Probable Involvement of the 5-Hydroxytryptamine₄ Receptor in Methotrexate-Induced Delayed Emesis in Dogs

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ABSTRACT

Delayed emesis in cancer patients undergoing chemotherapy remains a significant problem. The pathogenesis of delayed emesis is still obscure. It was recently demonstrated that methotrexate (MTX), an anticancer drug, evoked delayed emesis in dogs in a manner similar to its actions in humans. We evaluated the antiemetic activity of FK1052, a potent antagonist for both the 5-hydroxytryptamine (HT)₃ and 5-HT₄ receptors, on delayed emesis induced by MTX in beagle dogs. Animal behavior was recorded for 3 days using a video camera. Delayed emesis lasting up to 72 h was observed in dogs treated with MTX (2.5 mg/kg i.v.), but acute emesis did not occur. The following antiemetics, at the dose that prevents cisplatin-induced acute emesis in dogs, were administered i.v. as multiple injections every 12 h during days 2 to 3. FK1052 (1 and 3.2 mg/kg) significantly reduced the emetic episodes caused by MTX, whereas ondansetron (1 mg/kg), a selective 5-HT₃ receptor antagonist, was not effective. The emetic episodes induced by MTX were also inhibited by another 5-HT₃ receptor antagonist, tropisetron (1 mg/kg). CP-122,721 (0.1 mg/kg), a potent selective tachykinin NK₁ receptor antagonist, significantly reduced the emetic responses to MTX. Copper sulfate-induced emesis in dogs was also prevented by FK1052, tropisetron, and CP-122,721 but not by ondansetron. FK1052, tropisetron, and ondansetron had negligible affinity for the NK₁ receptor at 1 μM. These results suggest that the 5-HT₄ receptor may be in part involved in the production of delayed emesis induced by MTX in dogs and that FK1052 may be a useful drug against both acute and delayed emesis induced by cancer chemotherapy.

Nausea and vomiting are the most common distressing side effects of cancer chemotherapy. Acute emesis, occurring within the first 24 h of chemotherapy, is dramatically prevented by ondansetron, a selective 5-hydroxytryptamine (5-HT)₃ receptor antagonist (Marty et al., 1990). In contrast to acute emesis, delayed emesis, which occurs 24 h or later after the start of chemotherapy, is poorly controlled with antiemetic regimens, including the 5-HT₃ receptor antagonists (Goedhals et al., 1998; Kris et al., 1992). For some individuals, the severity of symptoms is great enough to cause discontinuation of further chemotherapy (Laszlo, 1983). Animal models of chemotherapy-induced acute emesis successfully predicted the clinical efficacy of the 5-HT₃ receptor antagonists for the control of vomiting. Indeed, the cisplatin-induced acute emesis model in the dog and ferret has been extensively used to identify the antiemetic potential of novel drug therapies (as reviewed by Kilpatrick et al., 1990). Although the role of central 5-HT₃ receptors in the induction of cisplatin-induced acute emesis is controversial (Andrews et al., 1990; Fukui et al., 1992; Gidda et al., 1995), it seems probable that mechanisms of action of 5-HT₃ receptor antagonists are complex and primarily involve inhibitory interaction with 5-HT₃ receptors present in both the peripheral and central sites (Karim et al., 1996). In contrast to the case of acute emesis, advancement in the understanding of the mechanisms activated during delayed emesis is limited by ethical considerations in humans and an absence of suitable animal models. Two animal models for chemotherapy-induced delayed emesis have recently been developed. Cisplatin, at a lower dose than that for the screening of acute emesis, induced a less intense emetic response that declined after 16 h but reappeared at approximately 32 h to reveal a delayed emesis in the ferret (Rudd et al., 1994). In the piglet, cisplatin also induced a biphasic pattern of vomiting consisting of an acute phase and a delayed phase (Milano et al., 1995). However, the cisplatin-induced delayed emesis in both ferrets and piglets was significantly prevented by selective 5-HT₄ receptor antagonists such as ondansetron and granisetron (Rudd and Naylor, 1994; Grelot et al., 1996). Thus, it is unlikely that these animal models accurately represent the clinical profile of delayed emesis as mentioned. In a recent report, Fukui and Yamamoto (1999) showed that methotrex-
ate (MTX), an anticancer drug, could induce delayed emesis in the dog and that this effect was in part inhibited by ondansetron. It is suggested that MTX-induced delayed emesis in dogs may be a useful model for studies on the mechanisms of delayed emesis induced by chemotherapy in humans.

