BIOMOLECULES & THERAPEUTICS

Original Article
Biomol Ther 23(5), 449-457 (2015)

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; Cascinu, 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; Yamakuni *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

Open Access **http://dx.doi.org/10.4062/biomolther.2015.034**

Received Mar 25, 2015 **Revised** Apr 29, 2015 **Accepted** May 6, 2015
Published online Sep 1, 2015

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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 Bruijn, 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*., 2013). 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.

MATERIALS 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 spelenocytes. 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).

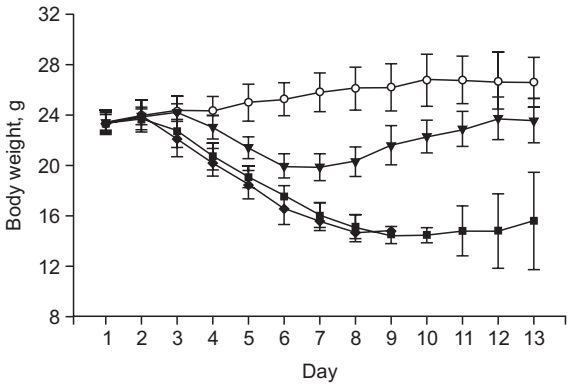

Fig. 1. Change in the body weights of mice intraperitoneally administered with cisplatin 4 times on days 1-4. ○, normal control; ▼, 3.5 mg/kg cisplatin; ■, 5 mg/kg cisplatin; ◆, 6.5 mg/kg cisplatin.

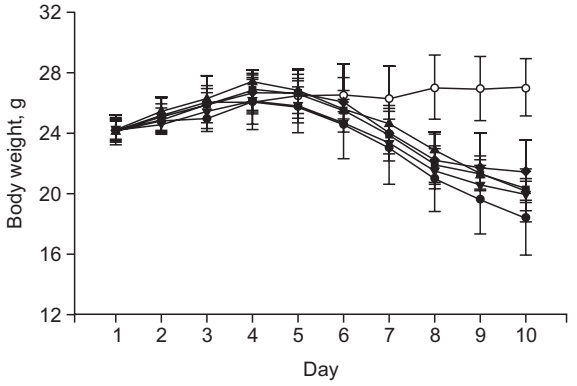

Fig. 2. Change in the body weights of mice orally administered with MB12662 for 10 days and intraperitoneally challenged with cisplatin (3.5 mg/kg) on days 4-7. ○, normal control; ●, cisplatin alone; ▼, cisplatin + 5 mg/kg MB12662; ■, cisplatin + 10 mg/kg MB12662; ◆, cisplatin + 25 mg/kg MB12662; ▲ cisplatin + 50 mg/kg MB12662.

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 ($p<0.05$).

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).

