The lethal dose (LD50) and the dosing period was too long (8 of free fatty acid (FFA)-release in isolated rat adipocytes.9,10) Tropolonato–Zn(II) Complexes with Zn(S2O2) Coordination Mode mimetic activity.5) When an aqueous solution of ZnCl2 was of insulin. This zinc-based activity was described as insulin-mimetic activity than does free Zn(II) ion, with respect to inhibition of free fatty acid (FFA) release and enhancement of glucose uptake in isolated rat adipocytes treated with adrenaline. On comparing investigations of the antidiabetic effect in vivo, ZT2 was found to exhibit potent hypoglycemic activity and improve insulin resistance in type 2 diabetic KK A mice at a low orally administered daily dose. Di(tropolonato)zinc(II) (ZT1), which has the Zn(O4) coordination mode, had a lesser effect at the same dose. In a pharmacokinetic analysis based on the 65Zn tracer method, ZT2 was found to be absorbed at a significantly slower rate with a longer half-life than was ZT1. These results suggest that the potent hypoglycemic activity of ZT2 might be attributed to its long half-life.

Key words inorganic medicine; anti-diabetic effect; Zn(II) complex; structure–activity relationship; tropolone derivative

The numbers of diabetic patients have been increasing globally as a result of recent drastic changes in both lifestyle and diet. Diabetes mellitus (DM) is associated with increased risks of several serious complications such as retinopathy, renal disorders, and nerve disorders.1) Zinc is known as one of the most important essential trace elements of all biological systems, and is less toxic than the other trace elements.2,3) Among the many physiological and nutritional roles of zinc,4) Zn(II) ion was found to stimulate lipogenesis in rat adipocytes in a manner similar to the action of insulin. This zinc-based activity was described as insulin-mimetic activity.5) When an aqueous solution of ZnCl2 was administered to streptozotocin-induced diabetes rats (type 1 DM) or hereditary diabetes ob/ob mice (type 2 DM), normalization of the high blood glucose levels of the animals was observed. However, the doses were higher than 50% of the lethal dose (LD50) and the dosing period was too long (8 weeks); therefore, this ZnCl2 study was significantly flawed with regard to its safety profile.6,7)

In order to increase the hypoglycemic activity and to reduce the side effects, many researchers have investigated various Zn(II) complexes with respect to insulin-mimetic and hypoglycemic activity.8-17) We previously reported that a di(maltolato)zinc(II) (Zn(mal)2) and di(ethyl 2,5-dihydro-4-hydroxy-5-oxo-1H-pyrole-3-carboxylato)zinc(II) with a Zn(O4) coordination mode exhibits higher insulin-mimetic activity than does free Zn(II) ion, with respect to inhibition of free fatty acid (FFA)-release in isolated rat adipocytes.8,9) We found that di(alloxinato)zinc(II) (Zn(alx)2), which has an allixin ligand and a Zn(O4) coordination mode, exhibits higher insulin-mimetic activity than Zn(mal)2, because Zn(alx)2 has a long alkyl side chain that causes its oil–water partition coefficient to be very high.11,12) Moreover, it was also reported that di(thioallixin-N-methyl)zinc(II) (Zn(tanm)2) and di(1-oxy-2-pyridine-thiolato)zinc(II) (Zn(opt)2, with a Zn(S2O2) coordination mode, exhibits not only the highest in vivo insulin-mimetic activity but also a potent hypoglycemic effect in vivo.13-15) We also reported that di(pyrrolidine-N-dithiocarbamato)zinc(II) with a Zn(S4) coordination mode exhibits higher insulin-mimetic activity than does Zn(tann)2, with a Zn(S2O2) coordination mode. However, it did not exhibit a very potent hypoglycemic effect in vivo.16,17) These findings support our proposal that insulin-mimetic activity of Zn(II) complexes are related to the coordination modes of Zn(II) complexes as well as the lipophilicity of the complexes. Zn(II) complexes with coordinating sulfur ligands might have high insulin-mimetic activity, making them appropriate candidates for a new class of anti-diabetic drugs.

We focused on di(hinokitiolato)zinc(II) (Zn(hkt)2), a complex with a Zn(O4) coordination mode that has been reported to have higher insulin-mimetic activity relative to Zn(II) complexes with the Zn(O4) coordination mode.17) Hinokitiol is the ligand of Zn(hkt)2, and is known to be a tropolone-related compound with an aromatic 7-membered ring. It was reported that the hinokitiol and tropolone obtained from Aomori cypress tar acids have antibacterial,18) antioxidant,19) and antitumor20) activity. In this study, tropolonato–Zn(II) complexes with the Zn(O4) coordination mode, including hinokitiol or tropolone, were converted into complexes with Zn(S2O2) or Zn(S4) coordination modes, and their insulin-mimetic activity was evaluated by measuring the inhibition of FFA release and enhancement of glucose uptake in isolated rat adipocytes. Next, we investigated the antidiabetic effects and the pharmacokinetic properties of di(2-mercaptotropolonato)zinc(II) (ZT2) and compared them with di(tropolonato)zinc(II) (ZT1).