FK1052, a 5-HT₃ receptor antagonist, exhibited a potent antiemetic action against cisplatin-induced vomiting in dogs (Yamakuni et al., 1992). FK1052 is not a selective 5-HT₃ receptor antagonist, but the actions of this compound on intestinal function apparently differed from those of ondansetron and granisetron (Kadowaki et al., 1993; Nagakura et al., 1993). Hence, the present study was designed to investigate the antiemetic efficacy of FK1052 against MTX-induced delayed emesis in dogs and to characterize the delayed emesis by MTX using other antiemetic agents.

**Materials and Methods**

**Animals.** Beagle dogs of either sex weighing 8.0 to 18.5 kg were used in the study. The dogs were individually housed in an animal room under standard controlled environmental conditions. In all experiments, dogs were removed from their home cages and transferred to observation cages in a quiet room. In some cases, animals received the emetics twice at the intervals mentioned later, using a different antiemetic.

**Cisplatin-Induced Acute Emesis in Dogs.** After the administration of cisplatin (3.2 mg/kg/ml), animals were observed continuously for 5 h, and the incidences of emesis were counted. The presence of vomiting separated from the next bout by at least 1 min was considered as a single emetic episode. FK1052 (1 mg/kg), ondansetron (1 mg/kg), tropisetron (1 mg/kg), CP-122,721 (0.1 mg/kg), or vehicle (0.5 ml/kg) was administered i.v. 10 min before the injection of cisplatin. A few dogs without obvious toxicity were dosed twice with cisplatin with a rest period of at least 4 weeks between the two doses.

**MTX-Induced Delayed Emesis in Dogs.** Dogs were injected i.v. with MTX (2.5 mg/kg/ml) at 7:30 AM. The animal behavior was recorded using a video camera with an automatic night photographing system for up to 72 h and analyzed at the end of the experiment. FK1052 (1 and 3.2 mg/kg), ondansetron (1 mg/kg), tropisetron (1 mg/kg), CP-122,721 (0.1 mg/kg), or vehicle (0.5 ml/kg) was administered i.v. 24, 36, 48, and 60 h after MTX treatment. Episodes of emesis occurring within a few minutes were defined as a single emetic episode. A 12-h artificial light cycle (lights on between 7:30 AM and 7:30 PM) was used throughout the study. Dogs were given a standard laboratory dog chow (300 g/day) and water ad libitum. The animals were retested with MTX at least 6 weeks later.

**Copper Sulfate-Induced Emesis in Dogs.** Dogs were deprived of food overnight. Copper sulfate solution (20 mg/kg/ml) was rapidly flushed into the stomach via an orogastric tube. Animal behavior was observed for 6 h. FK1052 (1 mg/kg, 3.2 mg/kg, ondansetron (1 mg/kg), tropisetron (1 mg/kg), CP-122,721 (0.1 mg/kg), or vehicle (0.5 ml/kg) was administered i.v. 10 min before the administration of copper sulfate. Two weeks later, the animals were retested with copper sulfate.

