A Dunnione Compound MB12662 Improves Cisplatin-Induced Tissue Injury and Emesis

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Abstract

The present study was aimed to investigate the effects of MB12662, a synthetic dunnione compound, on cisplatin-induced vomiting reflexes and intestinal, renal, immune system, and hematopoietic toxicities in ferrets and mice, respectively. Male ICR mice were orally administered MB12662 (5, 10, 25 or 50 mg/kg) for 10 days, during which intraperitoneally challenged with cisplatin (3.5 mg/kg) from day 4 to 7, and sacrificed on day 10 for the pathological examination. Male ferrets were orally administered MB12662 (25, 50 or 100 mg/kg) for 7 days, subcutaneously challenged with cisplatin (5 mg/kg), and monitored for vomiting reflexes and survival of the animals. Four-day injection of cisplatin (3.5 mg/kg) to mice caused body weight loss and degeneration and atrophy of intestinal villi, reducing villi/crypt ratio to a half level of control animals. Cisplatin also induced renal and hepatic toxicities, and depletion of splenocytes and bone marrow progenitor cells. The systemic toxicities including decreased villi/crypt ratio, immune system atrophy, splenocyte depletion, and decreased cellularity in bone marrow were improved by MB12662. Cisplatin (5 mg/kg) induced retching and emetic responses of ferrets, which were remarkably attenuated by MB12662 in a dose-dependent manner. All the ferrets pretreated with MB12662 survived the challenge of cisplatin, in comparison with 40% mortality in vehicle-treated animals, and blood parameters of nephrotoxicity and hepatotoxicity were markedly recovered. It is expected that MB12662 could be a candidate for the body protection against burden, including emesis, of chemotherapeutic agents.

Key Words: Cisplatin, Emesis, Intestinal injury, Nephrotoxicity, Dunnione, MB12662

INTRODUCTION

Anti-cancer chemotherapy and radiotherapy cause cytotoxicity of cancer cells as well as normal cells in the immune system, bone marrow, kidneys, and liver (Talmadge et al., 1994; Jo et al., 2000; Kobayashi et al., 2006; Lee et al., 2008) by affecting the DNA-synthesis process of proliferating cells (Farrell et al., 1998; De Martinis and Bianchi, 2001; Gibson et al., 2002). Bone marrow toxicity induces hematopoietic dysfunction and anemia, immunotoxicity causes immune dysfunction, and intestinal and renal injuries lead to malnutrition, diarrhea, and dehydration (Talmadge et al., 1994; Casciniu, 1995; Ku et al., 1997; Husain et al., 1998; Kobayashi et al., 2006; Lee et al., 2008). Chemotherapeutic agents including cisplatin, cyclophosphamide, and doxorubicin also induce vomiting reflexes by damaging the intestinal mucosa (Husain et al., 1998; De Martinis and Bianchi, 2001; Jeong et al., 2005; Rudd et al., 2006; Yarnakuni et al., 2006; Lee et al., 2008; Warr, 2008). Nausea and emesis are one of the most-distressing adverse-effects that limit optimal dosage of chemotherapeutic agents, and thereby reduce therapeutic efficacy. The emetic problems are mainly mediated by serotonin type 3 (5-hydroxytryptamine 3, 5-HT3) receptors (Andrews et al., 1988; Tyers, 1991). Serotonin released from enterochromaffin cells following mucosal injury activates vagus and sympathetic afferent neurons as well as chemoreceptor trigger zone in postrema (Andrews et al., 1990; Minami et al., 1997) which induce reflex of vomiting.

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center in the medulla (Rudd et al., 1994; Rudd et al., 1996).

It was reported that selective 5-HT3 receptor antagonists including ondansetron, granisetron, and tropisetron substantially alleviate nausea and emesis in animals (Rudd et al., 1996) and humans (Andrews et al., 1990; de Brujin, 1993; Morrow et al., 1995; Mantovani et al., 1996). However, they are effective for the elimination of early-phase vomiting reflexes, and have relatively-short duration, requiring repeated administration for enough effectiveness (Jeong et al., 2005).

Renal and hepatic toxicities of cisplatin were found to be attenuated by anti-oxidants and anti-inflammatory compounds (Husain et al., 1998; Lee et al., 2008). We also reported that ginseng intestinal metabolite-I attenuated the testicular toxicity of doxorubicin (Kang et al., 2002). Recently, the anti-emetic and gastroprotective effects of ginger were reviewed to establish clinical effectiveness (Haniadka et al., 2013).

