Liver Protection by Bis(Maltolato)Zinc(II) Complex

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Abstract: The aim of this study was to perform screening of a novel drug for treating liver injury. Bis(maltolato)zinc(II) complex [Zn(Mal)2], which was previously reported to possess insulinomimetic activity, was found to have potency against experimentally induced liver injury both in vitro and in vivo. Cultured rat hepatocytes were treated with bromobenzene for 24 h to induce cellular injury. Zn(Mal)2 of various concentrations was added along with bromobenzene in order to evaluate the hepatoprotective activity of Zn(Mal)2 in vitro. The number of viable hepatocytes decreased by 42% in the culture with bromobenzene. However, hepatocyte viability was maintained when Zn(Mal)2 was added to the bromobenzene culture. The hepatoprotective activity of Zn(Mal)2 in vivo was investigated using a concanavalin A-induced liver injury model in BALB/c mice. Changes in serum aminotransferase activities and the secretion of several cytokines were measured. The hepatoprotective effect of Zn(Mal)2 was also demonstrated in vivo by the suppression of serum aspartate aminotransferase and alanine aminotransferase elevation. No significant changes in serum cytokines associated with the induction of hepatic damage were observed in the concanavalin A-induced injury model. However, examination of concanavalin A-treated mouse splenocytes revealed a dose-dependent suppression of cytokine secretions by Zn(Mal)2. Zn(Mal)2 possessed hepatoprotective activity and might exert its effect by a number of mechanisms.

Key words: complex, concanavalin A, cytokine, hepatoprotection, zinc

Introduction

The liver is important in maintaining homeostasis. Therefore, the disruption of liver functions may be fatal. The pathological changes observed with hepatotoxicity are often associated with the production of free radicals, which interfere with numerous metabolic processes. Cytotoxins such as bromobenzene damage cells via free radical attack and induce lipid peroxidation, enzyme inactivation, and finally cellular...
necrosis [16, 36]. Therefore, bromobenzene is an occasionally used hepatotoxin, as well as carbon tetrachloride (CCl₄).

Liver injury may also be induced by activated T cells as in viral hepatitis [8, 19, 26]. An autoimmune disease is another case that affects the human liver by inducing the infiltration of CD4⁺ T cells [18]. A T cell mitogen, concanavalin A (ConA), may lead to liver injury when injected into mice. Mice with severe combined immunodeficiency (SCID) and athymic nude mice are completely insensitive to ConA injection, indicating the involvement of T cells in the development of liver injury [25, 37]. Therefore, ConA-induced liver injury is often used to investigate the pathogenesis of human liver diseases. ConA has a high affinity for the hepatic sinuses, which results in the activation of T cells within the liver tissue. This activation of T cells results in the secretion of several cytokines that mediate inflammation and organ reconstitution [9, 25, 34, 35, 38].

To date, many efforts have been made to seek out hepatoprotective agents, which may facilitate the treatment of liver failure. We previously reported a catechin, epigallocatechin-3-gallate (EGCG), is an effective liver-protecting agent and the complex formation of EGCG with zinc enhances the potency of EGCG against cytotoxic-induced hepatotoxicity [14]. Based on this result, the zinc complex was considered to play a protective role in hepatotoxicity. Zinc is an essential trace element in all living systems. It has four coordinating arms and plays a structural role in many proteins and enzymes. It was also reported to have insulinomimetic activity [6, 7, 12, 28, 31], as far back as 1980. In the development of insulinomimetic agents, synthetic low-molecular weight zinc complexes, such as bis(maltolato)zinc(II) [Zn(Mal)₂], have exhibited more promise in insulinomimetic activity than free zinc [29, 39, 42, 43]. In the present study, we examined the zinc complex, Zn(Mal)₂, as a potential liver-protecting agent. First, we investigated the pharmacological activity of this complex following bromobenzene-induced hepatotoxicity using rat hepatocytes in vitro. Then, we evaluated the hepatoprotective activity of Zn(Mal)₂ against ConA-induced hepatitis in mice in vivo, which is a model of human hepatitis.