**NK₁ Receptor Binding.** Chinese hamster ovary (CHO) cells stably transfected with the human tachykinin NK₁ receptor were kindly provided by Prof. Nakashima (Kyoto University, Japan). The CHO cells were harvested and homogenized with a Dounce homogenizer at 4°C in a buffer [0.25 M sucrose, 25 mM Tris-HCl, pH 7.4, 10 mM MgCl₂, 5 μg/ml p-amidinophenyl methanesulfonfyl fluoride HCl (p-APMSF), and 1 mM EDTA]. The homogenate was centrifuged (500g, 10 min), and the pellet was resuspended in the same buffer, homogenized, and centrifuged. The two supernatants were combined and centrifuged (100,000g, 1 h). The crude cell membranes thus isolated were resuspended in buffer (25 mM Tris-HCl, pH 7.4, 10 mM MgCl₂, 5 μg/ml p-APMSF, and 1 mM EDTA) and stored at −80°C until use. Cell membranes (6 μg/ml) were incubated with 125I-BH-Substance P (0.1 nM; New England Nuclear, Boston, MA) in the absence and presence of test compounds in 0.25 ml of medium (50 mM Tris-HCl, pH 7.4, 5 mM MnCl₂, 20 μg/ml chymostatin, 40 μg/ml bacitracin, 4 μg/ml leupeptin, 5 μg/ml p-APMSF, and 200 μg/ml BSA) for 90 min at room temperature. At the end of the incubation period, the contents were quickly filtered over a Blue Mat 11740 filter (Skatron; Sterling) that had been presoaked with 0.1% polyethyleneimine using a cell harvester. The filter was then washed with buffer (50 mM Tris-HCl, pH 7.4, and 5 mM MnCl₂). The radioactivity was counted by using an auto γ-counter. Nonspecific binding was determined using excess unlabeled Substance P (3 μM). Experiments were carried out in duplicate.

**Drugs.** Cisplatin (Sigma Chemical Co., St. Louis, MO) was prepared in normal saline at 70°C followed by gradual cooling to 40°C and administered immediately. MTX (Takeda Chemical, Osaka, Japan) was dissolved in 5% glucose solution. Copper sulfate pentahydrate (Wako Pure Chemicals, Osaka, Japan) was dissolved in distilled water. Tropisetron was purchased from Research Biochemicals Inc. (Natick, MA). FK1052 ([+]-8,9-dihydro-10-methyl-7-(5-methyl-1H-imidazol-4-yl)methyl)pyridin[1,2-α][1,3]indol-6(7H)-one hydrochloride, ondansetron, and CP-122,721 ([+]-2S,3S)-3-(2-methoxy-5-trifluoromethoxybenzyl)amino-2-phenylpiperidine) were synthesized at the Medicinal Chemistry Laboratories of Fujisawa Pharmaceutical Co. (Osaka, Japan). They were freshly dissolved in 5% glucose solution for in vivo experiments and in DMSO for in vitro experiments.

**Statistical Analysis.** Group results are expressed as mean ± S.E. Dunnett’s test was used as a measure of significance. Values of P < .05 were regarded as statistically significant.

**Results**

**Cisplatin-Induced Acute Emesis in Dogs.** Antiemetics were compared for their ability to prevent cisplatin-induced emesis after i.v. administration to beagle dogs. All four antiemetics at the dose used in this model, as shown in Table 1, significantly antagonized cisplatin-induced acute emesis. Pretreatment with FK1052 (1 mg/kg i.v.) and CP-122,721 (0.1 mg/kg i.v.) produced an increase in the latency of the emetic response to cisplatin but failed to reach statistical significance. The latency period was significantly prolonged by ondansetron (1 mg/kg i.v.) and tropisetron (1 mg/kg i.v.), and the former and the latter completely prevented emesis in two of three and one of three dogs, respectively.

