Zinc(II) complexes with allixin-derivatives as oral therapeutics for type 2 diabetes

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Abstract
During the investigation on the development of Zn(II) complexes with a blood glucose-lowering effect in experimental diabetic animals, we found a potent bis(maltolato)Zn(II) complex (Zn(ma)2) which exhibited an excellent blood glucose lowering effects in a type 2 diabetic animal model by daily intraperitoneal (i.p.) injections. In order to find orally active Zn(II) complexes in treating type 2 diabetic mellitus, we examined the in vitro and in vivo structure-activity relationships of Zn(II) complexes by using bis(3-hydroxypropionate)Zn(II) complex (Zn(3hp)2) as a leading compound. Zn(II) complex (Zn(alx)2) with allixin (Halx) isolated from garlic, exhibited the relatively high in vitro insulin-mimetic activity, as determined by the inhibition of free fatty acid (FFA)-release in isolated rat adipocytes treated with epinephrine. The insulin-mimetic activity of Zn(II) complexes examined strongly correlated with the partition coefficient of the ligand, indicating that the activity of Zn(II) complexes depends on the lipophilicity of the ligand. In type 2 diabetic KKAy mice, Zn(alx)2 exhibited higher anti-diabetic activity than Zn(ma)2 by daily i.p. injections for 2 weeks. In addition, daily oral administrations of Zn(alx)2 lowered the high blood glucose levels in KKAy mice, however the effect was not so high. In order to find more active Zn(II) complexes than Zn(alx)2, three Zn(alx)2-related complexes were newly prepared and a Zn(II) complex (Zn(tanm)2) with 1,6-dimethyl-3-hydroxy-5-methoxy-2-pentyl-1,4-dihydropyridine-4-thionato was found to have extremely high in vitro insulin-mimetic activity.

Keywords: Zn(II) complex, allixin, in vitro insulin-mimetic effect, anti-diabetic effect, KKAy mice

Introduction
The number of patients suffering from diabetes mellitus (DM) was recently reported to be gradually increasing worldwide [1]. DM, which will be the most significant disease in the 21st century, is generally classified into two main types, insulin-dependent type 1 DM and non-insulin-dependent type 2 DM; the former is known to be the result of absolute insulin deficiency, and the latter is characterized by low insulin sensitivity in insulin-targeting tissues, according to the definition of WHO. Although several types of insulin preparations have been developed for type 1 diabetic patients, and synthetic therapeutics are available for clinical use in type 2 diabetic patients, both types of treatment are associated with problems such as physical and mental pain due to daily insulin injections and severe side effects, respectively [2, 3]. Therefore, the development of new types of anti-diabetic agent is indispensable not only to treat DM but also to improve the quality of life (QOL) in DM patients. Owing to the worldwide necessity for the development of new types of therapeutics, we have
prepared several Zn(II) complexes with different coordination modes.

In 1980, Coulston and Dandona reported that zinc chloride (ZnCl₂) stimulated lipogenesis in rat adipocytes similarly to the action of insulin [4]. Zn(II) is known to be an essential trace element in relation to the physiology of insulin, and it is ubiquitously found in many proteins and metalloenzymes. In recent years, the insulin-mimetic effects of Zn(II) have attracted much interest in not only bioorganic chemist but also biologist. Several groups have attempted to determine whether or not ZnCl₂ exhibits in vivo insulin-mimetic activity. The blood glucose-lowering effects of ZnCl₂ in type 1 and 2 diabetic animals have been observed; where extremely high doses and/or the long-term administration of ZnCl₂ were used [5, 6]. Accordingly, we developed Zn(II) complexes with coordination modes such as Zn(O₄), Zn(N₂O₂), and Zn(S₂O₂). They exhibited more effective than ZnCl₂ in terms of both the absorption from the gastrointestinal system and the toxicity of Zn(II). Moreover, the advantages of these complexes were proposed that they were effective at lower doses and by short-term administration [7, 8].