Reagents
Bromobenzene and 3-hydroxy-2-methyl-4-pyrone (maltol) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Zinc sulfate (ZnSO₄·7H₂O) and dimethylsulfoxide (DMSO) were obtained from Nacalai Tesque (Kyoto, Japan). The Zn(Mal)₂ complex was prepared using the method of Yoshikawa et al. [42]. Briefly, an aqueous solution of zinc sulfate was added to an aqueous solution of an appropriate ligand maltol, and LiOH·H₂O, followed by stirring for 5 h at room temperature. The chemical structure of Zn(Mal)₂ is shown in Fig. 1. The coordination of zinc to maltol in a 1:2 ratio was confirmed by fast atom bombardment (FAB)-mass spectrometry. 1,1-Diphenyl-2-picryl-hydrazyl (DPPH) was purchased from Wako Pure Chemical Industries (Osaka, Japan). ConA was purchased from Honen (Tokyo, Japan). All other chemicals were obtained from local commercial sources, and used without further purification.

Media
Medium A consisted of William’s E medium (WE, ICN Biochemical, Costa Mesa, CA), 100 U/ml penicillin G, 100 µg/ml streptomycin, 50 ng/ml amphotericin B, 100 ng/ml aprotinin (Nacalai Tesque, Kyoto, Japan) and 10% (v/v) fetal bovine serum (ICN Biochemical, Costa Mesa, CA). Medium B consisted of Medium A, supplemented with 1 nM insulin and 1 nM dexamethasone (Nacalai Tesque, Kyoto, Japan). Medium C consisted of RPMI1640 medium (Nissui Pharmaceutical Co., Tokyo, Japan), 100 U/ml penicillin G, 100 µg/ml streptomycin, 10% (v/v) fetal bovine serum and 55 µM 2-mercaptoethanol (Nacalai Tesque, Kyoto, Japan).
Isolation of rat hepatocytes

Rat hepatocytes were isolated from male Sprague-Dawley rats (SLC:SD) weighing 150–200 g (SLC, Shizuoka, Japan) by perfusing the liver with collagenase (from *Clostridium histolyticum*; Sigma-Aldrich, St. Louis, MO) using the method of Seglen [30]. A hepatocyte preparation of more than 90% viability at the time of isolation was used for the experiments. The cells were seeded at a density of $1 \times 10^5$ cells/cm$^2$ into 12-well polystyrene culture plates (Nippon Becton and Dickinson, Tokyo, Japan), and were incubated for the first 6 h in Medium B under humidified air with 5% CO$_2$ at 37°C. After 6 h, Medium B was replaced by Medium A. The animal experiments were conducted according to the ethical guidelines of the Faculty of Pharmaceutical Sciences, Osaka University.

Treatment of rat hepatocytes with cytotoxin in the presence of Zn(Mal)$_2$

One day after the isolation of the rat hepatocytes, the medium was replaced by fresh Medium A containing 0.8% (v/v) bromobenzene dissolved in DMSO. The final concentration of bromobenzene was adjusted to 1 mM. In the experiments investigating the hepatoprotective effect of Zn(Mal)$_2$, Zn(Mal)$_2$ was also added to the medium to achieve a final concentration of 0.1 or 0.5 mg/ml. The cells were incubated under each set of conditions for another 24 h, and the number of viable cells in each culture was determined by trypan blue exclusion assay. Briefly, 0.2% (w/v) trypan blue solution was added to trypsinized cell suspensions and the viable cells, those that were not stained, were counted. A control run was performed using the medium containing only 0.8% (v/v) DMSO.

Antioxidant determination

Zn(Mal)$_2$ of various concentrations was dissolved in 0.1 M acetate buffer (pH 6.0), after which 1 ml of each solution was added to the same volume of 500 μM DPPH in ethanol (to give a final concentration of 250 μM). The mixture was vortexed and left to stand for 20 min at room temperature in the dark. The decrease in the amount of DPPH was measured based on its absorbance at 517 nm using a BioSpec-1600 spectrophotometer (Shimadzu, Kyoto, Japan) [21, 40].