**MTX-Induced Delayed Emesis in Dogs.** The pattern of emesis induced by MTX in dogs is shown in Fig. 1. Intravenously administered MTX (2.5 mg/kg) caused emesis in all 5% glucose-treated animals, with a latency to vomit of 35.3 ± 3.7 h lasting up to 72 h; however, acute emesis within 24 h

<table>
<thead>
<tr>
<th>TABLE 1</th>
<th>Effects of antiemetics on cisplatin-induced acute emesis in the dog</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Treatment</strong></td>
<td><strong>Dose</strong></td>
</tr>
<tr>
<td>mg/kg</td>
<td>i.v.</td>
</tr>
<tr>
<td>Control</td>
<td>6/6</td>
</tr>
<tr>
<td>FK1052</td>
<td>1</td>
</tr>
<tr>
<td>Ondansetron</td>
<td>1</td>
</tr>
<tr>
<td>Tropisetron</td>
<td>1</td>
</tr>
<tr>
<td>CP-122,721</td>
<td>0.1</td>
</tr>
</tbody>
</table>

*a* Antiemetics were administered i.v. 10 min before the injection of cisplatin. 

*If a dog did not vomit, the latency period was taken to be equal to the observation period (300 min).* 

Compared with the control, *P < .05, **P < .01.*
after the injection of MTX did not occur. In these animals, the total number of vomits and emetic episodes were 36.8 ± 1.4 and 17.0 ± 2.0, respectively. The following antiemetics at the dose that significantly prevented cisplatin-induced acute emesis in dogs as mentioned were administered i.v. as multiple injections every 12 h during days 2 to 3. FK1052 (1 mg/kg i.v. ×4) apparently reduced delayed emesis caused by MTX and increased, but not significantly, the time for onset of emesis (Table 2 and Fig. 2a). Furthermore, increasing the dose to 3.2 mg/kg of FK1052 also significantly inhibited the number of the emetic episodes induced by MTX, of which the action was more effective than the treatment with FK1052 at 1 mg/kg (Table 2). Tropisetron (1 mg/kg i.v. ×4) also dramatically inhibited MTX-induced emesis, but this compound failed to affect the latency to vomit (Table 2 and Fig. 2b). Emetic responses to MTX, both latency and emetic episodes, were unaffected by ondansetron (1 mg/kg i.v. ×4) (Table 2 and Fig. 2c). CP-122,721 (0.1 mg/kg i.v. ×4) produced a significant increase in the latency to the first vomiting and reduced significantly the emetic episodes by 78% (Table 2 and Fig. 2d).

All dogs treated with MTX showed a gradual decrease in feeding, and most of them showed heavy anorexia by day 3. The body weight of dogs treated with MTX decreased by about 15% of their weight during the 3 days; their food intake and body weight returned to normal within 1 week and 1 month after the end of experiments, respectively. The frequency of diarrhea gradually increased in most of the dogs during the 3 days, regardless of treatment with or without antiemetics.

**Discussion**

A previous study by Fukui and Yamamoto (1999) showed that i.v. MTX at 2.5 mg/kg, a nonlethal dose, caused delayed emesis in dogs. In this study, we confirmed that MTX evoked delayed emesis lasting up to 72 h, despite its failure to cause acute emesis. MTX is classified as a low-risk emetogenic agent, in contrast to cisplatin, which has the highest potential for inducing emetic responses in humans (Borison and McCarthy, 1983). Most studies have used cisplatin as the anticancer agent of choice in their models for the induction of emesis. It is generally accepted that stimulation of the abdominal vagal afferent nerves via the 5-HT₃ receptor is important to trigger acute emesis induced by cisplatin and other antineoplastic agents (Andrews et al., 1990). Interestingly, it is unlikely that MTX provokes its emetic reflex through the pathway associated with the 5-HT₃ receptor that is activated by cisplatin, because repeated administration of ondansetron (1 mg/kg) at the dose that strongly antagonized cisplatin-induced acute emesis in dogs failed to inhibit MTX-induced emesis in this study. Thus, it seems that the MTX model with dogs may be a suitable model in which to study the pathogenesis of delayed emesis induced by chemotherapy in humans compared with the models in ferrets and piglets that were sensitive to 5-HT₃ receptor antagonists (Rudd and Naylor, 1994; Grelot et al., 1996). The reason why MTX failed to cause emesis within the first 24-h period is unknown, but it may have been in part due to a species difference.

