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# Development of a novel antidiabetic zinc complex with an organoselenium ligand at the lowest dosage in KK-A$^y$ mice

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ABSTRACT

Diabetes mellitus (DM) is a considerably diagnosed metabolic disease and a serious problem worldwide. We prepared various zinc complexes and studied their potential for use as new antidiabetic agents. In this study, we synthesized a seleniferous zinc complex, di(2-selenopyridine-*N*-oxidato)zinc(II) ([ZPS]) that has a $Zn(Se_2O_2)$ coordination mode. Analyses of structure-activity relationships between its insulin-like activity and the coordination mode of [ZPS]-related complexes showed that it had high insulin-like activity. Hypoglycemic effects of [ZPS] on type 2 diabetic KK-A$^y$ mice were exerted at the lowest dose administered (1.25–2.5 mg Zn/kg body weight), unlike previously synthesized zinc complexes. Furthermore, [ZPS] afforded us a new advantage; we were able to investigate the tissue distribution of the ligand by measuring the amount of selenium in the organs of [ZPS]-treated mice. Gastrointestinal absorption and tissue penetration of zinc derived from [ZPS] in ddY mice, which was monitored using an isotope tracer technique, was significantly increased compared to that of $ZnCl_2$. These results suggest that [ZPS] has superior antidiabetic effects compared to previously reported zinc complexes, and is thus a potential novel antidiabetic agent that facilitates the possibility of organoselenium ligands as new metal delivery systems for treating DM.



## 1. Introduction

The number of diabetic patients has been globally increasing. This increase has been associated with lifespan extension, increased lifestyle-related cases of obesity, and increased stress [1]. Diabetic mellitus (DM) is broadly classified as type 1 DM (T1D), which is caused by the destruction of pancreatic β cells, and type 2 DM (T2D), which is caused by insulin resistance or degradation of secreted insulin. T2D is a common modern disease that is remarkable, not only because it accounts for the most part of DM [2], but also because of its associated risk factors—aging, environmental factors, and an affluent lifestyle—which can lead to lack of physical exercise, excessive nutrient intake, and increased spiritual stress [3]. The main treatment for T1D is subcutaneous injection of insulin, which is an invasive procedure. For T2D, the treatment includes diet modification, exercise therapy, and administration of various antidiabetic agents; however, existing treatments have some side effects, including pain at the site of injection, weight gain, hypoglycemia, a feeling of fullness in the abdomen, and poorly controlled blood glucose levels (BGL). Thus, novel antidiabetic agents that can be administered using a less-invasive approach are needed. On the other hand, several metals reportedly possess antidiabetic effects, and their future application in the treatment of DM is anticipated [4–7].

Zinc is an essential trace element; body tissues contain about 2 g of zinc as part of metalloproteins or metalloenzymes [8,9]. Zinc is closely related to insulin with regard to its biosynthesis, stability, and secretion [10]. In addition, studies measuring the insulin-like activity of zinc in adipocytes have been conducted by Coulston et al. [11]. Other groups have evaluated the in vivo antidiabetic effects of oral $ZnCl_2$ in streptozotocin-induced diabetic rats and obese (ob/ob) mice [12,13]. Many studies have also reported mechanisms related to the insulin-like activity of zinc. These include induction of the GLUT translocation to the plasma membrane [14], inhibition of intracellular glycogen synthase kinase-3β (GSK-3β) and protein tyrosine phosphatases (PTPs) [15,16], and modulation of insulin receptor tyrosine kinase activity [17]. Furthermore, clinical studies on the pathology of DM have reported zinc malabsorption, hyperzincuria, and decreased serum zinc concentrations. These studies have also provided evidence with regard to correlations between zinc deficiency and DM [18]. Consequently, zinc supplementation in diabetic patients may be an effective approach for preventing the onset of DM [19,20].

For several years, we have shown that various zinc complexes are effective for treating DM in experimental animal models. On the basis of our previous results, we have concluded that the insulin-like activity of zinc complexes is related to the lipophilicity and the coordination mode of each zinc complex [21]. Among these complexes, di(2-mercaptopyridine-*N*-oxidato)zinc(II) ([ZPM]) exerted the most

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potent antidiabetic effects in KK-$A^y$ mice, but [ZPM] needed to be administered at high doses compared to clinical doses of zinc preparations [22].

In this study, we use di(2-selenopyridine-*N*-oxidato)zinc(II) ([ZPS]), which is an analog of [ZPM] and has the $Zn(Se_2O_2)$ coordination mode. [ZPS] is unique in that no other antidiabetic zinc complex is composed of the organoselenium ligand. Selenium is an essential trace element and its importance is indicated by the fact that it is the only trace element to be specified in the genetic code as selenocysteine [23]. Selenium contributes to the protection of tissues and membranes from oxidative stress. It also controls the cell redox status as a crucial component of several functional selenoproteins such as glutathione peroxidases, thioredoxin reductases, iodothyronine deiodinases, and selenoprotein P [24]. Evidence from literature data suggests that selenium could enhance insulin sensitivity by mediating insulin-like activity [25]. Moreover, in vitro and in vivo studies have indicated that inorganic and organic selenium compounds can attenuate pathological conditions of DM [6,26,27]. In our present study, we evaluated the insulin-like activity as well as the antidiabetic effects of [ZPS]. Analysis of the disposition of [ZPS] demonstrates that this zinc complex is more effective as an antidiabetic compared to previously evaluated zinc complexes and thus has potential as an antidiabetic agent.