The authors declare no conflict of interest.
Experimental

Reagents Reagents used in this study were following: zinc acetate dehydrate was purchased from Wako Pure Chemical Ind., Ltd. (Osaka, Japan), tropolone and hinokitiol were purchased from Tokyo Chemical Industry Co. (Tokyo, Japan), and Lawesson’s reagent, collagenase (Type II), (±)-adrenaline hydrochloride, and bovine serum albumin (BSA: fraction V) were purchased from Sigma Chemical Co. (St. Louis, U.S.A.). 2MBq [125Zn]ZnCl2–HCl solution was obtained from Japan Radioisotope Association Co. (Tokyo, Japan).

Analytical Instrumentation Elemental analyses were performed by a Perkin-Elmer 240CHN elemental analyzer (Perkin-Elmer, Tokyo, Japan). IR spectra were recorded by a Shimadzu IR-408 spectrometer with a KB disk. Mass spectra were recorded by a JEOL JMS-SX 102 AQQ mass spectrometer (JEOL, Tokyo, Japan). iH-NMR spectra were measured by JEOL GX-400 FT-NMR spectrometers (JEOL, Tokyo, Japan) using tetramethylsilane as an internal standard. Zinc concentrations were measured by a graphite furnace atomic absorption spectrometer, AA-6300 and GFA-EX7i (Shimadzu Co., Kyoto, Japan). Radioactivity due to gamma ray of 125Zn was determined with an auto well gamma counter, ARC-360 (ALOKA, Tokyo, Japan).

Animals Animals used in this study were following: male Wistar rats were purchased from Shimizu Experimental Laboratory Inc. (Kyoto, Japan), male KKA mice were purchased from CLEA Japan Inc. (Tokyo, Japan). They were allowed free access to solid food and tap water, and housed in an air-conditioned room at a temperature of 23±1°C, with lights on from 8:00 to 20:00. The animal studies were approved by the Experimental Animal Research Committee at Kyoto Pharmaceutical University (KPU) and were performed according to the Guidelines for Animal Experimentation at KPU.

Synthesis of Di(tropolonato)zinc(II) (ZT1) A reaction mixture of tropolone (1.2 g, 10 mmol) and zinc acetate dihydrate (2.2 g, 10 mmol) in methanol was stirred at room temperature for 10 min and the organic layer was dried and concentrated, producing a black solid. This solid was dissolved in a water–ethanol mixture, and the insoluble fraction was removed by filtration. To the filtrate was added an aqueous solution of 10% NaOH, and the resulting solutions were incubated at 37°C for 2 h and filtered. The resulting pale-yellow precipitate was washed with methanol and dried at 60°C for 1 h. The mixture was stirred at room temperature for 2 h and filtered. The resulting pale-yellow precipitate was washed with methanol and dried at 60°C for 1 h.

Synthesis of Di(tropolonato)zinc(II) (ZT2) and Di(2-mercapto-tropolonato)zinc(II) (ZT3) To a solution of tropolone (5 g, 41 mmol) and acetic anhydride (5 g, 50 mmol) in acetic acid was heated at 60°C for 1 h. To the mixture were added water and CHCl3, and the organic layer was concentrated. After cooling to room temperature, a black precipitate of the mixture was removed by filtration, and concentrated, producing a black solid. This solid was dissolved in a water–ethanol mixture, and the insoluble fraction was removed by filtration.

Selective Synthesis of ZT2 (Chart I) A reaction mixture of tropolone (5 g, 41 mmol) and acetic anhydride (5 g, 50 mmol) in acetic acid was heated at 60°C for 1 h. The mixture was stirred at room temperature for 1 h. Then, CHCl3 was added and the mixture was acidified with 5 M HCl. The organic layer was dried and concentrated to give 2-mercaptotropolone as a brown oil. The acetyl-tropolone in toluene, Lawesson’s reagent (1.5 g, 3.7 mmol) dissolved in hot toluene was added slowly. The solution was heated at 90°C for 20 min and concentrated. After cooling to room temperature, a black precipitate of the mixture was removed by filtration, and concentrated, producing a dark-brown solid. This solid was dissolved in a water–ethanol mixture, and the insoluble fraction was removed by filtration.

