Potential Insulinominetic Agents of Zinc(II) Complexes with Picolinamide Derivatives: Preparations of Complexes, *in Vitro* and *in Vivo* Studies

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Following the finding of *in vitro* insulinominetic activities of new prepared Zn(II) complexes with amide ligands (2-picolinamide (pa-a) and 6-methyl-2-picolinmethylamide (6mpa-ma)) in isolated rat adipocytes treated with epinephrine in terms of inhibition of free fatty acid release, their blood glucose normalizing effects were observed on daily intraperitoneal injections for 14 d in a type 2 diabetes mellitus model animal, KK-A^y mice. The blood glucose levels of KK-A^y mice were maintained in a normal range during the administration of both complexes. After the administration of each complex for 14 d, the improvement of glucose metabolism was confirmed as judged by the glucose tolerance test.

Key words Zinc(II) complex; picolinamide derivative; type 2 diabetes mellitus; insulinominetic activity

Diabetes mellitus (DM), one of the life-style related diseases, is a metabolic disease which shows the symptom of hyperglycemia and causes many complications.[1,2] Recently, DM is becoming a serious social problem not only in Japan but also around the world, and the number of people suffering from DM has increased to over 14 million including the estimate of potential patients in Japan. Many researchers have enthusiastically studied the development of antidiabetic agents. However, according to reports, many therapeutics have side effects, therefore new antidiabetic agents with high effectiveness and low side effects are eagerly sought all over the world.

During the endeavor, a very important fact was revealed that several metal ions show insulinomimetic activity.[3—12] For example, Schwarz and Mertz. reported that the chromium ion stimulates insulin function and improves glucose tolerance.[3] Tuvemo *et al.*[4] and Paolisso *et al.*[5] proposed that hypomagnesemia is related to DM and thus the insulin sensitivity is improved by magnesium administration. Since the 1980s it has been recognized that vanadium has normoglycemic activity in streptozotocin-induced type 1 DM rats (STZ rats),[6—9] and the investigations on vanadium are energetically extended. In 1980, Coulston and Dandona found that Zn(II), one of the essential elements in animals and humans, stimulates lipogenesis in rat adipocytes similarly to the action of insulin.[10]

There is a close relation between Zn(II) and insulin, because Zn(II) is essential for the stabilization of the insulin precursor (proinsulin) and taken into the pancreas and secreted to the blood with insulin.[13] In addition, it was observed that Zn(II) concentration in the fingernails of the patients with DM decreases[14] and consequently the excretion of Zn(II) into the urine increases.[15] Therefore, the study on the relationship between Zn(II) and DM is very important and many researchers have investigated insulinomimetic activity of Zn(II).[11,12] Shisheva *et al.* reported that Zn(II) ions administered orally to STZ rats reduce the blood glucose levels as much as 50%.[11] Song *et al.* observed that when STZ rats are given drinking water containing Zn(II) with cyclo(His-Pro), the blood glucose levels were lower than those of the rats given Zn(II) alone.[12] Furthermore, previous studies have demonstrated that zinc acts on adipocytes and promotes the induction of leptine, and also acts on the pancreas, therefore it helps insulin to combine with insulin receptor, resulting in improvement of the conditions of type 2 DM.[16,17]

On the basis of these observations, Zn(II) is expected to be less toxic than other metal ions. Generally, it is known that the complexation of free metal ions lowers the toxicity of the metal ions and promotes their absorption into the blood.[18,19] We indicated that Zn(II) complexes with maltol, picolinic acid and amino acids have higher insulinomimetic activity than $ZnSO_4$ in *in vitro* experiments, and the administrations of the Zn(II) complexes to KK-A^y mice, a type 2 DM model animal, were found to show normoglycemic activity.[20—23] However, such Zn(II) complexes were all molecular complexes. In recent years, many researchers have proposed metal-containing therapeutic agents of cationic complexes such as BBR3364 of cisplatin derivatives[24] and ^{99m}Tc-tetrofosfin complex of diagnostic radiopharmacuetics.[25] In this paper, we have planned to synthesize new cationic Zn(II) complexes with picolinamide and its derivative and to estimate both *in vitro* insulinomimetic activities and *in vivo* blood glucose normalizing effects in KK-A^y mice. Four Zn(II) complexes, Zn(pa-a)$_3$Cl$_2$ **1**, Zn(pa-a)$_3$(ClO$_4$)$_2$ **2**, Zn(6mpa-ma)$_2$Cl$_2$ **3**, and Zn(6mpa-ma)$_2$SO$_4$ **4** of 2-picolinamide (pa-a) and 6-methyl-2-picolinemethylamide (6mpa-ma) ligands exhibited higher insulinomimetic activities than those of $VOSO_4$ and $ZnSO_4$ as standard.

