary of the inhibition induced by M4 at the indicated holding membrane potentials. Values are means ± S.E.M. (n = 10-15 oocytes).

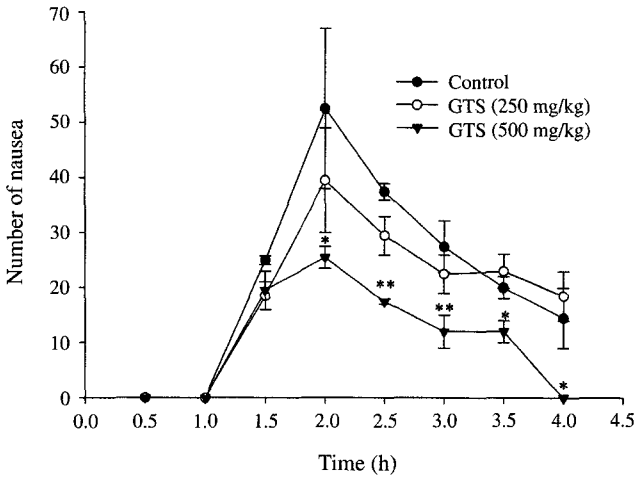


Fig. 5. The effect of administration of GTS on cisplatin-induced nausea. Control animal groups were administered with saline, whereas GTS-treated animal groups were administered with GTS (500 mg/kg, oral). After 60 min cisplatin (7.5 mg/kg, i.v.) was administered to both groups (7.5 mg/kg, i.v.). Number of nausea in the cat induced by a single dose of cisplatin was counted every 30 min intervals during 4 h. Results represent the mean ± S.E.M. (*$p < 0.01$ compared with saline treatment alone, n = 7 - 8).

However, it is not known precisely how CK and M4 inhibit $I_{5\text{-}HT}$. One possibility might be that they are open channel blockers but this is probably not the case since inhibition by M4 was not voltage-dependent whereas inhibition by open channel blockers is strongly voltage-dependent owing to the charge they possess in the trans-membrane electrical field [16-18]. Another possibility is that CK or M4 act as competitive inhibitors by interfering with binding of 5-HT to its binding site(s) on the 5-HT_{3A} receptor. In competition experiments, we observed that the presence of M4 did not significantly shift the dose-response curve (IC_{50}, from 7.3 ± 2.2 to 5.9 ± 0.6 μM and Hill coefficient, from 3.7 to 4.2 (Fig. 3B), suggesting that M4 inhibted $I_{5\text{-}HT}$ in a non-competitive manner. Thus, the voltage-independent and non-competitive effect of M4 on 5-HT_{3A} receptor channel activity together suggest that M4 has its own binding or interaction site(s) on the 5-HT_{3A} receptor. However we do not completely understand why pretreatment with M4 is more inhibitory than with CK. On the other hand, it is known that nausea and vomiting result from activation of 5-HT_{3A} receptors in the central nervous system and that antagonists of the 5-HT_{3A} receptor such as ondansetron block nausea or vomiting induced by anticancer agents such as cisplatin[19-21]. Our findings that CK and M4, which is a metabolite of PT ginsenosides, inhibit $I_{5\text{-}HT}$ show the possibility that PT ginsenosides and their metabolites such as M4 could be used as natural agents against *in vivo* nausea and vomiting.

To test this possibility we investigated *in vivo* effect of GTS on cisplatin-induced emesis in cats. In this study, administration of cisplatin (5 mg/kg, i.v.) did not induce vomiting and nausea (data not shown). When we increased the dose of cisplatin (7.5 mg/kg), we could observe cisplatin-induced emesis including nausea and vomiting[22]. As shown in Figures 5-7, cisplatin-induced nausea and vomiting episodes appeared after 60 min, reached to peak after 2 h, and persisted until 4 h. As a

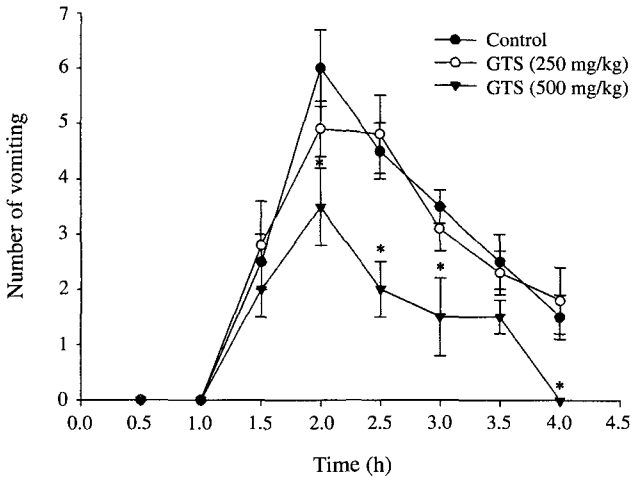


Fig. 6. The effect of administration of GTS on cisplatin-induced vomiting. Control animal groups were administered with saline, whereas GTS-treated animal groups were administered with GTS (500 mg/kg, oral). After 60 min cisplatin (7.5 mg/kg, i.v.) was administered to both groups (7.5 mg/kg, i.v.). Number of vomiting in the cat induced by a single dose of cisplatin was counted every 30 min intervals during 4 h. Results represent the mean ± S.E.M. (*$p < 0.01$ compared with saline treatment alone, n = 7 - 8).