Since the insulin-mimetic effect of Zn(II) was discovered, the action mechanisms of Zn(II) have been examined by numerous groups. In 1982, May et al. reported that the effects of Zn(II) on both glucose oxidation and lipolysis stimulation were inhibited by extracellular catalase, largely resulting from the generation of H₂O₂ [9]. In 1989, Ezaki demonstrated that Zn(II) stimulated both lipogenesis and glucose transport in adipocytes [10]. In 2002, Roint et al. observed that the in vivo insulin-mimetic activity of Zn(II) was mediated through the direct inhibition of endogenous glycogen synthase kinase-3β [11]. On the other hand, we examined the action mechanism of insulin-mimetic Zn(II) complexes in terms of free fatty acid (FFA) release in isolated rat adipocytes treated with epinephrine (adrenalin). By using several inhibitors in the insulin-signaling pathway, Zn(II) complexes have been shown to exert the effect on multiple intracellular sites; PI3-kinase (phosphatidylinositol-3-kinase), GLUT-4 (glucose transporter-4), and PDE (phosphodiesterase) [12], which was named as an “ensemble mechanism” in cell’s level. Based on previous observations, it is likely that the incorporation of the Zn(II) compound into the cells through the cell membrane is essentially important for developing the insulin-mimetic complexes.

Previously, a mixture of maltol and Zn(II) was reported to enhance absorption of Zn(II) in erythrocytes more than free Zn(II) did [13]. In contrast, we found that bis(maltolato)Zn(II) (Zn(ma)₂) complex with a Zn(O₄) coordination mode exhibited not only higher in vitro insulin-mimetic activity than free Zn(II) [14], but also blood glucose lowering effects in KKAY mice, which is an excellent animal model for human type 2 DM with obesity, by daily intraperitoneal (i.p.) injections for 2 weeks [15]. However, no blood glucose lowering effects in the KKA₂ mice were observed on the oral administration of Zn(ma)₂ [16]. In order to develop orally active Zn(II) complexes in type 2 diabetic KKA₂ mice, we then examined the in vitro and in vivo structure-activity relationships of Zn(II) complex with 3-hydroxypyrone (Zn(3hp)₂) or its related compounds (Fig. 1). In this paper, we propose that Zn(II) complex with allxin (Zn(alx)₂), which is isolated from dried garlic, and its related complexes are candidates as orally active therapeutics for type 2 DM.

**Experimental**

**Materials**

Zinc sulfate (ZnSO₄·7H₂O), maltol (3-hydroxy-2-methyl-4-pyrone, Hma), and kojic acid (5-hydroxy-2-hydroxymethyl-4-pyrone, Hka) were purchased from Wako Pure Chemical Co. (Osaka, Japan). Ethyl maltol (2-ethyl-3-hydroxy-4-pyrone, Hema) was obtained from Tokyo Kasei Co. (Tokyo, Japan). Allixin (3-hydroxy-5-methoxy-6-methyl-2-pentyl-4-pyrone, 3-Hydroxypyrone (H₃hp)): R₁=H, R₂=H, R₃=H

Maltol (Hma): R₁= -CH₃, R₂=H, R₃=H

Ethyl maltol (Hema): R₁= -CH₂CH₃, R₂=H, R₃=H

Kojic acid (Hka): R₁=H, R₂=H, R₃= -CH₂OH

Allixin (Halx): R₁= -CH₂CH₂CH₂CH₂CH₃, R₂= -OCH₃, R₃= -CH₃

![Fig. 1](image-url) Chemical structures of 3-hydroxypyrone and its related compounds.
Anti-diabetic Zn(II)-allixin related complexes

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Halx), thioallixin (3-hydroxy-5-methoxy-6-methyl-2-pentyl-4-pyran-4-thione: Htalx), allxin-N-methyl (1,6-dimethyl-3-hydroxy-5-methoxy-2-pentyl-1,4-dihydropyridine-4-one: Hanm), and thio-allixin-N-methyl (1,6-dimethyl-3-hydroxy-5-methoxy-2-pentyl-1,4-dihydropyridine-4-thion: HTanm) were the products of Wakunaga Pharmaceutical Co. (Hiroshima, Japan). All Zn(II) complexes were prepared according to the previously reported methods [14, 17, 18].