In Vitro Insulin-Mimetic Activity of Zn(II) Complexes Isolated adipocytes (1.0×105 cells/mL) of male Wistar rats, prepared as previously described,20 were incubated at 37°C for 30 min with various concentrations of Zn(II) complex in Krebs–Ringer bicarbonate (KRB) buffer, pH 7.4 (120 mM NaCl, 1.27 mM CaCl2, 1.2 mM MgSO4, 4.75 mM KCl, 1.2 mM KH2PO4, 24 mM NaHCO3, and 5 mM glucose) containing 2% BSA. To the reaction mixtures, 0.1 mM adrenaline was added, and the resulting solutions were incubated at 37°C for 180 min. The mixtures were centrifuged at 4°C and 3000 rpm for 10 min. FFA levels for the outer solution of the cells were determined with an FFA kit (Wako Pure Chemical Ind., Ltd.). The IC50 values were obtained on the basis of the concentration of Zn(II) complex required to inhibit 50% of the total amount of FFA released from the adipocytes. Glucose levels were determined with an FFA kit (Wako Pure Chemical Ind., Ltd.).
in the extracellular solutions were determined using an automatic glucose analyzer (Fuji Dry Chem; Fuji, Tokyo, Japan). The glucose uptake activity of the complexes was evaluated according to the EC_{50} value, which is 50% of the enhancing concentration of the complex required for maximal glucose uptake during a 180-min incubation period. Glucose uptake was defined as the difference between the initially added glucose concentration and the residual glucose concentration in the medium after a 180-min incubation period.

**Administration of Zn(II) Complexes** KKA\(^{+}\) mice were used as a type 2 DM model (12 weeks old) and received daily oral doses of Zn(II) complexes, ZT1 and ZT2, dissolved in PEG400. The doses were provided at approximately 11:00 a.m. for 25 d. The blood samples used for glucose level analysis were obtained from the tail vein of each mouse, and the blood glucose levels were measured using a Glucocard (Arkray, Kyoto, Japan) just before administration of the Zn(II) complexes. The body mass, intake of solid food, and drinking water of the KKA\(^{+}\) mice were measured daily during the period of administration of the Zn(II) complexes. The doses of ZT1 and ZT2 were 10 mg Zn/kg body weight (BW) (47 mg ZT1/kg BW and 52 mg ZT2/kg BW). The rate of BW gain (RBWG) was calculated in accordance with the following formula:

\[
\text{RBWG} (%) = \left( \frac{\text{BW}(x) - \text{BW}(0)}{\text{BW}(0)} \right) \times 100
\]

where \(\text{BW}(x)\) = BW on an arbitrary day, and \(\text{BW}(0)\) = BW at day 0. After administration of Zn(II) complexes for 25 d, the mice were fasted for 12 h and then glucose was administered orally at a dose of 1 g/kg BW for the oral glucose tolerance test (OGTT). Blood samples were obtained without anesthesia from the tail vein at 10, 20, 30, 45, 60, 90, and 120 min after glucose administration and the blood glucose levels were measured with a Glucocard. The level of hemoglobin A1c (HbA1c) was measured 24 h after the final administration of the complexes by an immunoassay method using the DCA 2000 (Bayer-Sankyo Co., Ltd., Tokyo, Japan). In addition, with the animals under ether anesthesia, serum samples for analyses of the serum parameters were obtained from the cavernous sinus 24 h after the final administration of Zn(II) complexes. Serum levels of insulin, leptin, and adiponectin were determined using a Glazyme insulin-EIA test (Wako Pure Chemical Ind., Ltd., Osaka, Japan), a leptin immunoassay kit (R&D Systems Inc., Minneapolis, MN, U.S.A.), and an adiponectin immunoassay kit (R&D Systems Inc., Minneapolis, MN, U.S.A.), respectively. Serum concentrations of alkaline phosphatase (ALP), triglyceride (TG), and total cholesterol (TCHO) were determined using a Fuji Dry Chem system (Fuji Medical Co., Tokyo, Japan).

After sacrifice, blood was removed from the KKA\(^{+}\) mice, several tissue samples (from the liver, kidney, spleen, pancreas, muscle, bone, and adipose tissue) were obtained for determination of zinc concentrations. The isolated tissue samples were thoroughly washed with saline, dried under reduced pressure, and weighed. The samples were repeatedly heated to 160°C with addition of 60% HNO\(_3\), 60% HClO\(_4\), and 30% H\(_2\)O\(_2\) in 50 mL beakers until the sample turned white. The dried samples were dissolved in 1% HNO\(_3\). Then, the concentrations of zinc were determined using a graphite furnace atomic absorption spectrometer, AA-6300 and GFA-EX7i (Shimadzu Co., Kyoto, Japan).