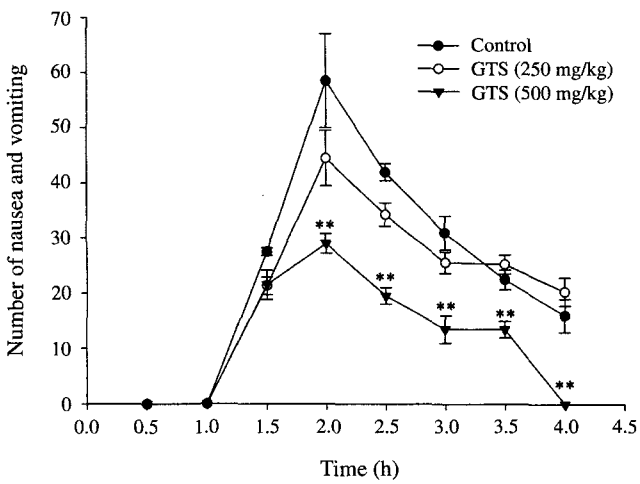


Fig. 7. The effect of administration of GTS on cisplatin-induced nausea and vomiting. Control animal groups were administered with saline, whereas GTS-treated animal groups were administered with GTS (500 mg/kg, oral). After 60 min cisplatin (7.5 mg/kg, i.v.) was administered to both groups (7.5 mg/kg, i.v.). Number of nausea and vomting in the cat induced by a single dose of cisplatin was counted every 30 min intervals during 4 h. Results represent the mean ± S.E.M. (*$p < 0.01$ compared with saline treatment alone, n = 7 - 8).

next step, we tested the effect of GTS on cisplatin-induced emesis. When we pre-administered GTS into cat with less than 250 mg/kg via oral route, we could not observe significant inhibitory effect of GTS on cisplatin (7.5 mg/kg, i.v.)-induced emesis (data not shown). On the other hand, the oral administration of GTS (500 mg/kg) significantly blocked cisplatin (7.5 mg/kg)-induced emesis in both nausea and vomiting (Figs. 5-7). Thus, pre-treatment of GTS (500 mg/kg, p.o.) significantly reduced cisplatin-induced nausea and vomiting by 51 ± 8.4 and 48.8 ± 6.4% during 4 h compared to GTS-untreated group, respectively. However, when we increased GTS concentrations more, we could observe a slight side-effect of GTS itself such as salivations in cat, although GTS attenuated cisplatin (7.5 mg/kg)-induced emesis in both nausea and vomiting and we did not use higher concentration of GTS than 500 mg/kg (data not shown). These results indicates that GTS attenuates cisplatin-induced both nausea and vomiting with dose- and time-dependent manners.

In present study, we performed *in vitro* and *in vivo* studies to know whether ginsenosides obtained from Korean Red Ginseng have anti-emetic activity in cats. We found that one of ginsenoside metabolites, M4, was more potent than CK, another metabolite of ginsenosides *in vitro* inhibition of 5-HT_{3A} receptor channel activity. Furthermore, we could also observe that oral administration of GTS attenuated cisplatin-induced nausea and vomiting in cat by relatively high concentration. It might be questioned how *in vitro* inhibition of 5-HT_{3A} receptor channel activity by ginsenosides could be coupled with *in vivo* inhibition of cisplatin-induced emesis. There might be two possible explanations for this argument. One possibility is that some of orally administered GTS might be directly absorbed into plasma and penetrated into blood-brain barrier and reached to the central nervous system, and finally attenuated cisplatin-mediated emesis by blocking 5-HT_{3A} receptor channel activity as shown in the previous report[23]. The other possibility is that GTS might be undergone metabolism process by microorganism in intestines and their metabolites such as CK or M4 are absorbed into plasma and inhibit 5-HT_{3A} receptor channel activity after penetration of blood-brain-barrier. At present, it is not clear how GTS could affect the anti-emetic action. But ginsenoside metabolites inhibit 5-HT_3 receptor channel activity expressed in *Xenopus* oocytes, suggesting that the anti-emetic potential by GTS is mediated by inhibition of 5-HT_3 receptor channel activity. This action may be also relevant to the *in vivo* anti-emetic effects observed in the present study. However, it is not

yet determined exactly whether ginsenosides and their metabolites could penetrate blood-brain barrier and further studies will be needed to clarify the role of blood-brain barrier in ginsenoside-induced anti-emetic efficacy.

In summary, we show the possibility that *in vitro* inhibition of 5-HT_{3A} receptor channel activity by ginsenosides and their metabolites might be coupled to *in vivo* anti-emetic activity.

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