As cell survival factors, NAD+/NADH regulators have been emerged, since sirtuin enzymes are NAD+-dependent in regulation of cell defense system against stress, apoptosis, and inflammation (Yang and Sauve, 2006). DNA-damaging agents such as radicals and chemotherapeutics activate poly (ADP-ribose) polymerase (PARP), leading to consumption of NAD+ (Yang and Sauve, 2006; Chen et al., 2013; Kauppinen et al., 2015). However, excessive activation of PARP depletes NAD+ for glyceraldehyde-3-phosphate dehydrogenase in glycolytic pathway, resulting in cell death due to ATP depletion (Sheline et al., 2003). Accordingly, it was suggested that NAD+ enhancers could be a promising candidate for neuroprotection in neurodegenerative diseases including stroke and Alzheimer's disease (Bedalov and Simon, 2004; Ying, 2008).

Recently, we have synthesized dunniones and β-lapachones, the anti-fungal orange-red pigments of Streptocarpus dunnii Mast (Khambay et al., 2003). The synthetic β-lapachone and dunnione greatly increased NAD+ via NADH oxidation (Hwang et al., 2009; Lee et al., 2012), alleviated cyanide cytotoxicity of preoligodendrocytes (unpublished results), and improved alcohol- and stress-induced gastric ulcers (Park et al., 2011; Jo et al., 2013). Such results led us to investigate the effects of MB12662, a dunnione compound, on damages of target tissues including intestinal, immune, and hematopoietic systems in mice as well as vomiting reflexes in ferrets following cisplatin administration.

MATTERIAls AND METHODS

Materials

A dunnione compound (2,3,3-trimethyl-2,3-dihydronaphtho[1,2-β]furan-4,5-dione; Mazence code No.: MB12662) was synthesized in Mazence Co., Ltd. (Suwon, Korea), and stored at 4°C until use. MB12662 was homogeneously dispersed in 1% carboxymethylcellulose (CMC), and orally administered. Control animals were given the same volume of the vehicle.

Animals

Five-week-old male mice (23-24 g) and 6-month-old male ferrets (1.5-1.6 kg) were obtained from the Marshall Co. (North Rose, USA), and acclimated to the housing environment for 1 week. The animals were housed in mouse or ferret cages in each room with temperature of 23 ± 2°C, relative humidity of 55 ± 5%, a 12-hour light/dark cycle of 150-300 lux, and Purina Rat Chow® and water available ad libitum. The experiments were conducted according to the ‘Standard Operation Procedures’ of Laboratory Animal Research Center, Chungbuk National University, Korea, and the protocol was approved by the Institutional Animal Care and Use Committee of the Center.

Dose-range-finding of cisplatin in mice

Based on information from references and a preliminary study, the dose of cisplatin was set at 3.5, 5 and 6.5 mg/kg, and administered according to the recipe of the manufacturer (Yuhan Co., Gunpo, Korea). Cisplatin was intraperitoneally injected at 10:00-11:00 for 4 days.

Mice were examined for clinical signs and mortality at 10:00 everyday morning, and weighed prior to cisplatin challenge. The animals were sacrificed on day 14 (10 days after the final injection of cisplatin). Small intestine was removed and fixed in neutral formalin solution. Paraffin-embedded tissue slides were stained with hematoxylin-eosin, and examined under a light microscope for the tissue injuries. To quantify the degree of intestinal damage, ratio of villi length/crypt depth was analyzed. In brief, the height of the villi and the depth of the crypts in the small intestine were measured by digital morphometry, at ×100 magnification: i.e., at least 6 villi and 6 crypts per slide sample were analyzed with Image analyzer (Focus Technologies, New Orleans, LA, USA). Then, the ratio of the height of the villi to the depth of the crypts was calculated. An optimal dose (3.5 mg/kg) inducing body weight loss by about 15% as well as damage of organs including intestinal mucosa without mortality were adopted for the assessment of the protective efficacy of MB12662.

Assessment of protective effects of MB12662 in mice

Mice were weighed, and orally administered MB12662 (5, 10, 25 or 50 mg/kg), dissolved in CMC, in a volume of 5 mL/kg for 10 days at 10:00. On days 4-7, 3.5 mg/kg of cisplatin was intraperitoneally injected 1 hour after the administration of MB12662. The animals were examined for clinical signs and mortality at 10:00 everyday morning, and weighed prior to MB12662 administration. The animals were sacrificed on day 10, at nadir of body weight loss (3 days after the final injection of cisplatin), 2 hours after MB12662 treatment.