**Pharmacokinetic Study of Zn(II) Complexes** A [\(^{65}\)Zn]ZnCl\(_2\)—HCl solution was gently dried using a mantle heater, and the residue was dissolved in dimethyl sulfoxide (DMSO) (specific activity = 0.1 µCi/µL). ZnCl\(_2\), ZT1, and ZT2 were mixed with the prepared [\(^{65}\)Zn]ZnCl\(_2\)—HCl solution dissolved in DMSO, and stirred at room temperature overnight to exchange the cold Zn with [\(^{65}\)Zn]ZnCl\(_2\), [\(^{65}\)Zn]ZT1, and [\(^{65}\)Zn]ZT2. Wistar rats (7-week-old, weighing 200–220 g) fasted for more than 12 h were intravenously (1 µCi/1 mg Zn/0.2 mL/kg BW) or orally (10–15 µCi/10 mg Zn/2 mL/kg BW) administered a single dose of [\(^{65}\)Zn]ZnCl\(_2\), [\(^{65}\)Zn]ZT1, and [\(^{65}\)Zn]ZT2, respectively, and the blood samples were drawn from the jugular vein over time. The plasma samples were prepared by centrifuging the blood samples at 4°C and 3000 rpm for 10 min. In addition, blood was removed from the rats 24 h after the administration of [\(^{65}\)Zn] compounds, and several tissues (the liver, kidney, muscle, adipose tissue, pancreas, bone, jejunum, and brain) were isolated from the rats after sacrifice. The radioactivity was determined in each case by an auto well gamma counter, ARC-360 (ALOKA,
Tokyo, Japan). Pharmacokinetic parameters were calculated in accordance with statistical moment analysis.22)

Erythrocyte concentrations of zinc and blood cellular uptake ratios of zinc (R) were calculated in accordance with following formula:

\[ R = \frac{C_{Wb} \times H_t + C_p \times (1 - H_t)}{C_{Wb} \times 100} \]

R: the blood cellular uptake ratio of zinc; C_{Wb}: the whole blood concentration of zinc; C_p: the erythrocyte concentration of zinc; H_t: hematocrit value (=0.45) in the blood of rats.

Statistical Analysis Data are expressed as the mean (standard deviations (S.D.s)). Statistical differences among the groups were detected by a Dunnett’s test.

**Results**

**Chemicals** In order to prepare the samples for an examination of the structure–activity relationship based on the coordination modes, we attempted to convert the Zn(O4) mode into the Zn(S2O2) coordination mode of tropolonato–Zn(II) complexes by using Lawesson’s reagent, which converts a carbonyl group to a thiocarbonyl group23) (Chart 1).

**In Vitro Insulin-Mimetic Activity of Tropolonato–Zn(II) Complexes** The in vitro insulin-mimetic activity of tropolonato–Zn(II) complexes were evaluated with respect to inhibition of FFA release and enhancement of glucose uptake in isolated adrenaline-treated rat adipocytes. These activities were compared with those of ZnSO4 as a positive control. All the 6 complexes (Table 1). Among the 6 tropolonato–Zn(II) complexes, ZT2, which has a Zn(S2O2) coordination mode, exhibited the highest activity with respect to the IC_{50} value, which was approximately 9 times higher than that of ZT1 with a Zn(O4) coordination mode (Table 1). The order of inhibition of FFA release was found to be Zn(S2O2)>Zn(O4)>Zn(S2) and the order of enhancement of glucose uptake was found to be Zn(S2O2)>Zn(S2)>Zn(O4) with or without the propyl group included in the side chain (Table 1). On the other hand, the glucose uptake effect of ZT2 was also the highest among the 6 complexes (Table 1).

**Anti-diabetic Effects of Zn(II) Complexes in Vivo** ZT2 with the Zn(S2O2) coordination mode exhibits the highest in vitro insulin-mimetic activity among the 6 tropolonato–Zn(II) complexes, and ZT1 with the Zn(O4) coordination mode as a control complex were daily administrated to type 2 diabetes KKA^v mice. At a dose of 10 mg Zn/kg BW, ZT2 was found to exhibit a significant hypoglycemic effect within 5 d. The blood glucose level was lowered to about 200 mg/dL (Fig. 1A). On the other hand, ZT1 did not have the same effect at the same dose. In untreated KKA^v mice, the HbA1c levels, which reflect the average blood glucose levels over a long period of time, were 9.9%, whereas in the KKA^v mice treated with ZT1 and ZT2 for 25 d, the levels were 7.6% and 4.2%, respectively. Treatment with ZT1 and ZT2 significantly improved the HbA1c levels (Table 2). These results indicate that ZT2 exhibits its significant hypoglycemic activity and is capable of normalizing chronic hyperglycemia.

Serum insulin and serum adiponectin levels, both of which are indicative of insulin resistance, were also measured, and an OGTT was carried out after ZT1 and ZT2 administration for 25 d. The untreated KKA^v mice given the vehicle (PEG400) alone exhibited hyperinsulinemia, hypoadiponectinemia, and glucose intolerance. Treatment with ZT1 and ZT2 was found to significantly lower the serum insulin levels of the KKA^v mice, and improve the hyperinsulinemia (Table 2). Furthermore, glucose intolerance was improved by treatment with ZT1 and ZT2, as estimated by the OGTT (Fig. 2). However, no changes in the serum adiponectin levels of the KKA^v mice treated with ZT1 and ZT2 were observed (Table 2). These results indicate that ZT1 and ZT2 could improve hyperglycemia, hyperinsulinemia, and glucose intolerance and consequently improve insulin resistance.