Experimental

Materials Zinc sulfate ($ZnSO_4 \cdot 7H_2O$), NEFA-C test Wako, and acacia were purchased from Wako Pure Chemicals (Osaka, Japan). D-(+)-Glucose was obtained from Nakalai Tesque Inc. (Kyoto, Japan). (±)-Epinephrine hydrochloride, collagenase and bovine serum albumin (BSA) were from Sigma Chemical Co. (St. Louis, U.S.A.). All other reagents were of analytical reagent quality and were used without further purification. Purity of $ZnSO_4 \cdot 7H_2O$ was determined by chelatometry using Cu-Pan (Cu-1-(2-pyridyl-azo-2-naphthol) (Dojindo, Kumamoto, Japan) as indicator.

Instruments Elemental analyses were carried out on a Perkin-Elmer 240C Elemental Analyzer (MA, U.S.A.). Fourier transform (FT)-IR spectra were recorded on a Jasco FT/IR-420 (Tokyo, Japan) spectrophotometer. ^{1}H-NMR spectra were recorded on a JEOL LA-300 WB FT-NMR spectrometer (Tokyo, Japan). FAB-MS was obtained with a JEOL AX-500 (Tokyo,

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Japan). Melting points were taken with Yanako MP-J3 Micro Point Aparatus (Kyoto, Japan). Hemoglobin A_{1c} (HbA_{1c}) levels were measured with DCA2000 System (Bayer Sankyo Co. Ltd., Tokyo, Japan). The blood glucose levels were measured with a Glucocard (Arkray Co. Ltd., Kyoto, Japan). The serum concentrations of blood urea nitrogen (BUN), glutamic-oxaloacetic transaminase (GOT), glutamic-pyruvic transaminase (GPT), and total cholesterol (TCHO) were determined by a Fuji Dry Chem (Tokyo, Japan).

Preparation of 6-Methyl-2-picolinmethylamide (6mpa-ma) Twenty-five milliliters of 98% H_2SO_4 was added by drops to 6-methyl-2-picolinic acid (5.48 g, 40 mmol) in 100 ml of methanol in an ice bath. The reaction mixture was stirred at 50 °C for 3 d. After the reaction mixture was poured into saturated $NaHCO_3$ and extracted with $CHCl_3$ three times, the $CHCl_3$ layer was dried over anhydrous Na_2SO_4, and evaporated to give methyl 6-methyl-2-picolinate (4.95 g, 82.0%). A 40% CH_3NH_2 (25.5 g, 328 mmol) in methanol was added to methyl 6-methyl-2-picolinate (4.95 g, 32.8 mmol). The mixture was stirred at room temperature for 3 d, and then evaporated. The residue was purified as a pale brown oil by sephadex LH-20 using methanol (4.55 g, 92%). *Anal.* Calcd for $C_8H_{10}N_2O \cdot 0.8H_2O$: C, 58.38; H, 7.10; N, 17.02. Found: C, 58.20; H, 6.61; N, 16.98. ^{1}H-NMR ($CDCl_3$) δ: 2.56 (3H, s), 3.03 (3H, d, J=5.1 Hz), 7.27 (1H, d, J=7.7 Hz), 7.72 (1H, dd, J=7.7, 7.9 Hz), 8.00 (1H, d, J=7.7 Hz), 8.08 (1H, br). IR (neat) cm^{-1}: 1667 for $\nu_{C=O}$. FAB-MS: m/z: 151 $(M+H)^+$.