Small intestine, kidneys, liver, thymus, spleen and femoral bone were removed, and the weights of the kidneys, liver, thymus and spleen were recorded. The excised tissues were fixed in neutral formalin solution. Paraffin-embedded tissue slides were stained with hematoxylin-eosin, and examined under a light microscope for the tissue injuries. Villi length/crypt depth ratio was analyzed to quantify the degree of intestinal injury.

In addition, we analyzed the number of splenocytes. Splenocytes were collected from the spleen, and the numbers of live cells were counted using a Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan) for the assessment of cellularity.

Measurement of anti-emetic efficacy in ferrets

Since ferrets administered 10 mg/kg of cisplatin did not survive for 24 hours in a preliminary study, the dose of cisplatin adjusted to 5 mg/kg. The animals were weighed, and orally administered MB12662 (25, 50 or 100 mg/kg), dissolved in CMC, in a volume of 5 mL/kg for 7 days at 10:00. Immediately after the final administration of MB12662, 5 mg/kg of cisplatin was subcutaneously injected to induce retching and vomiting.
reflexes. The reflexes were monitored with a CCTV at an angle of 60°, and the numbers to 4 and 24 hours were counted.

The survival rate of the animals to 24 hours was recorded, and blood samples were collected. Serum was analyzed with an automatic analyzer (Hitachi 7080, Hitachi Korea, Seoul, Korea) for the parameters of renal and hepatic toxicities; blood urea nitrogen (BUN) and creatinine for nephrotoxicity, and aspartate transaminase (AST), alanine transaminase (ALT), lactate dehydrogenase (LDH), alkaline phosphatase (ALP) and total bilirubins (TB) for hepatotoxicity.

**Statistical analysis**

The results were expressed as the mean ± S.D. Tests of significance were performed using Duncan’s multiple-range test after one-way analysis of variance, with $p<0.05$ as a criterion of difference.

**RESULTS**

**Dose determination of cisplatin in mice**

The treatment period of cisplatin was set at 4 days according to a clinical anti-cancer therapeutic schedule, and its dose inducing intestinal injury without mortality was considered as an optimal dosage. The body weights of male mice intraperitoneally challenged with 3.5 mg/kg of cisplatin for 4 days gradually decreased, reaching a nadir of 15.1% loss on day 7 (3 days after the final administration), and then recovered to a similar level on day 12 to that before treatment (Fig. 1). However, the body weight did not further increase to the level of normal animals. The mice challenged with 5 mg/kg of cisplatin showed abrupt body weight loss after the 2nd administration reaching 68.5% of control on day 7, resulting in mortality of 16.7% and 50% on days 8 and 19, respectively. The animals treated with a high dose (6.5 mg/kg) of cisplatin displayed 35% loss of body weights. At this dosage, 33.3% of the mice exhibited bloody diarrhea, and 16.7%, 33.3%, 83.3% and 100% of the animals died on days 7, 8, 9 and 10.

In order to quantitatively analyze the intestinal damage, we measured the villi length and crypt depth. In normal animals, villi length and crypt depth were 595.4 μm and 112.1 μm, respectively, leading to a villi/crypt ratio of 5.31 (Table 1). However, 3.5 mg/kg of cisplatin caused considerable atrophy of the villi without affecting crypt depth, reducing villi/crypt ratio by 47.1% to 2.81. On the other hand, 5 mg/kg of cisplatin induced severe atrophy and degeneration of the villi without influence on the crypt depth, lowering villi/crypt ratio by 54.2% to 2.43. A high dose (6.5 mg/kg) of cisplatin caused more severe atrophy and degeneration of the villi and light damage on the crypt (93.7 μm), resulting in a marked decrease (61.0%) in villi/crypt ratio to 2.07.

Based on the results, the dose of cisplatin for the evaluation of efficacy of MB12662 was set at 3.5 mg/kg/day, a dose inducing damage of internal organs including intestine along with 15% decrease in body weights. The necropsy was done at nadir of body weight loss, i.e., on day 7 (3 days after the final administration).