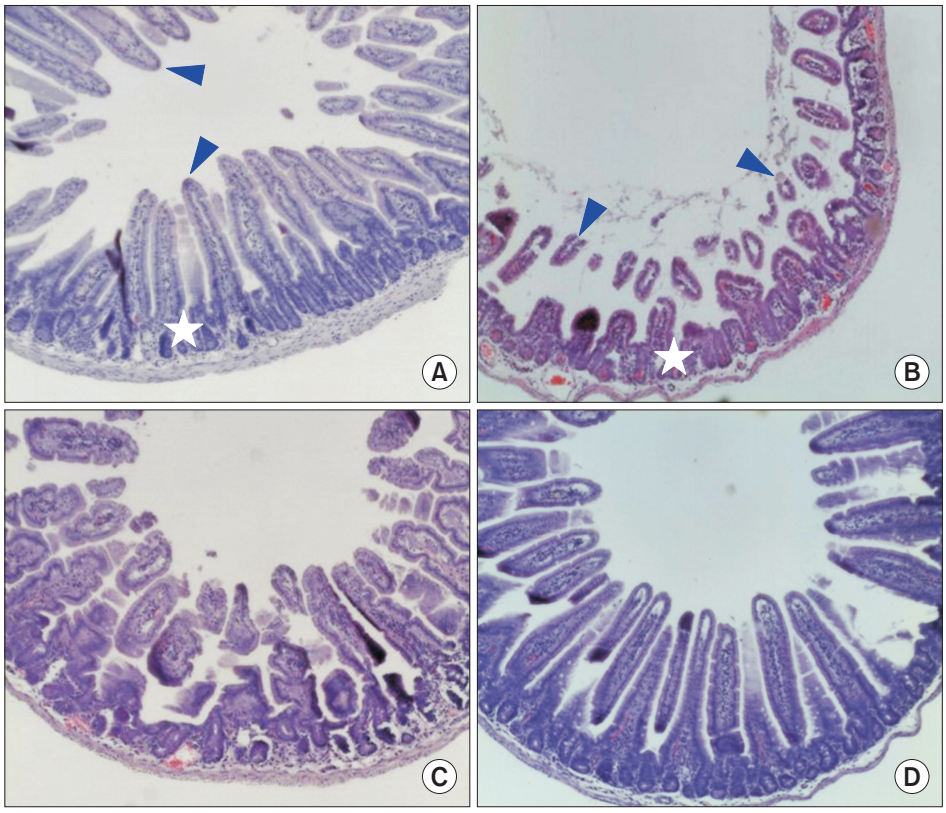

Fig. 3. Representative findings of the small intestine of mice orally administered with MB12662 for 10 days and intraperitoneally challenged with cisplatin (3.5 mg/kg) on days 4-7. (A) normal control; (B) cisplatin alone; (C), cisplatin + 10 mg/kg MB12662; (D) cisplatin + 50 mg/kg MB12662. Note the severe 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.

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

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 adminstration 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. 3B), 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).

In normal mice, the villi length and crypt depth were 604.0 μm and 117.4 μm, respectively, leading to a villi/crypt ratio of 5.14 (Table 3). Cisplatin (3.5 mg/kg) induced a marked atrophy of the villi (306.0 μm) with a light effect on crypt depth (115.6 μm), reducing villi/crypt ratio by 48.4% to 2.65. On the contrary, MB12662 significantly improved both the decrease in crypt depth and, especially, atrophy of villi induced by cis-

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 (mg/kg)	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#	118.0 ± 1.9#	3.21 ± 0.10#
+MB12662 (10)	414.0 ± 20.7#	118.6 ± 2.2#	3.49 ± 0.16#
+MB12662 (25)	491.4 ± 7.4#	118.2 ± 2.1#	4.16 ± 0.10#
+MB12662 (50)	595.4 ± 5.0#	117.6 ± 2.5	5.06 ± 0.11#