The present study demonstrated that MTX-induced delayed emesis was significantly reduced by FK1052 (1 and 3.2 mg/kg) and tropisetron (1 mg/kg) at the dose that apparently prevented cisplatin-induced emesis in dogs. Furthermore, these drugs at the same dose reduced emetic episodes induced by copper sulfate but failed to reach statistical significance. It has come to be accepted that peripheral 5-HT₃ receptors play an important role in copper sulfate-induced emesis (Bhandari and Andrews, 1991; Fukui et al., 1994). FK1052 has been reported to be an antagonist for the 5-HT₃ receptor in addition to the 5-HT₃ receptor both in vitro and in vivo (Kadowaki et al., 1993; Nagakura et al., 1993). FK1052 inhibited the 5-methoxytryptamine (5-MeOT; a 5-HT₃ receptor agonist)-induced contractions of guinea pig isolated ileum in the presence of a high concentration of ondansetron; on the other hand, granisetron was not effective (Nagakura et al., 1993). Kadowaki et al. (1993) reported that FK1052 completely suppressed 5-MeOT-induced diarrhea in mice. 5-HT₃-induced diarrhea in mice was also completely prevented by FK1052, whereas the inhibition produced by ondansetron and granisetron was only about 70% (Kadowaki et al., 1993).
The mechanism of action of FK1052 on intestinal secretory response to 5-HT agonists is consistent with the finding that the combination of a selective 5-HT₃ receptor antagonist YM060 with SB204070, a selective 5-HT₄ receptor antagonist, inhibited 5-HT-induced diarrhea in mice more effectively than either drug alone (Nagakura et al., 1997). Because it was demonstrated that 5-HT₃ and 5-HT₄ receptors are located in the enteric nervous system (Craig and Clarke, 1990; Kilbinger and Wolf, 1992), it is suggested that FK1052, with both 5-HT₃ and 5-HT₄ receptor antagonist activity on gastrointestinal motor activity and emesis induced by copper sulfate, is more potent and effective than ondansetron and granisetron. Tropisetron also showed 5-HT₄ receptor antagonistic action both in vitro and in vivo (Dumuis et al., 1988; Villalon et al., 1990). Furthermore, previous studies have demonstrated that copper sulfate-induced emesis in ferrets and dogs is abolished by tropisetron but not by other 5-HT₃ receptor antagonists, such as ondansetron, granisetron, and MDL 72222 (Bhandari and Andrews, 1991; Fukui et al., 1994). Thus, these findings suggest that both FK1052 and tropisetron at the dose used in this study may in part reduce emetic episodes induced by MTX through the blockade of at least the 5-HT₄ receptors. MTX-induced delayed emesis in dogs was not affected by abdominal vagotomy or greater splanchnic nerve section (Fukui and Yamamoto, 1999), although vomiting induced by copper sulfate and 5-MeOT was significantly prevented by abdominal visceral nerve section (Fukui et al., 1994). These findings indicate that the abdominal visceral afferent fibers fail to participate in emetic responses evoked by MTX in dogs, suggesting that the antiemetic site of action of FK1052 and tropisetron against MTX-induced delayed emesis lies