**Table 1.** Villi length and crypt depth of the small intestine, examined on day 10, of mice intraperitoneally administered with cisplatin (3.5, 5 or 6.5 mg/kg) 4 times on days 1-4

| Treatment (mg/kg) | Villi length (μm)     | Crypt depth (μm) | Villi/crypt ratio |
|------------------|-----------------------|-----------------|------------------|
| Normal control   | 595.4 ± 21.8          | 112.1 ± 4.0     | 5.31 ± 0.65      |
| Cisplatin (3.5×4 days) | 298.8 ± 26.1*      | 106.3 ± 7.1     | 2.81 ± 0.53*     |
| Cisplatin (5.0×4 days) | 244.5 ± 17.5*      | 100.6 ± 10.3    | 2.43 ± 0.18*     |
| Cisplatin (6.5×4 days) | 194.0 ± 21.3*      | 93.7 ± 8.4*     | 2.07 ± 0.26*     |

*Significantly different from normal (vehicle) control ($p<0.05$).
Protective effects of MB12662 on cisplatin toxicity in mice

The body weights of vehicle-treated mice challenged with 3.5 mg/kg of cisplatin decreased by 23.9% on day 10 (3 days after the final challenge with cisplatin) (Fig. 2). Since the body weights of normal animals increased by 11.6% from 24.17 g to 26.97 g for 10 days, the real body weight loss in cisplatin-challenged mice from the weight of control animals was 31.8%. In comparison, treatment with MB12662 (10-50 mg/kg) tended

Table 2. Absolute (g) and relative (%) organ weights of mice orally administered with MB12662 for 10 days and intraperitoneally challenged with cisplatin (3.5 mg/kg) on days 4-7

| Treatment (mg/kg) | Body weight | Kidneys | Liver | Thymus | Spleen |
|------------------|-------------|---------|-------|--------|--------|
|                  | Absolute organ weights |            |   |        |        |        |
| Normal control   | 26.97 ± 1.90 | 0.296 ± 0.038 | 1.587 ± 0.233 | 0.128 ± 0.026 | 0.109 ± 0.009 |
| Cisplatin alone  | 18.39 ± 2.44* | 0.254 ± 0.028 | 0.970 ± 0.214* | 0.024 ± 0.013* | 0.039 ± 0.017* |
| +MB12662 (5)     | 19.89 ± 1.72 | 0.238 ± 0.012 | 0.972 ± 0.088 | 0.039 ± 0.013* | 0.052 ± 0.011* |
| +MB12662 (10)    | 20.22 ± 0.73 | 0.256 ± 0.017 | 1.056 ± 0.147 | 0.026 ± 0.016 | 0.049 ± 0.010 |
| +MB12662 (25)    | 21.44 ± 2.05 | 0.249 ± 0.023 | 1.129 ± 0.200 | 0.044 ± 0.025* | 0.062 ± 0.017* |
| +MB12662 (50)    | 20.13 ± 1.31 | 0.244 ± 0.027 | 0.982 ± 0.070 | 0.032 ± 0.018 | 0.044 ± 0.005 |
|                  | Relative organ weights |            |   |        |        |        |
| Normal control   | - | 1.095 ± 0.071 | 5.864 ± 0.542 | 0.478 ± 0.114 | 0.320 ± 0.184 |
| Cisplatin alone  | - | 1.406 ± 0.221* | 5.256 ± 0.355 | 0.127 ± 0.052* | 0.168 ± 0.101* |
| +MB12662 (5)     | - | 1.203 ± 0.086 | 4.888 ± 0.195 | 0.195 ± 0.059* | 0.212 ± 0.125 |
| +MB12662 (10)    | - | 1.266 ± 0.085 | 5.206 ± 0.566 | 0.129 ± 0.072 | 0.190 ± 0.112 |
| +MB12662 (25)    | - | 1.172 ± 0.102 | 5.252 ± 0.240 | 0.198 ± 0.057* | 0.224 ± 0.137 |
| +MB12662 (50)    | - | 1.208 ± 0.074 | 4.882 ± 0.312 | 0.157 ± 0.091 | 0.179 ± 0.103 |

*Significantly different from normal (vehicle) control (*p<0.05). #Significantly different from cisplatin alone (*p<0.05).

Fig. 3. Representative findings of the small intestine of mice orally administered with MB12662 for 10 days and intraperitoneally challenged with cisplatin (3.5 mg/kg) on days 4-7. (A) normal control; (B) cisplatin alone; (C) cisplatin + 10 mg/kg MB12662; (D) cisplatin + 50 mg/kg MB12662. Note the severe degeneration and atrophy of intestinal villi (arrow heads) and relatively-mild injury of crypts (asterisks) in B and C, in comparison with the normal features in A.
to increase the body weights, resulting in higher weights, by 1.8-3 g, at the terminal day.