Various parameters related to obesity and lipid metabolism were also measured. No changes in food intake and serum leptin levels, which tend to be high in untreated KKA^v mice, were observed between the treated and untreated KKA^v mice (Table 2). Figure 1B shows the changes in the rate of body weight gain (RBWG); the RBWG of the KKA^v mice treated with ZT1 continued to significantly increase, and the RBWG of the ZT2-treated KKA^v mice continued to increase slightly. The TG values of ZT2-treated KKA^v mice were more than twice those of the untreated mice; the ZT2-treated KKA^v mice unfortunately exhibited exacerbated hyperlipidemia.

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Table 1. Estimated IC_{50} and EC_{50} Values of Zn(II) Complexes for Inhibition of Free Fatty Acids Release and Enhancement of Glucose Uptake, Respectively, in Isolated Rat Adipocytes

<table>
<thead>
<tr>
<th>Compound</th>
<th>X</th>
<th>Y</th>
<th>R</th>
<th>IC_{50} (µM)</th>
<th>EC_{50} (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZT1</td>
<td>O</td>
<td>O</td>
<td>H</td>
<td>113±4</td>
<td>72±9</td>
</tr>
<tr>
<td>ZT2</td>
<td>S</td>
<td>O</td>
<td>H</td>
<td>12±1*</td>
<td>0.7±0.1*</td>
</tr>
<tr>
<td>ZT3</td>
<td>S</td>
<td>S</td>
<td>H</td>
<td>145±5</td>
<td>19±6*</td>
</tr>
<tr>
<td>ZT4</td>
<td>O</td>
<td>O</td>
<td>iPr</td>
<td>101±1</td>
<td>62±9</td>
</tr>
<tr>
<td>ZT5</td>
<td>S</td>
<td>O</td>
<td>iPr</td>
<td>34±1*</td>
<td>5±1*</td>
</tr>
<tr>
<td>ZT6</td>
<td>S</td>
<td>S</td>
<td>iPr</td>
<td>255±21*</td>
<td>31±3*</td>
</tr>
<tr>
<td>ZnSO4</td>
<td></td>
<td></td>
<td></td>
<td>440±18*</td>
<td>176±9*</td>
</tr>
<tr>
<td>Insulin</td>
<td></td>
<td></td>
<td></td>
<td>0.3±0.1*</td>
<td>0.009±0.003*</td>
</tr>
</tbody>
</table>

Data are expressed as the means±S.D.s for three runs. Significance: *p<0.01 vs. ZT1. a) This data was based on ref. 24. iPr: isopropyl.
differences in the zinc levels in the adipose tissue, bone, and spleen were determined by AAS (Table 3). The zinc levels observed in the liver, kidney in several tissues were lower than those in the tissues of healthy mice. 

Treatment with ZT2 significantly increased relative to those of the control KKAy mice. The zinc levels observed in the tissues of KKAy mice were lower than those in the tissues of healthy mice. On the other hand, more than 90% of [65Zn] ZnCl2 and [65Zn] ZT2 was immediately transferred to blood cells after intravenous administration and thereafter, blood radioactivity was maintained at a steady state at approximately 40%. These results indicate that ZT2 is retained in the blood cells for a longer time, and therefore, blood radioactivity was higher than that of ZnCl2 and ZT1 (Table 4).

Figure 4 shows the plasma concentrations of total radioactivity (Fig. 4A) and the cellular uptake ratio of zinc (Fig. 4B) after intravenous administration of 1 mg Zn/kg BW of [65Zn] ZnCl2, [65Zn] ZT1, and [65Zn] ZT2 to fasted rats. ZnCl2 was gradually transferred to blood cells, and the blood cell uptake ratio of zinc was maintained at a steady state at approximately 40%. On the other hand, more than 90% of ZT2 was immediately transferred to blood cells after intravenous administration and then gradually released into the plasma. The blood cell uptake ratio of zinc was not increased in the terminal phase was found to be extremely long, and the blood concentration did not change significantly over 3 d (Fig. 3B). The fractional absorption values for [65Zn] ZnCl2, [65Zn] ZT1, and [65Zn] ZT2 calculated from the area under the curve (AUC) ratios for the radioactivity after oral and intravenous administration of these compounds to fasted rats in consideration of both doses, were 23.5%, 68.4%, and 19.1%, respectively (Table 5). In summary, ZT1 has higher gastrointestinal absorption and more rapid elimination, whereas ZT2 has lower absorption including first-pass effect and undergoes elimination at a slower rate.