ConA-induced liver injury model of mice

Male BALB/c mice (BALB/cAnNCrj) of 8–10 weeks old (25–30 g in weight) were obtained from Charles-River Co. Ltd. (Yokohama, Japan). All the mice were maintained under specific pathogen-free conditions in an environmentally controlled clean room at the Institute of Experimental Animals, Shinshu University. ConA was dissolved in phosphate-buffered saline (PBS) and administered (20 mg/kg body weight) to the mice via the tail vein. To examine the effect of Zn(Mal)$_2$, Zn(Mal)$_2$ (2.70 mg/kg body weight) in PBS was injected intraperitoneally 2 h before ConA administration. In the control mice, only the vehicle solution (PBS) was injected. The animal experiments were conducted according to the ethical guidelines of the Shinshu University School of Medicine.

Serum aminotransferase and cytokine determinations

In order to determine aminotransferase and cytokine levels, blood samples were drawn from the orbital sinus of the mice under ether anesthesia. Blood samples were left to stand for 1 h at room temperature and centrifuged at 3,000 rpm for 10 min. Then, the serum supernatants were recovered and stored at –20°C until use for assay.

Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities were assayed according to the method recommended by the Japan Society of Clinical Chemistry (JSCC), using a Model 7150 Automatic Analyzer (Hitachi, Tokyo, Japan). Tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ) and interleukin-6 (IL-6) levels were determined using commercially available ELISA kits (TECHNE Corp., Minneapolis, USA), according to the manufacturer’s instructions.

Preparation of conditioned culture medium of mouse splenocytes

Splenocytes were prepared from a male BALB/c mouse (BALB/cCrSlc) (SLC, Shizuoka, Japan) weighing 22–25 g. The cells were seeded at a density of 5 × 10$^6$ cells/ml in 24-well polystyrene culture plates (Nippon Becton and Dickinson, Tokyo, Japan), and incubated in Medium C. The splenocytes were stimulated by ConA (10 μg/ml) for 24 h. Zn(Mal)$_2$ at a final concentration of 0.1 or 0.5 mg/ml, was also added to the medium. Aliquots of the conditioned culture me-
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Medium were obtained and centrifuged at 1500 rpm for 3 min. The supernatant was stored at –20°C and used for ELISA. The animal experiments were conducted according to the ethical guidelines of the Faculty of Pharmaceutical Science, Osaka University.

Morphological Observations

Confluent splenocytes in 1 ml of Medium C were treated for 24 h, followed by microscopic examination under a phase contrast Olympus LH20A microscope (Tokyo, Japan).

Statistics

All assays and quantitative measurements of the 3–5 samples were performed twice to confirm reproducibility. The hepatocytes or splenocytes were prepared from the same animal in order to avoid any effects due to differences in cell origin. The data were analyzed for statistical significance using Student’s t-test.

Results

Protective effects of Zn(Mal)₂ on bromobenzene-induced cell injury in vitro

The addition of bromobenzene significantly reduced the viability of hepatocytes, compared with that of the control (-BB) run, in which the hepatocytes were cultured in a medium without bromobenzene (Fig. 2). The solvent (0.8% DMSO) itself had no effect on cell viability (data not shown). To represent quantitatively the protective effects of Zn(Mal)₂ against bromobenzene toxicity, Zn(Mal)₂ of various concentrations was added to the cultured cells. Zn(Mal)₂ at a low concentration (≤ 0.1 mg/ml) exhibited a protective effect from bromobenzene by maintaining the number of viable cells, however the protective effect of Zn(Mal)₂ was not observed at a higher concentration (0.5 mg/ml). This result suggests that there is an optimum concentration of Zn(Mal)₂ in its hepatoprotective effect. Zinc sulfate (one of the raw materials used to make Zn(Mal)₂) had no protective effect.

Radical scavenging activity of Zn(Mal)₂

Chemicals such as bromobenzene damage hepatocytes via free radical reactions, resulting in the induction of cell necrosis. Therefore, we consider radical scavenging activity was the possible mechanism of protection against bromobenzene. The radical scavenging activity of Zn(Mal)₂ was examined using DPPH. This method has been widely used to test the ability of compounds to act as free radical scavengers or hydrogen donors, and to evaluate the antioxidative activity of food and plant extracts. Figure 3 shows the radical scavenging activity of Zn(Mal)₂ at various concentra-
tions and also that of zinc sulfate. The activity can be evaluated in terms of the observed decrease in absorbance of DPPH at 517 nm. The free radical scavenging activity of Zn(Mal)$_2$ was observed to increase in a dose-dependent manner, while that of zinc sulfate was quite limited.