Although absolute kidney weights of mice 3 days after 4-day administration of cisplatin (3.5 mg/kg) slightly decreased compared to those of control animals, relative weights rather increased, because body weights greatly reduced by 31.8% (Table 2). On the contrary, absolute liver weights markedly decreased to 61.0% of control group, resulting in the lowered relative weights, despite remarkable decrease in body weights. In particular, there were severe decreases in the absolute weights of immune systems, i.e., thymus and spleen, to 18.5% and 36.1% of control level, respectively. The relative organ weights of thymus and spleen were one-fourth to a half of control group, respectively, in spite of body weight loss.

The cisplatin-induced increase in the relative kidney weights was tended to be improved by MB12662 (5-50 mg/kg), but reduced liver weights were not reversed. Notably, decreased thymus and spleen weights following cisplatin challenge were considerably recovered by MB12662.

Cisplatin induced degeneration and severe atrophy of small intestinal villi (Fig. 3C), compared to the normal features in control animals (Fig. 3A). In contrast, the effect of cisplatin on the crypts was not remarkable. MB12662 improved the intestinal injuries in a dose-dependent manner. Although low doses (5 - 10 mg/kg) of MB12662 were less effective (Fig. 3C), 25 mg/kg of MB12662 exhibited a marked protective efficacy. Especially, a full recovery of the villi degeneration and atrophy was achieved with 50 mg/kg of MB12662 (Fig. 3D).}

Table 3. Villi length and crypt depth of the small intestine of mice orally administered with MB12662 for 10 days and intraperitoneally challenged with cisplatin (3.5 mg/kg) on days 4-7

| Treatment        | Villi length (μm) | Crypt depth (μm) | Villi/crypt ratio |
|------------------|-------------------|------------------|-------------------|
| Normal control   | 604.0 ± 11.4       | 117.4 ± 2.3      | 5.14 ± 0.22       |
| Cisplatin alone  | 306.0 ± 27.0*     | 115.6 ± 1.3      | 2.65 ± 0.20*      |
| +MB12662 (5)     | 378.8 ± 16.6*y    | 118.0 ± 1.9*y    | 3.21 ± 0.10*y     |
| +MB12662 (10)    | 414.0 ± 20.7*y    | 118.6 ± 2.2*y    | 3.49 ± 0.16*y     |
| +MB12662 (25)    | 491.4 ± 7.4*y     | 118.2 ± 2.1*y    | 4.16 ± 0.10*y     |
| +MB12662 (50)    | 595.4 ± 5.0*y     | 117.6 ± 2.5      | 5.06 ± 0.11*y     |

*Significantly different from normal (vehicle) control (p<0.05). *Significantly different from cisplatin alone (p<0.05).

Fig. 3D. Representative findings of the bone marrows of mice orally administered with MB12662 for 10 days and intraperitoneally challenged with cisplatin (3.5 mg/kg) on days 4-7. (A) normal control; (B) cisplatin alone; (C) cisplatin + 10 mg/kg MB12662; (D) cisplatin + 50 mg/kg MB12662. Note the decreased cellularity of bone marrow precursor cells compared to the normal features in A, resulting in porotic changes (asterisks) in B-D.
platin. Thus, the villi/crypt ratio was increased by MB12662 in a dose-dependent manner; i.e., MB12662 recovered villi/crypt ratio to 80% of control level and fully at 25 and 50 mg/kg, respectively.

In addition to the serious intestinal injury, cisplatin caused systemic toxicities including focal degeneration of renal proximal tubules, focal hepatocytic degeneration and inflammatory cell infiltration in a part of animals (data not shown), and decreased cellularity of bone marrow precursor cells, resulting in porotic changes (Fig. 4). Such multiple toxicities of cisplatin were improved by MB12662 treatment.

In normal animals, the mean number of splenocytes was counted to be 1,104.3 (×10^4) cells (Table 4). The splenocyte count was drastically decreased by cisplatin challenge to 302.2 cells (27.4% of control). However, the cisplatin-induced decrease in splenocytes was reversed by MB12662 to 49.7%, 44.1%, 56.0% and 75.2% of the control value at 5, 10, 25 and 50 mg/kg, respectively.

**Table 4.** Number of splenocytes of mice orally administered with MB12662 for 10 days and intraperitoneally challenged with cisplatin (3.5 mg/kg) on days 4-7

| Treatment (mg/kg) | Splenocytes×10^4 (%) |
|------------------|---------------------|
| Normal control   | 1,104.3 ± 220.4 (100) |
| Cisplatin alone  | 302.2 ± 62.7* (27.4) |
| +MB12662 (5)     | 548.3 ± 82.1* (49.7) |
| +MB12662 (10)    | 487.5 ± 75.8* (44.1) |
| +MB12662 (25)    | 618.8 ± 88.4* (56.0) |
| +MB12662 (50)    | 830.1 ± 102.4* (75.2) |

*Significantly different from normal (vehicle) control (*p*<0.05). *Significantly different from cisplatin alone (*p*<0.05).