Pharmacokinetic Study of Zn(II) Complexes 

Relationships between the disposition and the hypoglycemic effect of Zn(II) complexes were investigated by a pharmacokinetic analysis using [65Zn(II)]-labeled complexes. Figure 3 shows the blood concentrations of total radioactivity after intravenous and oral administration of 1 mg Zn/kg BW and 10 mg Zn/kg BW of [65Zn] ZnCl2, [65Zn] ZT1, and [65Zn] ZT2 to fasted rats. Tables 4 and 5 demonstrate the intravenous and oral pharmacokinetic parameters of total [65Zn(II)], respectively.

With intravenous administration, the blood concentration of ZT2 declined more slowly (half-life (t1/2) of 8.3 h) than did the blood concentrations of ZnCl2 and ZT1 (Fig. 3A, Table 4). After 24 h, most of the ZT2 disappeared from the blood (Fig. 3A; enclosed figure). The volumes of distribution (Vdss) and the total clearances (CLtot) were calculated to be 302 mL/kg and 93.1 mL·h−1·kg−1 for ZnCl2, 358 mL/kg and 74.7 mL·h−1·kg−1 for ZT1, and 98 mL/kg and 9.5 mL·h−1·kg−1 for ZT2 (Table 4). The Vdss value, which is indicative of the distribution into tissues, and the CLtot value, which is indicative of the decline per unit time, for ZT2 were found to be remarkably lower than for ZnCl2 and ZT1 (Table 4).

Fig. 1. (A) Changes of Blood Glucose Levels and (B) Rates of Body Weight Gain in KKAy Mice, Untreated (○, n=4), Treated with ZT1 (●, n=8), and Treated with ZT2 (▲, n=8) by Daily Oral Administrations for 25 d; Daily Doses Were 10 mg Zn/kg BW

Data are expressed as the means±S.D.s for 4–8 mice.

Fig. 2. Oral Glucose Tolerance Tests (OGTT) for the Untreated KKAy Mice (○), ZT1-Treated KKAy Mice (●), ZT2-Treated KKAy Mice (▲), and Untreated C57BL/6J Mice (□) after Oral Administrations of Zinc Complexes for 25 d

Data are expressed as the means±S.D.s for 4–8 mice. Data of the C57BL/6J mice were based on ref. 13.
It has been reported that many types of Zn(II) complexes with different coordination modes, such as Zn(N(NO)₃)₂,⁵ Zn(O₄)₆,¹¹ Zn(S₂O₂)₆,¹² and Zn(S₄)₆ exert insulin-mimetic and antidiabetic activity. However, few studies on the relationships between structure and activity based on coordination modes have been conducted. Therefore, we investigated the structure–activity relationships of Zn(II) complexes with troponoid ligands when the complexes were exchanged from Zn(O₄) to Zn(S₂O₂) and Zn(S₄) coordination modes.

The tropolonato–Zn(II) complexes were evaluated with respect to inhibition of FFA release and enhancement of glucose uptake in isolated rat adipocytes in the presence of adrenaline. It has been proposed that inhibition of FFA release is correlated with the oil–water partition coefficients for many Zn(II) complexes.¹¹ In particular, it has been found that Zn(II) complexes that include coordinating sulfur atoms exhibit higher activity.¹⁰ In the present study, ZT2 with the Zn(S₂O₂) coordination mode was found to exhibit the highest activity among tropolonato–Zn(II) complexes with different coordination modes. ZT2 has approximately 9 times higher activity in terms of IC₅₀ values than does ZT1, which has a Zn(O₄) coordination mode (Table 1). In the Zn(II) complexes with Zn–S bonds, the zinc and sulfur would effectively dispel the mutual charges because zinc is a soft acid and sulfur is a soft base. Therefore, the membrane permeability of the nonpolarized Zn(II) complex may be enhanced. Hence, ZT2, which was discovered in this study, exhibits higher activity than does ZT1 and comparable activity to the previously reported Zn(tannm), which has the highest in vitro insulin-mimetic activity (IC₅₀: 11 μM)² and the most potent in vivo hypoglycemic activity.¹³

In the present in vitro study, ZT2, which has the highest insulin-mimetic activity, and ZT1, as a control compound, were administered to KKA' mice prepared as a type 2 DM model with daily oral administration at a dose of 10 mg Zn/kg BW to compare the in vivo anti-diabetic effects. Although ZT1, which has a Zn(O₄) coordination mode, did not show hypoglycemic activity, the same dose of ZT2 was found to exhibit potent hypoglycemic activity (Fig. 1A), and improvements with respect to chronic hyperglycemia, hyperinsulinemia (Table 2), and glucose intolerance (Fig. 2) in KKA' mice. On the other hands, ZT1 may have some potential hypoglycemic activity, despite no potent effect on daily blood glucose levels, because of the improvements of HbA1c and insulin resistance. However, in spite of the higher hypoglycemic activity, the serum insulin level due to administration of ZT2 was maintained in the high values compared with treatment of ZT1. Recent studies have demonstrated that ZT2 increased in the secretion of insulin from rat derived RIN-5F pancreatic β cells (data not shown). Briefly, it leads to the suggestion that ZT2 has both insulinotropic effect and improvement effect of insulin resistance.