## 2. Experimental

### 2.1. Material

Zinc sulfate heptahydrate ($ZnSO_4 \cdot 7H_2O$), 2-hydroxypyridine-*N*-oxide (2-HPO), 2-tiopyridine-*N*-oxide (2-TPO), polyethylene glycol 400 (PEG-400), and DMSO were purchased from Wako Pure Chemicals Co., Osaka, Japan. 2-Bromopyridine (2-BP), barium hydroxide octahydrate ($Ba(OH)_2 \cdot 8H_2O$), and trifluoroacetic acid (TFA) were purchased from Tokyo Kasei Inc., Tokyo, Japan. Selenium powder and zinc acetate dihydrate ($Zn(CH_3COO)_2 \cdot 2H_2O$) were purchased from Nacalai Tesque Inc., Kyoto, Japan.

(±) Adrenaline hydrochloride, collagenase, and bovine serum albumin (BSA) were purchased from Sigma Aldrich Inc., St. Louis, MO, USA. $^{65}ZnCl_2$ in HCl was obtained from Japan Radioisotope Association Inc., Tokyo, Japan.

### 2.2. Experimental animal model

Male Wistar rats (8-week-old and weighing 240–260 g) and male ddY mice (5-week-old and weighing 24–26 g) were purchased from Shimizu Experimental Laboratory Inc. (Kyoto, Japan). Male type 2 diabetic KK-$A^y$ mice (5-week-old and weighing 22–25 g) were purchased from CLEA Japan Inc. (Kyoto, Japan) and used for in vivo studies when they were 9 weeks old. The KK-$A^y$ mouse strain was generated by crossing glucose-intolerant black KK female mice with yellow obese $A^y$ male mice. Since the $A^y$ allele causes massive obesity, KK-$A^y$ mice exhibit signs and symptoms of hyperglycemia, hyperinsulinemia, hyperleptinemia, and severe obesity at the age of approximately 8 weeks, and thus serve as a good model for T2D. All animals were housed in a temperature-controlled (23 ± 2 °C) and humidity-controlled (60 ± 10%) room with a 12-h light/dark cycle and were given free access to solid food and water. KK-$A^y$ mice were kept in individual cages, and the other animals were housed in groups. All 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.

### 2.3. Synthesis and characterization of [ZPS]-related zinc complexes ([ZPX])

#### 2.3.1. Di(2-hydroxypyridine-N-oxidato)zinc(II) ([ZPH])

An aqueous solution of $ZnSO_4 \cdot 7H_2O$ (431 mg, 1.5 mM) was added to an aqueous solution of 2-HPO (333 mg, 3.0 mM) and $Ba(OH)_2 \cdot 8H_2O$ (475 mg, 1.5 mM), and the solution was stirred at room temperature. After filtering out precipitated $BaSO_4$ and removing the solvent, the residue was washed with water.

#### 2.3.2. Di(2-mercaptopyridine-N-oxidato)zinc(II) ([ZPM])

A methanol solution of $Zn(CH_3COO)_2 \cdot 2H_2O$ (250 mg, 1.0 mM) was added to a methanol solution of 2-mercaptopyridine-*N*-oxide (2-MPO) (254 mg, 2.0 mM), followed by stirring at room temperature. The precipitate thus formed was collected by filtration and washed with methanol.

#### 2.3.3. Di(2-selenopyridine-N-oxidato)zinc(II) ([ZPS])

Trifluoroperacetic acid (TFAA) was produced from 30% aqueous $H_2O_2$ (9.0 mL, 80 mM) and TFA (6.0 mL, 80 mM). 2-Bromopyridine-*N*-oxide (2-BPO) was prepared by mixing TFAA and 2-BP (30 mL, 30.0 mM), followed by stirring for 6 h at 40 °C. The mixture was then extracted with chloroform (4 × 60 mL), and the organic layers were concentrated and separated by chromatography on silica gel. $Na_2SeH$ was prepared from gray selenium powder (2.1 g, 26.6 mM) and $NaBH_4$ (1.5 g, 36.9 mM) in ethanol (60 mL) by using the method described by Kienitz et al., with slight modifications [28]. After 30 min, 2-BPO was added, followed by stirring for 1.5 h at 70 °C under bubbling nitrogen gas. Subsequently, 2-selenopyridine-*N*-oxide (2-SPO) was prepared using the method of Barton et al., with slight modifications [29]. Glacial acetic acid (15.0 mL) was then added and concentrated. Next, benzene (15.0 mL) was added and evaporated to dryness. The resultant brown residue was extracted using chloroform (4 × 75 mL), and the organic layers were concentrated and separated via chromatography on silica gel. The resultant residue (100 mg) was dissolved using ether/ethanol as a mixed solvent, followed by the addition of $NaBH_4$ (25.0 mg, 0.66 mM), and finally $(CH_3COO)_2Zn \cdot 2H_2O$ (123 mg, 0.56 mM), all while stirring on ice. After removing the solvent, the residue was washed in the ether/ethanol mixed solvent. The physicochemical properties of the synthesized complexes were determined by elemental analysis, IR spectrometry, and mass spectrometry.

### 2.4. Measurement of the partition coefficients (log P) of [ZPX]

To determine log *P*, the shake flask method was performed in a 1-octanol/2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES) buffer system [30]. 1-Octanol and 10 mM HEPES buffer were mixed in equal amounts overnight at room temperature. The mixture was then allowed to stand for a few minutes, and the resultant two layers were then separated. [ZPX] was dissolved in the organic layer at a concentration of 50 μM or 100 μM. After shaking this solution for 1 h at 37 °C, the two resultant layers were separated again. Concentrations of [ZPX] in each layer were subsequently measured at wavelengths of 275 nm–312 nm by using a UV spectrometer (Agilent, Tokyo, Japan). Log *P* was then calculated using the following equation: $P = C_{org}/C_w$, where $C_{org}$ and $C_w$ are the equilibrium concentrations of the complex in the organic and aqueous layers, respectively.