Collagenase (Type II), bovine serum albumin (BSA; fraction V), and (~)-epinephrine hydrochloride (adrenaline) were obtained from Sigma Chemical (St. Louis, MO, USA). Other reagents were of the highest purity commercially available.

Animals
Male Wistar rats (7 weeks old) used for biological tests of Zn(II) complexes were obtained from Shimizu Experimental Material Co. (Kyoto, Japan). Male KKA\(^\,\) mice (type 2 diabetic mice) and C57black/6J (C57BL) (normal mice) were purchased from CLEA Japan, Inc. (Tokyo, Japan). All animals were maintained on a 12-hr light/dark cycle in our temperature-controlled central animal facility, and KKA\(^\,\) mice were individually housed in a cage. All animals were allowed free access to solid food (MF, Oriental Yeast Co., Tokyo, Japan) and tap water. All of the animal experiments were approved by the Experimental Animal Research Committee of Kyoto Pharmaceutical University (KPU) and were performed according to the Guidelines for Animal Experimentation at KPU.

Measurement of the partition coefficients of ligands
The partition coefficients (logP) of ligands (50 \(pM\)) were determined by using a 10 mM HEPES buffer (pH 7.4) / n-octanol system [13]. After incubation for 30 min at room temperature, the mixture was centrifuged at 3000 rpm for 10 min. The two resulting phases were separated. The concentrations of compounds in each phase were measured at the characteristic wavelength due to the pyrone ring. The partition coefficients were calculated by the equilibrium concentrations of the compounds in n-octanol and HEPES buffer.

Evaluation of the in vitro insulin-mimetic activity of Zn(II) complexes in isolated rat adipocytes treated with epinephrine
The in vitro insulin-mimetic activity of Zn(II) complex was determined by the inhibitory activity of FFA-release from isolated rat adipocytes treated with epinephrine, according to previously reported methods [19]. Briefly, epididymal fat pads, excised from male Wistar rats anesthetized with ether, were cut into appropriately sized pieces and were incubated with collagenase in Krebs Ringer Bicarbonate (KRB) buffer (120 mM NaCl, 1.27 mM CaCl\(_2\), 1.2 mM MgSO\(_4\), 4.75 mM KCl, 1.2 mM KH\(_2\)PO\(_4\) and 24 mM NaHCO\(_3\); pH 7.4) containing 2% BSA at 37°C with gentle shaking at 100 cycle/min for 1 hr. At the end of the incubation period, the prepared cells were filtered through sterilized cotton gauze and washed three times with the KRB buffer. The cells (1.5-2.0 \(x\) 10\(^6\) cells/ml) were incubated at 37°C for 30 min with Zn(II) complexes at various concentrations in KRB buffer containing 2% BSA and 2% DMSO. A 10 \(\mu\)M dose of epinephrine was then added to the reaction mixtures and the resulting solutions were incubated at 37°C for 3 hr. The mixtures were centrifuged at 3000 rpm for 10 min at 4°C. As regards the outer solution of the cells, FFA levels were then determined using a FFA kit (NEFA C-test Wako; Wako Pure Chemicals, Osaka, Japan). The inhibition of FFA-release was evaluated with respect to the apparent IC\(_{50}\) value, i.e., the 50% inhibitory concentration of Zn(II) complex on the release of FFA from isolated rat adipocytes treated with epinephrine during a 3 hr incubation period.

In vivo evaluation of Zn(II) complexes on blood glucose-lowering in KKA\(^\,\) mice
KKA\(^\,\) mice with type 2 DM received daily i.p. injections or oral administrations of Zn(II) complexes, and their blood glucose levels, body weight, food intake, and water consumption were monitored daily. Blood samples used for the analysis of blood glucose levels were obtained from the tail vein of KKA\(^\,\) mice, and blood glucose levels were measured using the glucose oxidase method (Glucocard; Arkray, Kyoto, Japan). HbA\(_1c\) levels in the blood obtained from the tail vein of the mice after the administration of Zn(II) complexes were determined by using a DCA 2000 system (Bayer Medical Co., Tokyo, Japan).