**TABLE 3**

Effects of antiemetics on copper sulfate-induced delayed emesis in the dog

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dosea</th>
<th>Animals</th>
<th>Latencyb</th>
<th>Emetic Episodes</th>
<th>Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>3/3</td>
<td>7.7 ± 1.8</td>
<td>10.7 ± 4.4</td>
<td></td>
</tr>
<tr>
<td>FK1052</td>
<td>1</td>
<td>3/3</td>
<td>7.4 ± 1.6</td>
<td>5.3 ± 2.8</td>
<td>50.5</td>
</tr>
<tr>
<td>Ondansetron</td>
<td>3.2</td>
<td>3/3</td>
<td>10.4 ± 3.7</td>
<td>2.3 ± 0.3</td>
<td>78.5</td>
</tr>
<tr>
<td>Tropisetron</td>
<td>1</td>
<td>3/3</td>
<td>8.6 ± 3.2</td>
<td>8.0 ± 2.0</td>
<td>25.2</td>
</tr>
<tr>
<td>CP-122,721</td>
<td>0.1</td>
<td>3/3</td>
<td>6.8 ± 1.5</td>
<td>6.3 ± 1.5</td>
<td>40.2</td>
</tr>
</tbody>
</table>

a Antiemetics were administered i.v. 10 min before the administration of copper sulfate.
within the brain. Indeed, it is well known that tropisetron crosses the blood-brain barrier, and FK1052 is also reported to readily penetrate the blood-brain barrier (unpublished data). 5-HT₄ receptors have been reported to be in the central nervous system (CNS), as well as the peripheral tissues mentioned earlier. The 5-HT₄ receptor was first characterized in the CNS as a 5-HT receptor that was positively coupled to adenylate cyclase and that displayed an unusual pharmacological profile (Dumuis et al., 1988; Bockaert et al., 1992). The expression of 5-HT₃ receptor mRNA and the existence of 5-HT₃ receptor binding sites in the brain have also been shown (Jakeman et al., 1994; Vilaro et al., 1996). Although the pathogenesis of delayed emesis remains poorly understood in comparison with the improved understanding of the pathophysiology of acute emesis, our study is the first to suggest the possibility that the 5-HT₁ receptor, probably in the CNS, may be in part involved in the production of delayed emesis induced by chemotherapy.

NK₁ receptor antagonists that penetrate the blood-brain barrier exhibited potent antiepileptic properties against a wide variety of emetic stimuli (Gardner et al., 1995; Watson et al., 1995; Tattersall et al., 1996). Several studies suggest that the site of the antiepileptic action of NK₁ receptor antagonists is located in the nucleus of the solitary tract (Gardner et al., 1994; Watson et al., 1995; Tattersall et al., 1996). On the other hand, a recent study suggests that NK₁ receptors that mediate the retching response are in the vicinity of the nucleus ambiguous but not the nucleus of the solitary tract (Fukuda et al., 1999). Gardner et al. (1994) demonstrated that the injection of Substance P into the hindbrain induced an emetic reflex in the ferret. Therefore, the central localization of NK₁ receptors in areas of the brain known to be associated with emetic reflex indicates that the antiepileptic activity of NK₁ receptor antagonists is centrally mediated.

CP-122,721 is a potent and selective NK₁ receptor antagonist that crosses the blood-brain barrier, suppresses vomiting caused by various emetic stimuli (Gonsalves et al., 1996; McLean et al., 1996), and prevents delayed emesis in patients receiving cisplatin (Kris et al., 1997). CP-122,721 (0.1 mg/kg) at the dose that prevented cisplatin- and copper sulfate-induced emesis significantly inhibited MTX-induced delayed emesis in dogs. Although the mechanism of action for delayed emesis induced by MTX is unclear, Substance P may exert a critical role in the MTX-induced emetic reflex pathway similar to other emetogens. Because FK1052 and tropisetron had negligible affinity for the NK₁ receptor, their antiepileptic activity against MTX-induced delayed emesis is unlikely to be due to their antagonism for NK₁ receptors.

In conclusion, the present study suggests that the 5-HT₄ receptor, not to mention the NK₁ receptor, may be in part involved in the incidence of delayed emesis evoked by MTX and that dual antagonists for 5-HT₃ and 5-HT₄ receptors, such as FK1052, may be useful against both acute and delayed emesis induced by cancer chemotherapy. Further studies will investigate the sites of 5-HT₄ receptors associated with the production of MTX-induced emesis using selective 5-HT₄ receptor antagonists with or without the ability to penetrate the blood-brain barrier.

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