**Table 5.** Effect of 7-day repeated oral pretreatment with MB12662 on the numbers of retching and emesis of ferrets induced by subcutaneous challenge with cisplatin (5 mg/kg)

| Treatment (mg/kg) | 4 hours (%) | 24 hours (%) |
|------------------|-------------|--------------|
| Cisplatin alone  | 190.2 ± 66.3 (100) | 609.3 ± 53.4 (100) |
| +MB12662 (25)    | 151.1 ± 11.2 (79.4) | 439.1 ± 29.6* (72.1) |
| +MB12662 (50)    | 134.2 ± 15.8 (70.6) | 359.4 ± 17.4* (59.0) |
| +MB12662 (100)   | 104.2 ± 7.9* (54.8) | 279.0 ± 23.1* (45.8) |

*Significantly different from cisplatin alone (*p*<0.05).

**Table 6.** Blood biochemistry of ferrets orally pretreated with MB12662 for 7 days, followed by subcutaneous challenge with cisplatin (5 mg/kg)

| Treatment (mg/kg) | BUN  | Creatinine | AST  | ALT  | LDH  | ALP  | TB   |
|------------------|------|------------|------|------|------|------|------|
| Cisplatin alone  | 36.1 ± 8.1 | 1.65 ± 0.76 | 47.5 ± 11.2 | 85.2 ± 21.7 | 376.5 ± 33.3 | 38.8 ± 9.4 | 0.14 ± 0.11 |
| +MB12662 (25)    | 332.0 ± 15.4* | 9.93 ± 2.11* | 673.7 ± 37.7* | 1,075.2 ± 242.1* | 641.9 ± 102.1* | 192.2 ± 26.6* | 1.22 ± 0.88* |
| +MB12662 (50)    | 191.9 ± 31.4* | 3.54 ± 1.21* | 96.9 ± 12.7* | 161.2 ± 33.1* | 329.7 ± 17.8* | 61.6 ± 10.6* | 0.11 ± 0.12* |
| +MB12662 (100)   | 151.9 ± 21.9* | 4.91 ± 0.86* | 103.7 ± 12.2* | 168.3 ± 56.2* | 421.9 ± 42.6* | 92.8 ± 13.0* | 0.23 ± 0.13* |

BUN, blood urea nitrogen; AST, aspartate transaminase; ALT, alanine transaminase; LDH, lactate dehydrogenase; ALP, alkaline phosphatase; TB, total bilirubin. *Significantly different from normal (vehicle) control (*p*<0.05). *Significantly different from cisplatin alone (*p*<0.05).

**Table 7.** Twenty-four-hour survival rate of ferrets after subcutaneous challenge with cisplatin (5 mg/kg)

| Treatment (mg/kg) | Survival (%) |
|------------------|--------------|
| Cisplatin alone  | 3/5 (60)     |
| +MB12662 (25)    | 4/5 (80)     |
| +MB12662 (50)    | 5/5 (100)    |
| +MB12662 (100)   | 5/5 (100)    |

*Significantly different from cisplatin alone (*p*<0.05).

**Protective effects of MB12662 on cisplatin toxicity in ferrets**

Ferrets subcutaneously challenged with 5 mg/kg of cisplatin displayed decreased body weights by 21.8%, from 1.73 kg to 1.36 kg. Although a low dose (25 mg/kg) of MB12662 did not prevent cisplatin-induced body weight loss, 50 and 100 mg/kg attenuated the body weight decrease, leading to loss by only 10.9% and 7.0%, respectively (data not shown).

Challenge with 5 mg/kg of cisplatin caused continuous emetic reflexes of ferrets, displaying mixed retching (without vomitus) and vomiting (with vomitus) during the first 4 hours and mainly retching response thereafter (Table 5). The numbers of retching and vomiting reflexes were counted to be 190.2 and 609.3 times during the first 4 hours and up to 24 hours, respectively. On the contrary, 7-day pretreatment with 25, 50 and 100 mg/kg of MB12662 decreased the numbers of retching and vomiting during 4 hours to 151.1 (79.4%), 134.2 (70.6%) and 104.2 (54.8%) times, respectively. Also, the numbers during 24 hours were reduced to 439.1 (72.1%), 359.4 (59.0%) and 279.0 (45.8%) times, respectively.