**Protective effect of Zn(Mal)$_2$ against ConA-induced liver injury in vivo**

To examine the hepatoprotective effect of Zn(Mal)$_2$ in vivo, serum aminotransferase levels were measured as shown in Fig. 4. ConA injection (20 mg/kg body weight) led to severe liver injury in mice at 12 and 24 h as assessed by a striking increase in serum AST and ALT activities. In contrast, injection of Zn(Mal)$_2$ (2.70 mg/kg body weight) 2 h before ConA administration significantly suppressed AST and ALT activities. These results suggest that Zn(Mal)$_2$ protects the liver from ConA-induced injury in vivo.

The mechanism of ConA-induced liver injury has been examined in detail and several cytokines have been implicated. Figure 5 shows serum cytokine levels following the induction of liver injury. TNF-$\alpha$, IFN-$\gamma$, and IL-6 levels were higher than those of control mice, following ConA administration at 12 and 24 h. These cytokines were also present at high levels when Zn(Mal)$_2$ was administered 2 h before ConA was given. Thus, Zn(Mal)$_2$ had little or no effect on serum cytokine levels during ConA-induced hepatitis.

**Effect of Zn(Mal)$_2$ on cytokine release from splenocytes**

The effect of Zn(Mal)$_2$ on cytokine release from immune T cells was also examined in vitro using mouse splenocytes. TNF-$\alpha$, IFN-$\gamma$, and IL-6 levels in splenocyte-conditioned medium were quantified by ELISA and the results are shown in Fig. 6. Unlike the in vivo study, Zn(Mal)$_2$ dose-dependently suppressed cytokine release from splenocytes stimulated with ConA.

Morphological observations were made and the images of the cells are presented in Fig. 7. ConA caused aggregation and bridging of splenocytes, which are the characteristic effects of lectins such as ConA (Fig. 7B). Splenocyte aggregation decreased upon the addition of 0.1 mg/ml Zn(Mal)$_2$ (Fig. 7C), and nearly no aggregation was observed following the addition of 0.5 mg/ml Zn(Mal)$_2$ (Fig. 7D), compared with the control (Fig. 7A).

**Discussion**

In the present study, we examined the protective effect of Zn(Mal)$_2$ on the liver. Our data revealed a hepatoprotective effect of Zn(Mal)$_2$. It has been reported that bromobenzene is metabolically activated via cytochrome P450 enzymes (CYPs) and that the resulting reactive intermediates induce covalent binding, enzyme inactivation and lipid peroxidation [16, 36].
Zn(Mal)$_2$ acted as a hepatoprotective agent when added at a concentration from 0.05 to 0.1 mg/ml (Fig. 2). Radical scavenging activity increased dose-dependently (Fig. 3). These results suggest that hepatoprotection by Zn(Mal)$_2$ was caused by not only simple radical scavenging, but also other biological functions of Zn(Mal)$_2$, because 0.05 mg/ml Zn(Mal)$_2$, which demonstrated limited radical scavenging activity, was also effective against cellular damage. Zinc is also known to induce the metal binding protein metallothionein (MT), which is involved in zinc homeostasis and scavenging of reactive oxygen species [3, 15, 17]. Zinc pretreatment has been shown to protect the liver in a number of mammal models [1, 4, 10, 11, 33], possibly via hepatic MT. However, in the present report (Fig. 2) and a previous report [14], the induction of MT did not appear to protect against hepatotoxicity, since zinc itself is unable to protect cells against bromobenzene-induced damage. This is supported by the study of McMillan et al. who demonstrated that bromobenzene metabolites labeled with radioactive $[^{14}\text{C}]$ were unable to bind to MT [23]. The reduction of CYPs which inhibits the metabolic activation of bromobenzene by zinc, as demonstrated by McMillan et al. in vivo [22, 23], clearly did not

![Fig. 5. Changes in serum (A) TNF-α, (B) IFN-γ, and (C) IL-6 levels measured by ELISA. Zn(Mal)$_2$ in PBS was injected intraperitoneally 2 h before ConA administration. In the control animals, only the carrier solution (PBS) was injected. Serum cytokine levels were assayed at the indicated time points. Open columns, control; solid columns, ConA (20 mg/kg body weight) alone; hatched columns, ConA (20 mg/kg body weight) plus Zn(Mal)$_2$ (2.70 mg/kg body weight). Results shown are means ± S.D. of five independent measurements.](image)