### 2.5. Analysis of in vitro insulin-like activity of [ZPX]

The inhibitory effect of [ZPX] on free fatty acid (FFA) release from adipocytes has been evaluated in a previous procedure [31]. Isolated male Wistar rat epididymal adipocytes ($1.0 \times 10^6$ cells/mL) were prepared and preincubated separately with various concentrations of the different zinc complexes in 5 mM glucose in Krebs–Ringer bicarbonate (KRB) buffer (120 mM NaCl, 1.3 mM $CaCl_2$, 1.2 mM $MgSO_4$, 4.8 mM KCl, 1.2 mM $KH_2PO_4$, 24 mM $NaHCO_3$, pH = 7.4) containing 2% BSA, with shaking at 100 cycles/min for 30 min at 37 °C. Adrenaline (15 μL, 0.2 mM) was then added to the mixtures, and the resultant solutions were then incubated for 3 h at 37 °C. The reaction mixture was subsequently centrifuged at 650 × *g* for 10 min at 4 °C.

FFA levels in the extracellular solutions were determined using an FFA kit (NEFA C-test; Wako, Osaka, Japan). Insulin-like activity of [ZPX] was determined on the basis of the $IC_{50}$ value—the 50% inhibition concentration of [ZPX] for FFA release from adipocytes.

### 2.6. Oral administration of [ZPS] to KK-A$^y$ mice

The antidiabetic effects of [ZPS] were examined in KK-A$^y$ mice. These mice were divided into control and treatment groups comprising 8 mice each. [ZPS] was then dissolved in PEG-400, and 1.25–2.5 mg Zn/kg body weight (a volume of 1 mL/100 g body weight) was orally administered to the treatment group (or PEG-400 alone to the control group) once a day for 28 days. BGL in the KK-A$^y$ mice were monitored daily by using a GLUCOCARD (ARKRAY Inc., Kyoto, Japan). After measuring their BGL, PEG-400 alone and the [ZPS] suspension were administered to the control and treatment groups, respectively, at about 11:00 a.m. In addition, their body weights, food intake, and water intake were measured daily before treatments.

### 2.7. Determination of blood parameters and organ distribution of zinc and selenium in KK-A$^y$ mice after treatment

After treatment, hemoglobin A1c (HbA1c) levels were measured in blood obtained from the mouse tail vein by using an immunoassay method with the DCA 2000 (Bayer-Sankyo Co., Ltd., Tokyo, Japan). The mice were then subjected to a 12-h fast, and blood samples were collected from the eye socket under ether anesthesia by using heparinized tools. Collected samples were then centrifuged at 650×*g* for 10 min at 4 °C, and the resultant plasma samples were used to analyze various biochemical parameters. The levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), blood urea nitrogen (BUN), triglyceride (TG), and total cholesterol (TCHO) in plasma samples were determined using a Fuji Dry Chem system (Fuji Medical Co., Tokyo, Japan). The plasma levels of insulin, leptin, and adiponectin were determined using a Glazyme insulin-EIA test (Wako Pure Chemicals Co., Osaka, Japan), a leptin immunoassay kit (R&D Systems Inc., Minneapolis, MN, USA), and an adiponectin immunoassay kit (R&D Systems Inc., Minneapolis, MN, USA), respectively.

After collecting the blood samples, the mice were subjected to dissection under ether anesthesia, and resultant tissue samples were washed in saline solution. Each of the samples were dehydrated for a few days in desiccators, weighed, and then heated in 7% $HNO_3$ (5 mL) for 2 h at 110 °C in a closed system. The resultant solution was centrifuged at 650×*g* for 15 min, and the supernatant liquid was diluted using 7% $HNO_3$. The amounts of zinc and selenium in the samples were then determined using ICPM-8500 (Shimadzu Inc., Kyoto, Japan).

### 2.8. Evaluation of gastrointestinal absorption and tissue penetration of $^{65}$Zn in ddY mice orally administered $^{65}$Zn-labeled compounds

Analysis of gastrointestinal absorption and tissue penetration of zinc was carried out using an isotope tracer technique [32]. $^{65}ZnCl_2$ in HCl solution was dried using a mantle heater, and the residue was then dissolved in DMSO. [ZPS] and $ZnCl_2$ were also separately dissolved in DMSO, and each solution was mixed with DMSO solution of $^{65}ZnCl_2$. The ligand substitution reaction converting [ZPS] to $^{65}$Zn-labeled [ZPS] ([$^{65}$ZPS]) proceeded for 3 h at room temperature under nitrogen gas. Mixtures were then diluted using DMSO, and the administered solutions containing 0.75 mg Zn/mL (3.0 Ci $^{65}$Zn/mL) were prepared. Male ddY mice were divided into 10 experimental groups and they received single oral doses of $^{65}ZnCl_2$ or [$^{65}$ZPS] at a dose of 7.5 mg Zn/kg body weight (a volume of 10 mL/kg body weight). At 2, 4, 8, 12, and 24 h after treatment, the mice were subjected to dissection in the same way as the KK-A$^y$ mice. Blood and organ samples were weighed and placed in containers for gamma counting. $^{65}$Zn radioactivity in each sample was measured using Wizard 1480 (PerkinElmer Inc., California, USA).

### 2.9. Statistical analysis

Data are expressed as the mean ± SE. Statistical analysis was performed using analysis of variance or the Bailer method [33].