Challenge with cisplatin (5 mg/kg) greatly increased BUN and creatinine to 9.2 and 6.0 folds of control, respectively, indicative of severe nephrotoxicity (Table 6). However, pretreatment with 25-50 mg/kg of MB12662 significantly attenuated the increases in BUN and creatinine, and especially, 100 mg/kg of MB12662 recovered the parameters to normal levels. Parameters of hepatocytotoxicity such as AST, ALT and LDH increased to 14.2, 12.6 and 1.7 folds of normal levels, respectively, following challenge with cisplatin (5 mg/kg). Cisplatin also enhanced ALP and TB, the parameters of cholestasis, to 5.0 and 8.7 folds of control values, respectively. In contrast, 25-50 mg/kg of MB12662 significantly attenuated the increases in the parameters of both hepatocellular injury and cholestasis. Interestingly, a high dose (100 mg/kg) of MB12662 exerted an excellent hepatoprotective effect, showing full recovery...
DISCUSSION

In the present study, 4-day treatment with 5 mg/kg of cisplatin decreased body weights of mice more than 30% 3 days after the final treatment, resulting in 50% mortality. Moreover, the survived animals could not recover their body weights. A high dose (6.5 mg/kg) of cisplatin led to 100% death of the animals, which may result from inability of feed intake, mal-absorption of nutrients and serious dehydration following severe intestinal injuries. In comparison, 3.5 mg/kg of cisplatin (4 consecutive treatments) induced typical degeneration and atrophy of intestinal villi (Jordan et al., 2007). Such injuries of intestinal epithelium usually cause dehydration, in which maximum 15-24% loss of body weights were observed. Interestingly, cisplatin targeted the villi rather than crypt, resulting in a severe decrease in villi/crypt ratio to near-half levels (50-54%). In the dosage schedule (3.5 mg/kg for 4 days), the mice survived the challenge and recovered their body weights to the initial weights 5 days after nadir. The recovery might be due to a rapid proliferation of cryptic cells. In fact, we showed that proliferating cell nuclear antigen (PCNA)-positive cells greatly increased after intestinal injury induced by 5-fluouracil, another chemotherapeutic agent, in contrast to a minimal PCNA-positive cells in normal intestine (unpublished results). Such phenomenon may come from stimulatory proliferation of cryptic stem cells. Thus, growth factors have been presented as an effective prescription for the attenuation and recovery of intestinal and dermal injuries induced by diverse toxicants including chemotherapeutic agents. It was demonstrated that growth factors such as keratinocyte growth factor, insulin-like growth factor-I, and epidermal growth factor (EGF) prevented intestinal damage from anti-cancer agents and radiotherapy, and that EGF stimulated dermal recovery (Farrell et al., 1998; Farrell et al., 2002; Gibson et al., 2002; Kwon et al., 2006; von Bultzing slown, 2006).

In a quantitative analysis of intestinal injuries, MB12662 recovered the villi/crypt ratio decreased (to 51.6% of control) by cisplatin to 80% and 100% at 25 and 100 mg/kg, respectively. Although additional information is required for the explanation on the efficacy, it is suggested that anti-oxidative activity of MB12662 may play a role. In general, anti-cancer agents exert their cytotoxicity via DNA alkylation as well as radical reactions (Husain et al., 1998; De Martinis and Bianchi, 2001; Jeong et al., 2005; Lee et al., 2008). Interestingly, MB12662 and its derivative MB12066 (β-lapachone; 3,4-dihydro-2,2-dimethyl-2H-naphthol[1,2-β][pyran-5,6-dione) markedly reduced gastric secretion and prevented alcoholic gastric ulcers accompanied by oxidative tissue damage (Park et al., 2011; Jo et al., 2013). In spite of anti-oxidative, anti-gastrosecretory, and anti-ulcer activities, it is believed that the major action mechanism of MB12662 might be its NAD+-preserving potential (Hwang et al., 2009), based on the much higher efficacy in alcoholic ulcers than in stress-induced ulcers (Jo et al., 2013).

Cisplatin also induces severe nephrotoxicity and hepatotoxicity (Lee et al., 2008). In the present study, 3.5 mg/kg of cisplatin for 4 days increased relative kidney weights by 28%, but decreased the liver weights by 10%. Cisplatin induced focal degeneration of renal tubular epithelium and hyaline droplets as well as focal hepatocytic degeneration and inflammatory cell infiltration (data not shown). MB12662 remarkably prevented these renal and hepatic toxicities. It was reported that cisplatin induced radical-mediated renal and hepatic toxicities, in which not only the increase in tissue malondialdehyde and decrease in glutathione, but also increases in blood nitric oxide and tumor-necrosis factor-α were observed, suggestive of oxidative stress and inflammatory responses (Kobayahi et al., 2006; Lee et al., 2008). Therefore, it seemed that the protective effects of MB12662 against renal and hepatic injuries might be due to its anti-oxidative potential as attained by ebselen, vitamin C, and Prunus persica extract (Husain et al., 1998; De Martinis and Bianchi, 2001; Lee et al., 2008).