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

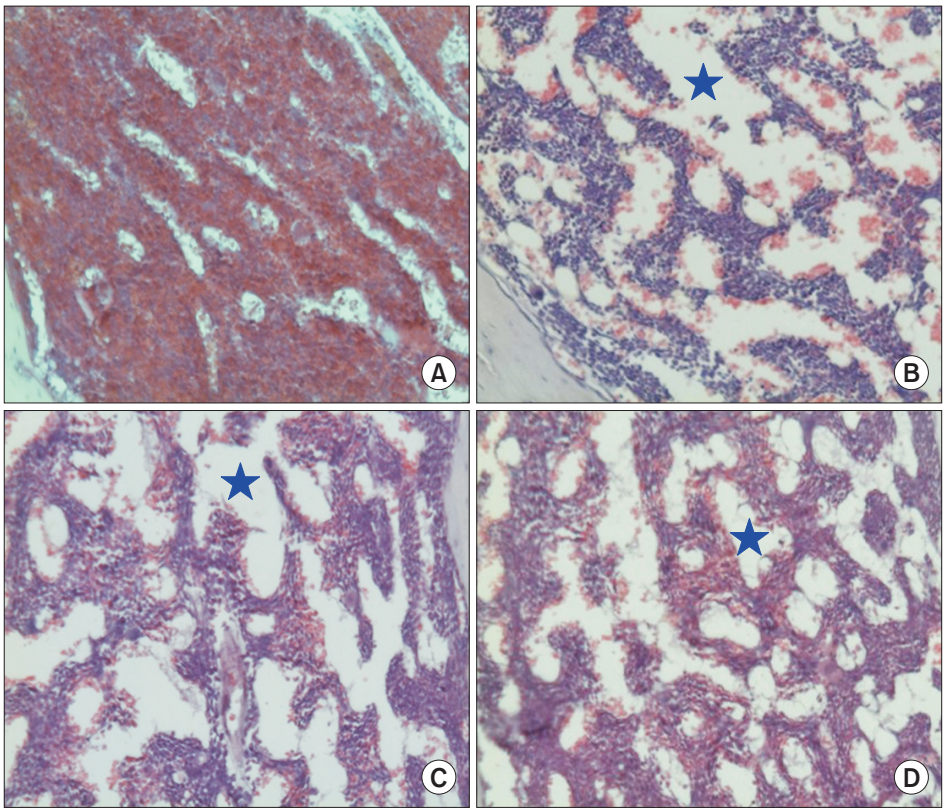

Fig. 4. Representative findings of the bone marrows of mice orally administered with MB12662 for 10 days and intraperitoneally challenged with cisplatin (3.5 mg/kg) on days 4-7. (A) normal control; (B) cisplatin alone; (C) cisplatin + 10 mg/kg MB12662; (D) cisplatin + 50 mg/kg MB12662. Note the decreased 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 ($\times 10^4$) 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$\times 10^4$ (%)
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).

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% of control) times, respectively. Also, the numbers during 24 hours were reduced to 439.1 (72.1%), 359.4 (59.0%) and 279.0 (45.8% of control) 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

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

Treatment (mg/kg)	Survival (%)
Cisplatin alone (5)	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).

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)	Normal	Cisplatin (5) alone	+MB12662 (25)	+MB12662 (50)	+MB12662 (100)
BUN	36.1 ± 8.1	332.0 ± 15.4*	191.9 ± 31.4#	151.9 ± 21.9#	53.6 ± 9.66#
Creatinine	1.65 ± 0.76	9.93 ± 2.11*	3.54 ± 1.21#	4.91 ± 0.86#	0.98 ± 1.20#
AST	47.5 ± 11.2	673.7 ± 37.7*	96.9 ± 12.7#	103.7 ± 12.2#	97.1 ± 18.8#
ALT	85.2 ± 21.7	1,075.2 ± 242.1*	161.2 ± 33.1#	168.3 ± 56.2#	89.5 ± 31.1#
LDH	376.5 ± 33.3	641.9 ± 102.1*	329.7 ± 17.8#	421.9 ± 42.6#	476.5 ± 30.1#
ALP	38.8 ± 9.4	192.2 ± 26.6*	61.6 ± 10.6#	92.8 ± 13.0#	43.1 ± 5.78#
TB	0.14 ± 0.11	1.22 ± 0.88*	0.11 ± 0.12#	0.23 ± 0.13#	0.04 ± 0.11#

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).

of the blood parameters.

Fourty percent of vehicle-treated animals died in 24 hours following challenge with ciaplatin (5 mg/kg) (Table 7). In comparison, 25 mg/kg of MB12662 reduced the mortality to 20%, and higher doses (50-100 mg/kg) fully protected the animals.

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 results from inability of feed intake, malabsorption 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-fluorouracil, 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 Bultzingslowen, 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 early phase of vomiting response (Roila *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.

ACKNOWLEDGMENTS

This research was supported by High Value-added Food Technology Development Program, Ministry of Agriculture, Food and Rural Affairs (MAFRA; grant number 113034-3).

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