## 3. Results

### 3.1. Physicochemical properties of [ZPX]

The physicochemical properties of [ZPX] are summarized in Table 1. For elemental analysis, both calculated and measured values of the percent concentrations of carbon (C), hydrogen (H), and nitrogen (N) were identical and within the estimated range of experimental error. Regarding the IR spectra, frequencies due to the [ZPX] $\nu_{N\text{-}O}$ band were found at 1184 $cm^{-1}$ to 1200 $cm^{-1}$ and each $\nu$(N–O) band shifted from that of the free ligand, indicating the coordination of oxygen atom to zinc ion. Based on results from the elemental analyses and mass spectra of [ZPX], all structures were identified as ligand:zinc complexes in a 2:1 ratio. The proposed structure for [ZPX] is shown in Fig. 1.

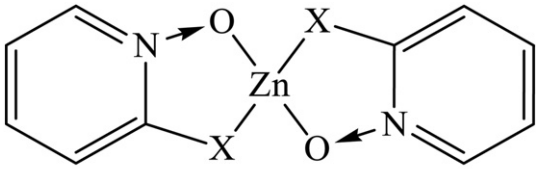
### 3.2. In vitro insulin-like activity of [ZPX]

To evaluate insulin-like activity of [ZPX], we examined the inhibitory effects of [ZPX] on FFA released from isolated rat adipocytes (Table 2). [ZPX] exerted insulin-like activity, which increased in a concentration-dependent manner as the softness of the ligand atoms increased. We speculate that differences in the activities of these complexes are partially attributable to their lipophilicity,

**Table 1**
Analytical and physicochemical properties of zinc complexes.

| Zinc complex | Elemental analysis (%) | | | | IR spectrum ($cm^{-1}$) | EI (+) MS | log*P* |
|---|---|---|---|---|---|---|---|
| | | H | C | N | $\nu$(N–O) (ligand) | (m/z) | ($P = C_{org}/C_w$) |
| [ZPH] | Found | 2.84 | 41.47 | 9.65 | 1184 (1226) | 284 $[M]^+$ | −0.75 ± 0.01 |
| | Calc. | 2.80 | 42.04 | 9.81 | | | |
| [ZPM] | Found | 2.13 | 38.00 | 8.73 | 1200 (1249) | 317 $[M+H]^+$ | 0.67 ± 0.02* |
| | Calc. | 2.54 | 37.77 | 8.81 | | | |
| [ZPS] | Found | 1.93 | 28.98 | 6.62 | 1198 (1244)# | 412 $[M]^+$ | 0.90 ± 0.04*† |
| | Calc. | 1.96 | 29.15 | 6.80 | | | |

Data are expressed as mean ± SE for 3 experiments.
Significance: *$p<0.0001$ vs. [ZPH], †$p<0.01$ vs. [ZPM].
#: The value of $\nu$(N–O) was measured using 2,2'-dipyridyldiselenide bis-*N*-oxide as an alternative for 2-selenopyridine-N-oxide.




| X | Zinc complex |
|---|---|
| O | [ZPH] |
| S | [ZPM] |
| Se | [ZPS] |

**Fig. 1.** Chemical structures of zinc complexes.

which is an indicator of their ability to permeate the cell membrane. This point of view is supported by the log *P* values of [ZPX] (Table 1).

### 3.3. Antidiabetic effects of [ZPS] on KK-A$^y$ mice

We evaluated the antidiabetic effects of [ZPS] by using a lower oral dose (1.25 mg–2.5 mg Zn/kg body weight) unlike previous studies on KK-A$^y$ mice [22]. Within the first 10 days of [ZPS] treatment, BGL in the treatment group were significantly decreased to approximately 250 mg/dL, and were then maintained at this level, while BGL in the control group remained around 400 mg/dL (Fig. 2(A)). The HbA1c levels in the treatment group decreased considerably compared to those in the control group (Fig. 2(B)). These results indicate that [ZPS] has the most effective hypoglycemic effects among the known zinc complexes. Daily food intake in the control group, treatment group, and healthy C57BL/6 J mice were 6.0 ± 0.5 g, 4.7 ± 0.6 g, and 3.4 ± 0.1 g, respectively [34], and the body weight gain in the treatment group was lower than that in the control group, demonstrating the suppression of obesity in KK-A$^y$ mice following the treatment. Serum levels of AST, ALT, and BUN were identical in each treatment group, indicating that there was no liver or kidney damage due to [ZPS] treatment (Table 3). Serum levels of TG, TCHO, insulin, leptin, and adiponectin did not significantly change between the two groups.

### 3.4. Measurement of zinc and selenium in organs of KK-A$^y$ mice

The amounts of zinc and selenium in the blood, plasma, liver, kidney, muscle, adipose, pancreas, spleen, and bone of KK-A$^y$ mice after the treatment period were measured using ICP-MS. As shown in Table 4, no significant differences were observed with regard to the zinc levels between the control and treatment groups in any of the organs, except for the bone tissue. In addition, no differences were observed in the blood and plasma concentrations of zinc between the two groups. With respect to selenium, the levels in the treatment group were notably increased in the liver, kidney, spleen, and bone. In the treatment group, blood concentrations of selenium also increased, but plasma selenium levels remained unchanged. Accumulation of the ligand was not