Chemotherapeutic agents cause shrinkage of immune system. In the present study, cisplatin reduced the relative weights of thymus and spleen to 26.6% and 52.5% of control. However, there were no injured or dead cells in microscopic examination, which may be due to the timing of examination. It was reported that thymocyte death following administration of cyclophosphamide was confirmed several hours after challenge (Ku et al., 1997). Thus, it is assumed that the injured or dead cells were cleared from the tissue by the time of sacrifice (3 days after the final cisplatin challenge) in the present study. It is of interest to note that MB12662 remarkably reversed the atrophy of immune system.

We examined the histopathological findings of bone marrow to see insight into the effects of cisplatin and MB12662 on hematopoietic function related to the changes in immune system. Cisplatin markedly decreased the cellularity of lymphoid, myeloid, and erythroid progenitor cells, which were somewhat attenuated by a high dose (50 mg/kg) of MB12662. Furthermore, in the analysis of survived splenocytes, the reduced number following cisplatin challenge (27.4% of control mice) was restored by MB12662 in a dose-dependent manner, leading to 75.2% of control at 50 mg/kg.

Although emesis is triggered by various stimuli including gastrointestinal irritation, gastric over-extension, and motion sickness, chemotherapy-induced severe nausea and vomiting are mediated by serotonin secreted from damaged enterochromaffin cells (Minami et al., 1997). Thus, 5-HT3 antagonists have been widely used for the attenuation of anti-cancer agent-induced vomiting reflexes (de Bruijn, 1993; Mantovani et al., 1996; Jordan et al., 2007). However, 5-HT3 antagonists including tropisetron are effective for the elimination of only agent-induced vomiting reflexes (Rola et al., 2006), cause pain during injection, and possess a relatively-short half-life of about 2 hours (Jeong et al., 2005). Therefore, the dosage regimen for 5-HT3 antagonists consists of intravenous administration before anti-cancer agent therapy, and follow-up oral treatments (Jordan et al., 2007). Otherwise, researches on the development of topical patches are also focused (Jeong et al., 2005).

We assessed the effectiveness of MB12662, an oral regimen, in the attenuation of emesis, the organ protection, and the survivability of ferrets up to 24 hours as described in a previous report that showed the efficacy of aprepitant, an oral anti-emetic prescription (Huskey et al., 2003). MB12662 effectively prevented the cisplatin-induced body weight loss and mortality as well as vomiting reflexes, in which delayed symp-
toms were more significantly alleviated than the early signs within 4 hours. Such an efficacy of MB12662 might be due to its intestine-preserving activity which lowers ensuing retching and vomiting, prevents dehydration and inability of food consumption, and recovers animals as demonstrated in mice. The protective effects of MB12662 on gastric ulcers induced by alcohol and stress were also confirmed in rats (Jo et al., 2013). Since radiotherapy also induces intestinal injury and emesis as in chemotherapy (Priestman et al., 1990; Farrell et al., 1998; Jo et al., 2000), it is expected that MB12662 would be effective for the relief of radiation-induced gastrointestinal problems. Besides anti-emetic effect, MB12662 displayed excellent protective activities against the renal and hepatic toxicities of cisplatin in ferrets as confirmed with blood biochemical parameters. Such beneficial effects were similar in mice, wherein most of the target organs of cisplatin were markedly preserved by treatment with MB12662.

In the present study, it was demonstrated that MB12662 attenuated cisplatin-induced retching and vomiting, body weight loss and death, and nephrotoxicity and hepatotoxicity. Although anti-emetic drugs acting on 5-HT3 or NK1 (substance P) receptors have been used (Andrews et al., 1988; Gardner et al., 1995; Morrow et al., 1995; Jeong et al., 2005; Warr, 2008), it is suggested that MB12662 blocks vomiting reflexes by preserving intestinal mucosa, as confirmed in mice and ferrets. Furthermore, the body-protective effects of MB12662 were also presented in tissue-injury models using cisplatin, alcohol, and stress, although its action mechanisms, especially on 5-HT3 and NK1 receptors, remain to be clarified.

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