It has been believed that the target organ for the hypoglycemic effect of Zn(II) complexes is adipose tissue, because these complexes have insulin-mimetic activity in isolated adipocytes in vitro. Actually, Zn(tannm), which exhibits high hypoglycemic activity, significantly accumulates in the adipose tissue, and improves the level of serum adiponectin secreted from adipocytes when administered daily orally.¹³ However, in the present study, ZT1 and ZT2 were not observed to accumulate in the adipose tissue (Table 3), and no improvement in the serum adiponectin level was observed (Table 2). On the other hand, the tissue concentrations of ZT2-treated KKA' mice in the muscle and liver were significantly higher than those of in the muscle and liver of untreated KKA' mice (Table 3). The
Fig. 3. Blood Concentrations of Radioactivity after Intravenous (A) or Single-Dose Oral (B) Administration of ⁶⁵Zn-Labeled Zinc Complexes to Male Rats

Each point represents the mean ± S.D. (n=3–4). The intravenous and oral doses were 1 and 10 mg Zn/kg BW, respectively. ○: [⁶⁵Zn]ZnCl₂, ●: [⁶⁵Zn]ZT₁, ▲: [⁶⁵Zn]ZT₂.

Table 4. Pharmacokinetic Parameters for Blood Concentrations of Radioactivity after Intravenous Administration of ⁶⁵Zn-Labeled Zinc Complexes to Fasted Rats at a Dose of 1 mg Zn/kg BW

<table>
<thead>
<tr>
<th>Compound</th>
<th>t₁/₂(β) (h)</th>
<th>AUCₜ→∞ (µg·h/mL)</th>
<th>MRT (h)</th>
<th>CLₘₐₜ (mL/h/kg)</th>
<th>Vdₜₜ (mL/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[⁶⁵Zn]ZnCl₂</td>
<td>2.57±0.38</td>
<td>13.7±2.2</td>
<td>3.26±0.62</td>
<td>93.1±12.9</td>
<td>302±49</td>
</tr>
<tr>
<td>[⁶⁵Zn]ZT₁</td>
<td>3.59±0.70</td>
<td>14.1±3.6</td>
<td>4.87±0.92</td>
<td>74.7±21.8</td>
<td>358±91</td>
</tr>
<tr>
<td>[⁶⁵Zn]ZT₂</td>
<td>8.26±0.28</td>
<td>110.6±15.6*</td>
<td>10.28±0.33*</td>
<td>9.52±1.64*</td>
<td>98±15*</td>
</tr>
</tbody>
</table>

Data are expressed as the means ± S.D.s for 3–4 rats. Significance: *p<0.05 vs. [⁶⁵Zn]ZnCl₂. MRT: mean residence time.

Fig. 4. Plasma Concentrations of Radioactivity (A) and Blood Cellular Uptake Ratio of Zinc (B) after Intravenous Administration of ⁶⁵Zn-Labeled Zinc Complexes to Male Rats

Each point represents the mean ± S.D. (n=3–4). The intravenous dose was 1 mg Zn/kg BW. ○: [⁶⁵Zn]ZnCl₂, ▲: [⁶⁵Zn]ZT₂.

Table 5. Pharmacokinetic Parameters for Blood Concentrations of Radioactivity after Single-Dose Oral Administration of ⁶⁵Zn-Labeled Zinc Complexes to Fasted Rats at a Dose of 10 mg Zn/kg BW

<table>
<thead>
<tr>
<th>Compound</th>
<th>tₘₚₜ (h)</th>
<th>Cₘₚₜ (µg/mL)</th>
<th>t₁/₂(β) (h)</th>
<th>AUCₜ→∞ (µg·h/mL)</th>
<th>Absorption (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[⁶⁵Zn]ZnCl₂</td>
<td>1.0±0.0</td>
<td>1.75±0.40</td>
<td>17.6±7.5</td>
<td>31.2±12.4</td>
<td>23.5±10.9</td>
</tr>
<tr>
<td>[⁶⁵Zn]ZT₁</td>
<td>3.5±1.0*</td>
<td>5.03±1.80*</td>
<td>11.9±1.6</td>
<td>94.8±30.3*</td>
<td>68.4±19.5*</td>
</tr>
<tr>
<td>[⁶⁵Zn]ZT₂</td>
<td>18.0±12.0*</td>
<td>1.60±0.45</td>
<td>108.4±39.5*</td>
<td>208.6±55.6*</td>
<td>19.1±5.3</td>
</tr>
</tbody>
</table>

Data are expressed as the means ± S.D.s for 4 rats. Significance: *p<0.05 vs. [⁶⁵Zn]ZnCl₂.
liver is known to be an important tissue for controlling the blood glucose level with the glycogen synthesis and gluconeogenesis. Thus, these results suggest that the optimal tissue for producing a significant ZT2-based hypoglycemic effect might be the liver rather than the adipose tissue.