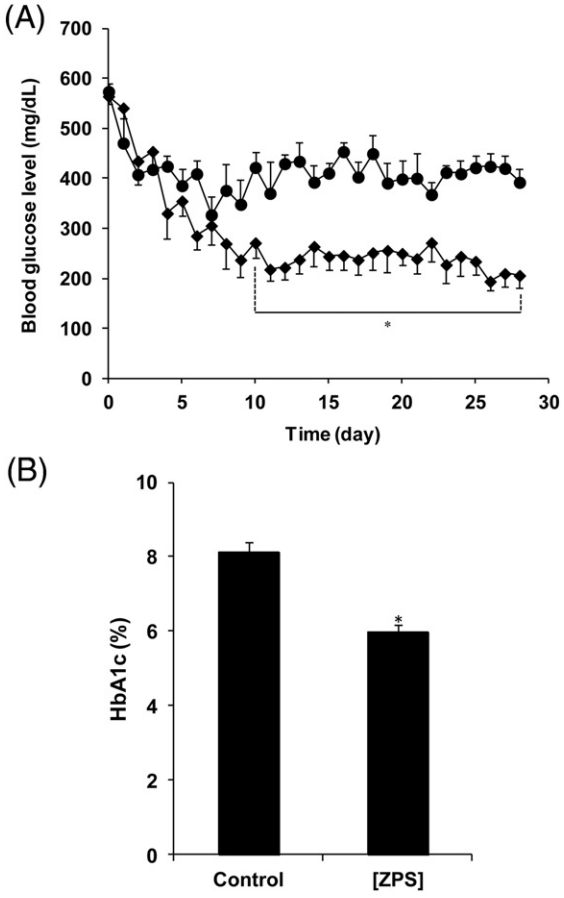



**Fig. 2.** Changes in blood glucose levels (A) and HbA1c levels (B) in KK-A$^y$ mice administered PEG-400 (control) (●) or [ZPS] (♦). Data are expressed as mean ± SE for 5–7 mice. Significance: $^*p<0.05$ vs. control.

observed in the brain (data not shown), suggesting the possibility of a metal delivery system—a system potentially with fewer side effects as it had no effect on the brain and caused minimal damage to the liver and kidney.

### 3.5. Improved $^{65}$Zn uptake from the gastrointestinal tract and tissue penetration of $^{65}$Zn in ddY mice orally administrated $^{65}$Zn-labeled compounds

To investigate the gastrointestinal absorption and tissue penetration of zinc following oral administration of zinc compounds, $^{65}$Zn-labeled [ZPS] ([$^{65}$ZPS]) or $^{65}$Zn-labeled $ZnCl_2$ ($^{65}ZnCl_2$) were orally administered to ddY mice in a single dose. Fig. 3 summarizes the resultant blood concentration and area under the curve (AUC) values for the $^{65}$Zn-labeled compound-treated mice. Blood $^{65}$Zn levels and AUC values of [$^{65}$ZPS]-treated mice were dramatically higher than those of the mice dosed with $^{65}ZnCl_2$, suggesting that [ZPS] greatly enhanced the gastrointestinal absorption of zinc. Organ $^{65}$Zn

**Table 2**
Coordination modes and $IC_{50}$ values of zinc complexes.

| Zinc complex | Coordination mode | $IC_{50}$ (μM) |
|---|---|---|
| [ZPH] | $O_4$ | 261.1 ± 4.3 |
| [ZPM] | $S_2O_2$ | 8.9 ± 1.1* |
| [ZPS] | $Se_2O_2$ | 7.4 ± 0.5* |

Data are expressed as mean ± SE for 3 experiments.
Significance: $^*p<0.0001$ vs. [ZPH].

**Table 3**
Plasma parameters in control diabetic KK-A$^y$ mice and those treated with [ZPS].

| | Control | [ZPS] |
|---|---|---|
| AST (U/L) | 55 ± 4 | 50 ± 3 |
| ALT (U/L) | 26 ± 2 | 22 ± 1 |
| BUN (mg/dL) | 23 ± 1 | 23 ± 2 |
| TG (mg/dL) | 150 ± 12 | 175 ± 16 |
| TCHO (mg/dL) | 158 ± 8 | 189 ± 12 |
| Insulin (ng/mL) | 6.9 ± 0.7 | 5.5 ± 0.9 |
| Leptin (ng/mL) | 42.5 ± 3.2 | 39.5 ± 3.4 |
| Adiponectin (μg/mL) | 7.7 ± 0.4 | 8.2 ± 0.4 |

Data are expressed as mean ± SE for 7–8 mice.

**Table 4**
Zinc and selenium concentrations of dry tissues (μg/g), blood (μg/mL), and plasma (μg/mL) in control diabetic KK-A$^y$ mice and those treated with [ZPS].

| Organ | Zn | | Se | |
|---|---|---|---|---|
| | Control | [ZPS] | Control | [ZPS] |
| Blood | 4.5 ± 0.2 | 4.5 ± 0.4 | 0.9 ± 0.0 | 2.6 ± 0.2** |
| Plasma | 2.1 ± 0.4 | 3.1 ± 0.9 | 0.7 ± 0.0 | 0.7 ± 0.0 |
| Liver | 84.1 ± 4.1 | 73.5 ± 4.0 | 6.6 ± 0.2 | 9.8 ± 0.3** |
| Kidney | 71.3 ± 0.9 | 73.0 ± 2.1 | 7.1 ± 0.2 | 10.0 ± 0.5** |
| Muscle | 37.6 ± 4.5 | 29.9 ± 2.8 | 2.5 ± 0.1 | 2.0 ± 0.2 |
| Adipose | 2.1 ± 0.1 | 1.3 ± 0.2 | 0.3 ± 0.0 | 0.3 ± 0.0 |
| Pancreas | 146.8 ± 6.2 | 141.6 ± 6.4 | 3.0 ± 0.3 | 4.0 ± 0.4 |
| Spleen | 78.6 ± 1.8 | 80.4 ± 2.7 | 2.9 ± 0.1 | 8.0 ± 0.4** |
| Bone | 117.4 ± 3.4 | 141.8 ± 3.1* | 0.6 ± 0.0 | 1.4 ± 0.1** |

Data are expressed as mean ± SE for 7–8 mice.
Significance: *$p$<0.005 and **$p$<0.0001 vs. control.