Statistical analysis
All experimental results are expressed as the mean values ± standard deviations (SD). Statistical analysis was performed by analysis of variance (ANOVA) at a 1 or 5% significance level of difference.
Results and Discussion

The leading complex Zn(3hp)$_2$, and its related complexes such as Zn(ma)$_2$, bis(ethylmaltolato)Zn(II) (Zn(ema)$_2$), bis(kojato)Zn(II) (Zn(ka)$_2$), and Zn(alx)$_2$ were prepared according to the previously reported methods [14], and determined to be all a four-coordinated complex at the binding ratio of Zn(II) : ligand = 1 : 2 by using elemental analysis, IR and mass spectra, as shown in Fig. 2 [17, 18]. The inhibitory activities of Zn(II) complexes on FFA-release were evaluated by using isolated rat adipocytes treated with epinephrine according to a previously reported method [19]. The concentration-dependent inhibitory effects of Zn(II) complexes on FFA-release were observed, from which the IC$_{50}$ values of the Zn(II) complexes were calculated as shown in Table 1. The inhibitory activities of Zn(3hp)$_2$, Zn(ma)$_2$, Zn(ema)$_2$, and Zn(alx)$_2$ were higher than that of ionic ZnSO$_4$, and no inhibitory activity was observed in Zn(ka)$_2$. In particular, Zn(alx)$_2$ exhibited the highest inhibitory activity among Zn(3hp)$_2$-related complexes examined.

In order to elucidate the relationship between the obtained in vitro insulin-mimetic activity and lipophilicity of the ligands in Zn(II) complexes, their reciprocal IC$_{50}$ values (1/IC$_{50}$) on FFA-release were plotted against logP of the ligands. A good linear correlation was observed, as shown in Fig. 3 (r = 0.99).

When epinephrine binds to the $\beta$-receptor of adipocytes, adenylate cyclase is activated to transform ATP to cyclic adenosin 3',5'-monophosphate (cAMP), which in turn activates certain protein kinases and lipase. The activated lipase then hydrolizes triglycerides to FFA, which are then released from the cell [20]. When insulin is added in this system, this hormone binds to the $\alpha$-subunit of the insulin receptor and catalyzes auto-phosphorylation, which in turn stimulates tyrosine kinase in the $\beta$-subunit of insulin receptor. The insulin receptor tyrosine kinase then phosphorylates the IRS (insulin receptor substrate). Following these reactions, signal information is conveyed to downstream locations such as PI3-K and PDE, and GLUT-4 is then translocated to the surface of the cell membrane. Consequently, glucose-uptake is enhanced and FFA-release is subsequently suppressed in isolated rat adipocytes treated with epinephrine [15, 21, 22].

Zn(II) is known to have effects similar to those of insulin [8, 9, 17]. Recently, we found that Zn(II) compounds act on multiple intracellular sites such as PI3-kinase, GLUT-4, and PDE [12]. In the present study, we examined the insulin-mimetic activity of Zn(3hp)$_2$-related complexes in terms of the inhibition of FFA release in isolated rat adipocytes treated with epinephrine, and found that the insulin-mimetic activity of Zn(II) complexes was strongly correlated with the partition coefficient of the ligand, indicating that the insulin-mimetic activity of Zn(II) complexes depended on the lipophilicity of the ligand (Fig. 3). These results suggest a possibility that the action sites of Zn(II) are primarily in the cells, supporting the hypothesis that the lipophilicity of the ligand in Zn(II) complexes is an important factor for developing the insulin-mimetic activity of these complexes. On the basis of these results, it was revealed that Zn(alx)$_2$.