Type 2 diabetes is closely related to obesity and lipid metabolism due to genetic predisposition and lifestyle. Body weights of type 2 diabetes KKA' mice were 1.5 times higher than those of normal C57BL mice, and KKA' mice have hyperlipidemia and hyperleptinemia (Table 2). Administration of Zn(tanm)2, which has potent hypoglycemic activity, was reported to cause a significant decrease in dietary intake and improve hyperlipidemia and hyperleptinemia. However, in the present study, ZT1 and ZT2 did not show these effects and instead were observed to promote an adverse increase in body weight (Fig. 1B). This result is in agreement with the finding that the present Zn(II) complexes have a similar mechanism of action vs. insulin, which is involved in storage of energy and in anabolic effects. Moreover, ZT2 exhibits a critical adverse effect in exacerbating hyperlipidemia (Table 2).

The mechanism of the aggravation of hyperlipidemia could be attributed to a lack of effect of ZT1 and ZT2 on adipocytes, unlike insulin or other Zn(II) complexes. In fact, because the Zn(II) complexes do not affect adipocytes with insulin-mimetic action, TG would not be taken up by adipocytes. This would most likely increase the TG levels in the blood. It was suggested that the effects on adipocytes might be extremely important for suppressing side effects in clinical applications.

On the other hand, ZT2 caused problems such as an increase in body weight and abnormal lipid metabolism. It was suggested that these problems could be attributed to the tissues that are accessible for ZT2 to act on. Thus, in order to reveal the disposition in blood and the tissue distribution of ZT1 and ZT2 in more detail, we investigated the disposition of these complexes using 65Zn as a tracer in the next experiment.

The main portion of the Zn(II) complexes responsible for the hypoglycemic activity is certainly the centrally located zinc atom (the tropolonato ligands themselves do not have insulin-mimetic activity). Hence, changes in the hypoglycemic activity as a result of the different coordination mode would be dependent on transferring zinc to the target organ. Differences in the dispositions were compared between ZT1, which has low hypoglycemic activity, and ZT2, which has potent hypoglycemic activity.

In an intravenous administration, ZT2 was found to have a much slower rate of elimination and levels of this complex in the blood remained constant for a longer time compared to ZT1 (Fig. 3A). The volume of distribution (Vdss) of ZT2 was found to be very low, and transfer to target organs is facilitated by the ion and the Zn(O4) mode over the Zn(S2O2) mode of the zinc complexes (Table 4).

In a single oral administration, ZT1 attained a high level of gastrointestinal absorption and rapid elimination, whereas ZT2 had a low level of absorption and slow elimination (Fig. 3B). Since the blood levels of ZT2 were constant over 3 d (Fig. 3B), and the half-life with intravenous administration was only 8 h (Table 4), it appears that residual ZT2 in the gastrointestinal tract was absorbed continuously. The concentration of zinc in the jejunum was at a high level 24 h after the single oral administration, but no significant differences were observed between ZnCl2 and ZT2 (Table 6).

As calculated for plasma (Fig. 4A) and total blood (Fig. 3A) levels of zinc after intravenous administration, the uptake ratio of zinc into blood cells was higher for ZT2 than for ZnCl2. Moreover, the data shown in Fig. 4B suggest that the zinc of ZT2 immediately enters blood cells after intravenous administration of ZT2 and then it is gradually released to the plasma. The long half-life and the long retention time for ZT2 in the blood explain the results of these experiments.

The concentrations of zinc in tissues 24 h after the single oral administration were not significantly different between ZnCl2 and ZT2 (Table 6). After administration of ZT2, the chemical species of the complex may be Zn(II) ion, when it is transferred from the blood cells to the tissues.

As a final comment, the most important thing is to reveal the action mechanism of ZT2 in the next step. Thus, elucidation of the action mechanism of ZT2 awaits further characterization.

Conclusion

The present study showed that both in vitro insulin-mimetic activity and in vivo hypoglycemic activity are improved by the introduction of zinc complexes that have sulfur ligands. ZT2, which has a Zn(S2O2) coordination mode, exhibits the most potent hypoglycemic activity. However, problems such as increased body weight and abnormal lipid metabolism are linked to administration of this complex, and it appears that these problems could be linked to the tissue distribution of ZT2. The pharmacokinetic analysis indicated that ZT2 has a relatively long retention time in blood accompanied by low gastrointestinal absorption, and this is similar to a sustained release formulation. It is expected that repeated administration of ZT2 will lead to a higher level of accumulation of the complex in blood. This might contribute to the potent hypoglycemic activity of ZT2.

References

3) Underwood E. J., “Trace Elements in Human and Animal