**Table 5**
Area under the curve (AUC) of radioactivity of $^{65}$Zn and the ratio of $AUC_{organ}$ to $AUC_{blood}$ in ddY mice given oral $ZnCl_2$ and [ZPS].

| Organ | AUC (nCi hr g$^{-1}$) | | $AUC_{organ}/AUC_{blood}$ | |
|---|---|---|---|---|
| | $ZnCl_2$ | [ZPS] | $ZnCl_2$ | [ZPS] |
| Blood | 52 ± 1 | 694 ± 64* | | |
| Liver | 184 ± 9 | 1782 ± 118* | 3.5 | 2.6 |
| Kidney | 164 ± 7 | 1367 ± 79* | 3.2 | 2.0 |
| Muscle | 25 ± 1 | 183 ± 14* | 0.5 | 0.3 |
| Adipose | 16 ± 2 | 141 ± 11* | 0.3 | 0.2 |
| Pancreas | 312 ± 14 | 2134 ± 157* | 6.0 | 3.1 |
| Spleen | 197 ± 7 | 1053 ± 80* | 3.8 | 1.5 |
| Bone | 148 ± 6 | 3494 ± 277* | 2.9 | 5.0 |

Data are expressed as the mean ± SE for 5–7 mice.
Significance: *$p$<0.05 vs. $ZnCl_2$.

concentrations were also exceedingly increased in [$^{65}$ZPS]-treated mice compared with those of $^{65}ZnCl_2$-treated mice (Table 5). However, based on the $AUC_{organ}/AUC_{blood}$ values, a lower tissue penetration ratio was observed for $^{65}$Zn derived from [$^{65}$ZPS] compared to that of $^{65}ZnCl_2$. These results suggest that [ZPS] was inferior to $ZnCl_2$ with regard to tissue penetration of zinc from circulating blood to the tissues.

## 4. Discussion

Based on the results from in vitro experiments, high antidiabetic effects of [ZPS] were expected. We tested our expectations by using a lower oral dose (1.25–2.5 mg Zn/kg body weight) unlike previous studies in KK-A$^y$ mice. The insulin-like activity of [ZPM] and [ZPS] were not significantly different (Table 2); nevertheless, the hypoglycemic effects of [ZPS] were shown to be the strongest in previously

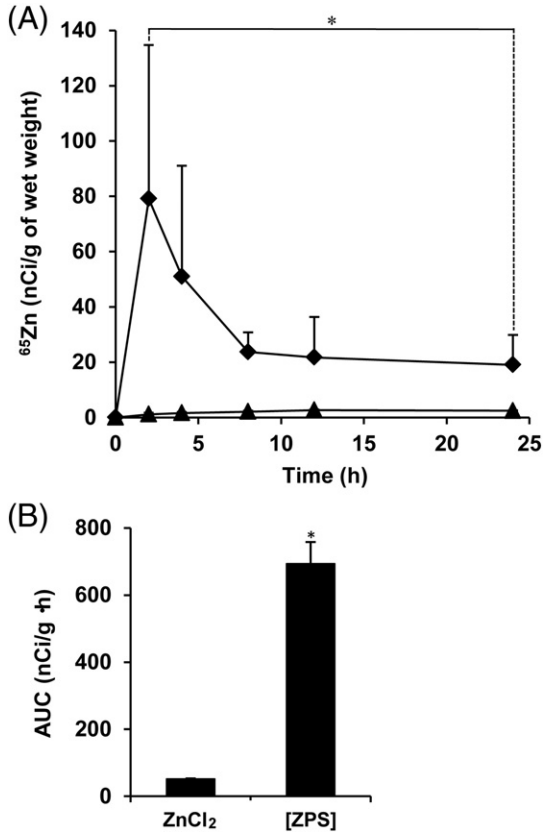



**Fig. 3.** Blood concentration (A) and area under the curve (AUC) (B) of $^{65}$Zn in ddY mice orally administered $^{65}ZnCl_2$ (▲) or [ZPS] (♦). Data are expressed as mean ± SE for 5–7 mice. Significance: *$p$<0.05 vs. $ZnCl_2$.

synthesized antidiabetic zinc complexes (Fig. 2(A)) [22,35–37]. Consequently, it was necessary to evaluate the antidiabetic effects of 2-SPO because 2-MPO, the ligand of [ZPM], has no insulin-like activity [22]; however, it was difficult to evaluate the antidiabetic effects of 2-SPO in vivo because it easily undergoes diselenide(Se-Se)-mediated dimerization via atmospheric oxidation. However, we expect that the ligand might have additional antidiabetic effects for [ZPS] in terms of higher hypoglycemic effects compared to that of [ZPM]. It was previously reported that selenium compounds such as Ebselen oxidize thiolate ligands in zinc clusters of metallothionein (MT) by the formation of a selenodisulfide (Se–S) and release zinc from MT [38,39]. Therefore, it may be possible for ligands dissociating from [ZPS] to interact with zinc/thiolate-coordination environment and catalyze the release of zinc from MT. A change in the distribution of zinc possibly contributes to the hypoglycemic effects of [ZPS]. Considering that the calculated p$K_a$ value of selenol in 2-SPO is −0.68 ± 0.70 at 25 °C [40], we propose that selenol exists in deprotonated state under the physiological pH range and interacts with any biomolecules.