![Fig. 6. Effect of Zn(Mal)$_2$ (0.1 or 0.5 mg/ml) on cytokine release from splenocytes treated with ConA (10 µg/ml). (A) TNF-α, (B) IFN-γ, (C) IL-6. Results shown are means ± S.D. of three independent measurements. **P<0.01 compared with the control.](image)
occur in the present in vitro study. This finding is also supported by the study of Iszard et al. [11].

It is possible that the biological functions of hepatocytes are promoted by the insulinomimetic effect of Zn(Mal)$_2$ [42, 43], including the stimulation of amino acid and glucose transport, the modulation of enzyme activities and the enhancement of DNA synthesis [27, 32, 41]. The major target organ of insulin is the liver, and insulin is also implicated in the liver regeneration process following partial hepatectomy [5, 24]. Thus, the insulinomimetic function of Zn(Mal)$_2$ is considered to play an important role in hepatoprotection. However, this is an area that warrants further investigation.

On the basis of the result of the in vitro experiment, we next attempted to examine the protective effect of Zn(Mal)$_2$ on the liver in vivo. High levels of cytokines, namely TNF-α, IFN-γ and IL-6, are released in the early phase of ConA-induced hepatic injury. TNF-α and IFN-γ are directly involved in the induction of apoptosis. IL-6 seems to both harm and protect hepatocytes during ConA-induced hepatitis [9, 25, 34, 35, 38]. In our study, Zn(Mal)$_2$ was noted to possess liver-protecting activity in vivo, as based on serum aminotransferase levels 12 and 24 h after ConA-induced injury (Fig. 4). However, Zn(Mal)$_2$ had almost no effect on serum cytokine levels in ConA-induced hepatitis (Fig. 5).

Serum cytokines are secreted from many sources in the body, and thus do not reflect accurately local cytokine levels in the liver. We, therefore, conducted the same cytokine assay in vitro using mouse splenocytes, which are rich in immune T cells. ConA activates T cells by binding to T cell antigen receptors, after which conformational changes of membrane proteins and bridging of cells are observed. This affects T cells like inflammatory stimulants in the body. In Fig.

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**Fig. 7.** Light micrographs of the splenocyte cultures treated with ConA and Zn(Mal)$_2$. (A) Control, (B) treated with ConA (10 µg/ml), (C) treated with ConA (10 µg/ml) plus 0.1 mg/ml Zn(Mal)$_2$ or (D) 0.5 mg/ml Zn(Mal)$_2$ concurrently for 24 h (magnification × 100).
7, we demonstrated inhibition of splenocyte aggregation by Zn(Mal)$_2$ in a dose-dependent manner. Zn(Mal)$_2$ was also found to suppress cytokine release from splenocytes stimulated by ConA (Fig. 6), probably due to the effect shown in Fig. 7. It has been reported that Ca$^{2+}$ is required for lymphocyte activation and A23187, a divalent cation ionophore, is a lymphocyte mitogen [20]. Cation-chelating compounds, such as citrate, EDTA [2] and flavonoid [13], have been shown to impede cell activation, which can be reversed by the addition of divalent cations (Fe$^{2+}$, Mn$^{2+}$, Mg$^{2+}$, Ca$^{2+}$) [13]. The suppression of cytokine release from splenocytes by Zn(Mal)$_2$ may also be the result of cytokine deprivation due to the presence of maltol in the medium.

Zn(Mal)$_2$ suppressed cytokine release from splenocytes as mentioned above. However, this effect was not observed in the in vivo experiment. We aim to clarify the difference between the in vitro and in vivo experiments in a future study. In conclusion, the results described here indicate that Zn(Mal)$_2$ is a potent hepatoprotective agent against experimentally induced liver injury. As mentioned above, Zn(Mal)$_2$ has multiple biological functions, some of which may have a synergistic effect on protecting the liver. This report is the first to demonstrate the hepatoprotective effect of Zn(Mal)$_2$.

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