![Fig. 2 Chemical structures of Zn(3hp)$_2$ and its related complexes.](image-url)
exhibited the highest insulin-mimetic activity among all of the complexes prepared; this relatively high level of activity was due to the high partition coefficient of the ligand ($\log P = 1.99 \pm 0.06$). These results indicate that the insulin-mimetic activity of Zn(3hp)$_2$-related complexes predominantly depends on the permeability of the complex through the cell membrane.

Then, we examined the blood glucose lowering effects of Zn(alx)$_2$ by daily i.p. injection for 2 weeks in type 2 diabetic KKA$^v$ mice by comparing with those of Zn(ma)$_2$ as reference. The blood glucose levels of KKA$^v$ mice treated with Zn(alx)$_2$ or Zn(ma)$_2$ were normalized after the treatment (Fig. 4). Concomitantly, HbA$_1c$ levels of KKA$^v$ mice treated with Zn(alx)$_2$ were significantly lowered than those of the mice treated with Zn(ma)$_2$ (Fig. 4A). Previously, Zn(alx)$_2$ was observed to improve the glucose tolerance in KKA$^v$ mice much more than Zn(ma)$_2$ [17]. From these results, Zn(alx)$_2$ was confirmed to be an excellent agent to treat type 2 diabetes in mice. KKA$^v$ mice were given daily oral administrations of Zn(alx)$_2$ for 2 weeks. Zn(alx)$_2$ complex lowered the high blood glucose levels (Fig. 5), however, the activity was relatively low. Therefore, the finding of more active complexes than Zn(alx)$_2$ was needed.

In attempting to develop more active Zn(II) complexes than Zn(alx)$_2$, the following hypotheses were used: 1) the concept of equivalent transformation of ligand atoms, from oxygen to sulfur, to alter the activity of the complex [23]; and 2) the substitution of ether oxygen at the O-1 position of allixin to N-CH$_3$ to change the lipophilicity of allixin [23] (Fig. 6). Zn(anm)$_2$, Zn(talx)$_2$, and Zn(tanm)$_2$ were then synthesized according to the previously reported method [18]. The insulin-mimetic activity of Zn(II) complexes

![Fig. 3](image-url) Relationship between the reciprocal IC$_{50}$ values of Zn(II) complexes and the partition coefficients of their ligands ($\log P$). The correlation coefficient of the linear repression was greater than 0.99 for a total of four points in triplicate measurements. Data are expressed as the means $\pm$ SD for 3 experiments. (Modified from reference [18])

![Fig. 4](image-url) Changes in blood glucose [A] and HbA$_1c$ [B] levels in type 2 diabetic KKA$^v$ mice (DM, 8 weeks) and KKA$^v$ mice treated with Zn(ma)$_2$ (DM-Zn(ma)$_2$) or Zn(alx)$_2$ (DM-Zn(alx)$_2$) by daily intraperitoneal (i.p.) injections for 2 weeks (Doses were 4.5 mg (69 µmol) Zn/kg of the body weight for the first two days, and the doses were adjusted to 2.0-4.5 mg (31-69 µmol) Zn/kg according to daily changes in blood glucose levels). Data are expressed as the means $\pm$ SD for 4-5 mice. Significance ; *$p < 0.01$ (Modified from reference [18])
Changes in blood glucose [A] and HbA1c [B] levels in type 2 diabetic KKA\textsuperscript{+} mice (DM, 16 weeks) and KKA\textsuperscript{+} mice treated with Zn(alx\textsubscript{2}) (DM-Zn(alx\textsubscript{2})) by oral administrations for 2 weeks (Doses were 15 mg (228 \text{pmol}) Zn/kg of the body weight). Data are expressed as the means ± SD for 5 mice. Significance : *p < 0.01 (Unpublished data)