Distribution of zinc derived from [ZPS] was not clearly measured, but that of selenium was notably present in vivo (Table 4). This might be because of the large amount of zinc in the target organs relative to the low dose of zinc administered, as well as rapid dissemination or excretion by the body [41]. No differences were observed in the blood and plasma concentrations of zinc between the control and treatment groups. On the other hand, blood concentrations of selenium in the treatment group increased although plasma selenium levels remained unchanged. These results suggest that [ZPS] might partly dissociate into zinc ion and the ligand in blood cells, and that zinc derived from [ZPS] might then transfer from the blood cells to plasma or to several other tissues, while the ligand is held to some extent in the cells. Consequently, after gastrointestinal absorption of [ZPS], slower organ distribution of the ligand compared to zinc was suspected. Meanwhile, another speculation on the existing form of the complex might be possible. Oral administration of [ZPS] remarkably increased the gastrointestinal absorption of zinc (Fig. 3), suggesting that most of the chelation of administered [ZPS] is maintained during the absorption process. To the best of our knowledge, these results are the first report of the relations between dissociation and distribution of zinc and the ligand derived from an antidiabetic zinc complex.

Interestingly, in this study, fasting BGL of mice treated with [ZPS] was likely to increase relative to the control group; fasting BGL of the control and treatment groups were 108.5 ± 29.3 and 138.0 ± 37.1 mg/dL, respectively. This result is supported by previous studies; a significant increase in fasting BGL was observed in KK-A$^y$ mice that were administered a high dose of [ZPS] (2.5–5.0 mg Zn/kg body weight) for 14 days. Fasting BGL of the control and treatment groups were 84.6 ± 14.7 and 129.3 ± 36.5 mg/dL, respectively. These results suggest that [ZPS] is capable of regulating BGL within normal limits and that its properties are advantageous as a potential antidiabetic agent.

In patients and animal models of T2D, abnormalities in homeostasis, such as zinc deficiency, are well documented [18], and organ zinc concentrations decrease in KK-A$^y$ mice relative to healthy C57BL mice, particularly in the pancreas and muscle, as these are the main organs responsible for glucose metabolism [32]. Such a low zinc status might be caused by impaired zinc absorption in the gastrointestinal tract [20]. If such abnormal zinc metabolism affects the pathogenesis of T2D, reducing zinc deficiency might be important in the treatment of DM. With regard to efficient zinc supplementation and administering zinc complexes, the relationship between zinc distribution and the stability of zinc complexes is highly important, as indicated by findings in this study as well as a previous study [42]. Therefore, a zinc complex with moderate stability and lipophilicity is required to transport zinc efficiently to the organs responsible for glucose metabolism.

Using the isotope tracer technique, the lower $AUC_{organ}/AUC_{blood}$ values of zinc derived from [ZPS] compared to that of $ZnCl_2$ was calculated. In other words, free zinc is superior to zinc complexes in terms of tissue penetration. However, gastrointestinal absorption of free zinc is lower than that of zinc complexes [43], and greater $^{65}Zn$ levels were measured in organs treated with [$^{65}$ZPS] compared to those treated with $^{65}ZnCl_2$. Considering a patient's quality of life, oral supplementation of zinc is useful, and complexation of zinc is totally advantageous for more efficient distribution of oral zinc to any tissue; i.e., achievement of high zinc uptake by administering [ZPS] orally might be useful in the treatment of DM.

Several organoselenium compounds are already reported as potential therapeutic agents [44]. This study, however, is the first report that an organoselenium compound as a metal ligand has potential as a therapeutic agent for DM. Although dietary levels of the desired amount of selenium fall within a very narrow range, organoselenium compounds are characterized by low toxicity, even when used at supra pharmacological doses [45–47]. Furthermore, the experimental dose of zinc derived from [ZPS] is less than the clinical dose of zinc acetate used to treat Wilson's disease [48]. Thereby, it is concluded that [ZPS] is a potential novel antidiabetic agent.

## 5. Conclusion

The hypoglycemic effects of [ZPS] in KK-A$^y$ mice were apparent at lower doses than previously studied zinc complexes. In addition, greatly elevated gastrointestinal absorption of zinc by [ZPS], and no intravital toxicity of [ZPS] was observed, suggesting the possibility of the organoselenium ligand as a new metal delivery system for treating DM.

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## References

[1] J.E. Shaw, R.A. Sicree, P.Z. Zimmet, Diab. Res. Clin. Pract. 87 (2009) 4–14.
[2] A. Amos, D.G. McCarthy, P. Zimmet, Diabet. Med. 14 (1997) S5–S85.
[3] R.M. Sapolsky, L.C. Krey, B.S. McEwen, Endocr. Rev. 7 (1986) 284–301.
[4] C.E. Heyliger, A.G. Tahiliani, J.H. McNeill, Science 22 (1985) 1474–1477.
[5] M. Nishida, H. Sakurai, J. Kawada, M. Koyama, J. Takada, Naturwissenschaften 76 (1989) 220–222.
[6] J.H. McNeill, H.L.M. Delgatty, M.L. Battell, Diabetes 40 (1991) 1675–1678.
[7] R.A. Anderson, N. Cheng, N.A. Bryden, M.M. Polansky, J. Chi, J. Feng, Diabetes 46 (1997) 1786–1791.
[8] E.J. Coleman, Annu. Rev. Biochem. 61 (1992) 897–946.
[9] C.T. Chasapis, A.C. Loutsidou, C.A. Spiliopoulou, M.E. Stefanidou, Arch. Toxicol. 86 (2012) 521–534.
[10] P. Zalewski, S. Millard, I. Forbes, O. Kapaniris, S. Slavotinek, W. Betts, A. Ward, S. Lincoln, I. Mahadevan, J. Histochem. Cytochem. 42 (1994) 877–884.
[11] L. Coulston, P. Dandona, Diabetes 29 (1980) 665–667.
[12] A. Shisheva, D. Gefel, Y. Shechter, Diabetes 41 (1992) 982–988.
[13] M.D. Chen, S.J. Liou, P.Y. Lin, V.C. Yang, P.S. Alexander, W.H. Lin, Biol. Trace Elem. Res. 61 (1998) 303–311.
[14] O. Ezaki, J. Biol. Chem. 264 (1989) 16118–16122.
[15] H. Haase, W. Maret, Exp. Cell Res. 291 (2002) 289–298.
[16] R. Ilouz, O. Kaidanovich, D. Gurwitz, H. E-Finkelman, Biochem. Biophys. Res. Commun. 295 (2002) 102–106.
[17] S.F. Simon, C.G. Taylor, Exp. Biol. Med. 226 (2001) 43–51.
[18] J.J. Cunningham, A. Fu, P.L. Mearkle, R.G. Brown, Metabolism 43 (1994) 1558–1562.
[19] E. Ho, N. Quan, Y.H. Tsai, W. Lai, T.M. Bray, Exp. Biol. Med. 226 (2001) 103–111.
[20] M.J. Salgueiro, N. Krebs, M.B. Zubillaga, R. Weill, E. Postaire, A.E. Lysionek, R.A. Caro, T. De Paoli, A. Hager, J. Boccio, Biol. Trace Elem. Res. 81 (2001) 215–228.
[21] Y. Yoshikawa, H. Yasui, Curr. Top. Med. Chem. 12 (2012) 210–218.
[22] Y. Yoshikawa, A. Murayama, Y. Adachi, H. Sakurai, H. Yasui, Metallomics 3 (2011) 686–692.
[23] L.A. Daniels, Biol. Trace Elem. Res. 54 (1996) 185–199.
[24] M.P. Rayman, Lancet 356 (2005) 233–241.
[25] S.R. Stapleton, Cell. Mol. Life Sci. 57 (2000) 1874–1879.
[26] O. Ezaki, J. Biol. Chem. 265 (1990) 1124–1128.
[27] N.B.V. Barbosa, J.B.T. Rocha, D.C. Wondracek, J. Perottoni, G. Zeni, C.W. Nogueira, Chem. Biol. Interact. 163 (2006) 230–238.
[28] C.O. Kienitz, C. Thöne, P.G. Jones, Inorg. Chem. 35 (1996) 3990–3997.
[29] D.H. Barton, D. Crich, Y. Herye, P. Potier, J. Thierry, Tetrahedron 41 (1985) 4347–4357.
[30] R.C. Hider, R. Ejim, P.D. Gale, E. Huehns, J.B. Porter, Biochem. Pharmacol. 39 (1990) 1005–1012.
[31] M. Nakai, H. Watanabe, C. Fujiwara, H. Kakegawa, T. Satoh, J. Takeda, R. Matsushita, H. Sakurai, Biol. Pharm. Bull. 18 (1995) 719–725.
[32] Y. Adachi, J. Yoshida, Y. Kodera, T. Kiss, T. Jakusch, E.A. Enyedy, Y. Yoshikawa, H. Sakurai, Biochem. Biophys. Res. Commun. 351 (2006) 165–170.
[33] J. Bailer, J. Pharmacokinet. Biopharm. 16 (1987) 303–309.
[34] S. Karmaker, T.K. Saha, Y. Yoshikawa, H. Sakurai, Chem. Med. Chem. 2 (2007) 1607–1612.
[35] Y. Adachi, J. Yoshida, Y. Kodera, T. Kiss, T. Jakusch, E.A. Enyedy, Y. Yoshikawa, H. Sakurai, Biochem. Biophys. Res. Commun. 351 (2006) 165–170.
[36] Y. Yoshikawa, Y. Adachi, H. Sakurai, Life Sci. 80 (2007) 759–766.
[37] H. Murakami, H. Yasui, Y. Yoshikawa, Chem. Pharm. Bull. 60 (2012) 1096–1104.
[38] C. Jacob, W. Maret, B.L. Vallee, Biochem. Biophys. Res. Commun. 248 (1998) 569–573.
[39] C. Jacob, W. Maret, B.L. Vallee, Proc. Natl. Acad. Sci. 96 (1999) 1910–1914.
[40] Calculated using Advanced Chemistry Development (ACD/Labs) Software V8.14 (1994-2010 ACD/Labs).
[41] N.F. Krebs, J. Nutr. 130 (2000) 1374S–1377S.
[42] R.M. Epand, A.R. Stafford, M. Tyers, E. Nieboer, Mol. Pharm. 27 (1985) 366–374.
[43] J.C. King, D.M. Shames, L.R. Woodhouse, J. Nutr. 130 (2000) 1360S–1366S.
[44] M.S. Garcia, Curr. Med. Chem. 11 (2004) 1657–1669.
[45] R. Fachinetto, L.A. Pivetta, M. Farina, R.P. Pereira, C.W. Nogueira, J.B. Rocha, Food Chem. Toxicol. 44 (2006) 588–594.
[46] J. Perottoni, F.C. Meotti, V. Folmer, L. Pivetta, C.W. Nogueira, G. Zeni, J.B.T. Rocha, Environ. Toxicol. Pharmacol. 19 (2005) 239–248.
[47] H. Masumoto, H. Hakusui, M. Takaichi, T. Yokota, T. Honda, Y. Esumi, Xenobiotic Metabol. Dispos. 12 (1997) 610–618.
[48] T. Mizuochi, A. Kimura, N. Shimizu, H. Nishiura, M. Matsushita, M. Yoshino, J. Pediatr. Gastroenterol. Nutr. 53 (2011) 365–367.