was evaluated by the inhibitory activity of the release of FFA from isolated rat adipocytes treated with epinephrine. All the Zn(II) complexes examined exhibited complex concentration-dependency, and the IC\textsubscript{50} value of each complex was calculated from these data (Table 1). Zn(II) complexes with Zn(S\textsubscript{2}O\textsubscript{4}) coordination mode (Zn(talx\textsubscript{2}) and Zn(tanm\textsubscript{2})) exhibited extremely high insulin-mimetic activities in comparison to those of Zn(II) complexes (Zn(alx\textsubscript{2}) and Zn(anm\textsubscript{2})) with Zn(O\textsubscript{3}) coordination mode, indicating that S\textsubscript{2}O\textsubscript{4} ligation to Zn(II) is better than O\textsubscript{3} ligation. When a N-CH\textsubscript{3} group was substituted in place of oxygen at the 0-1 position of allixin, the lipophilicity of the ligand Hanm almost unchanged (Table 1), being in agreement with their IC\textsubscript{50} values. By substitution of sulfur in place of oxygen at the ketone group of allixin, the lipophilicity of the ligand Htalx was not increased over that of allixin, however, N-CH\textsubscript{3} substitution (Htanm) at the O-1 position of ligand (Htalx) enhanced both the lipophilicity and high insulin-mimetic activity. Following the \textit{in vitro} experiments, we examined the blood glucose lowering effects of Zn(tanm\textsubscript{2}) by daily oral administrations in KKA\textsuperscript{+} mice. The treatment of Zn(tanm\textsubscript{2}) gradually lowered the high blood glucose level, and normalized the HbA1c level (data will be reported). Zn(tanm\textsubscript{2}) is concluded to be the most potent active complex among Zn(II) complex previously reported.

Acknowledgements

This study was supported in part by grants from the Ministry of Education, Culture, Sports, Science, and Technology of the Japanese government (Grants-in-Aid for Scientific Research (B), Scientific Research on
Table 1  
IC$_{50}$ value for inhibitory activity of FFA-release from rat adipocytes treated with epinephrine and partition coefficient (log$P$) of ligand (Modified from reference [18] and [19])

<table>
<thead>
<tr>
<th>Zn(II) complex</th>
<th>Coordination mode</th>
<th>IC$_{50}$(μM)</th>
<th>log$P$ of ligand (ligand name)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZnSO$_4$</td>
<td>Ionic</td>
<td>408 ±28*</td>
<td>-</td>
</tr>
<tr>
<td>Zn(3hp)$_2$</td>
<td>O$_4$</td>
<td>234 ± 27*</td>
<td>-0.49±0.01 (H3hp)</td>
</tr>
<tr>
<td>Zn(ma)$_2$</td>
<td>O$_4$</td>
<td>220 ± 28*</td>
<td>-0.12±0.06 (Hma)</td>
</tr>
<tr>
<td>Zn(ema)$_2$</td>
<td>O$_4$</td>
<td>179 ± 7*</td>
<td>0.69±0.00 (Hema)</td>
</tr>
<tr>
<td>Zn(ka)$_2$</td>
<td>O$_4$</td>
<td>Low activity</td>
<td>-0.84±0.01 (Hka)</td>
</tr>
<tr>
<td>Zn(alx)$_2$</td>
<td>O$_4$</td>
<td>151 ± 11</td>
<td>1.99±0.06 (Halx)</td>
</tr>
<tr>
<td>Zn(anm)$_2$</td>
<td>O$_4$</td>
<td>159 ± 26</td>
<td>1.73±0.01 (Hanm)</td>
</tr>
<tr>
<td>Zn(talx)$_2$</td>
<td>S$_2$O$_2$</td>
<td>31 ± 3*</td>
<td>1.36±0.06 (Htalx)</td>
</tr>
<tr>
<td>Zn(tanm)$_2$</td>
<td>S$_2$O$_2$</td>
<td>11 ± 1*</td>
<td>2.04±0.08 (Htanm)</td>
</tr>
</tbody>
</table>

Significance at *p< 0.01 vs. Zn(alx)$_2$, †p< 0.01 vs. Zn(talx)$_2$
Each datum is expressed as mean ± SD (n = 3).

References