Preparation of Zn(pa-a)$_3$Cl$_2$ 1 To a methanol solution of pa-a (0.37 g, 3.0 mmol), a methanol solution of $ZnCl_2$ (0.14 g, 1.0 mmol) was added and stirred for 3 h at room temperature. **1** was obtained as the residue by removing the solvent. Yield: 0.50 g (98%). *Anal.* Calcd for $C_{18}H_{18}N_6O_3Cl_2Zn \cdot 0.4H_2O$: C, 42.40; N, 16.48; H, 3.72. Found: C, 42.64; N, 16.40; H, 3.67. IR (KBr): 1667 cm^{-1} for $\nu_{C=O}$. Mp; 112—130 °C.

Preparation of Zn(pa-a)$_3$(ClO$_4$)$_2$ 2 To an aqueous solution of pa-a (0.49 g, 4.0 mmol), an aqueous solution of $ZnSO_4 \cdot 7H_2O$ (0.58 g, 2.0 mmol) and $Ba(ClO_4)_2 \cdot 3H_2O$ (0.78 g, 2.0 mmol) were added and stirred overnight at room temperature. After the filtration of $BaSO_4$ and removal of the solvent, **2** was recrystallized from a small amount of hot water. Yield: 0.23 g (27%). *Anal.* Calcd for $C_{18}H_{18}N_6O_{11}Cl_2Zn \cdot 0.8H_2O$: C, 33.51; N, 13.13; H, 3.06. Found: C, 33.53; N, 13.02; H, 3.10. IR (KBr): 1671 cm^{-1} for $\nu_{C=O}$. Mp; 265—270 °C.

Preparation of Zn(6mpa-ma)$_2$Cl$_2$ 3 The complex **3** was prepared by referring to the method of **1**. Yield: 0.39 g (96%). *Anal.* Calcd for $C_{16}H_{20}N_4O_2Cl_2Zn \cdot 0.8H_2O$: C, 42.40; N, 12.42; H, 4.83. Found: C, 42.72; N, 12.24; H, 4.88. IR (KBr): 1645 cm^{-1} for $\nu_{C=O}$. Mp; >300 °C.

Preparation of Zn(6mpa-ma)$_2$SO$_4$ 4 To an aqueous solution of 6mpa-ma (0.33 g, 2.0 mmol), an aqueous solution of $ZnSO_4 \cdot 7H_2O$ (0.29 g, 1.0 mmol) was added and following stirred for 3 h at room temperature. **4** was obtained as the residue by removing the solvent. Yield: 0.50 g (94%). *Anal.* Calcd for $C_{16}H_{20}N_4O_6SZn \cdot 4.3H_2O$: C, 35.64; N, 10.39; H, 5.35. Found: C, 35.68; N, 10.36; H, 5.25. IR (KBr): 1643 cm^{-1} for $\nu_{C=O}$. Mp; >300 °C.

Inhibition Effects of Zn(II) Complexes on Free Fatty Acid (FFA) Release from Isolated Rat Adipocytes Treated with Epinephrine Isolated male rat adipocytes (1.0×10^6 cells/ml) prepared as described in ref. 26 were preincubated at 37 °C for 30 min with various concentrations (10^{-4}—10^{-3} M) of Zn(II) complexes in KRB buffer (120 mM NaCl, 1.27 mM $CaCl_2$, 1.2 mM $MgSO_4$, 4.75 mM KCl, 1.2 mM KH_2PO_4, 24 mM $NaHCO_3$: pH 7.4) containing 2% BSA. A 10^{-4} M epinephrine was then added to the reaction mixtures and the resulting solutions were incubated at 37 °C for 3 h. The reactions were stopped by soaking in ice water and the mixtures were centrifuged at 3000 rpm for 10 min. For the outer solutions of the cells, FFA levels were determined with NEFA-C test Wako.

Blood Glucose Lowering Effects of Zn(II) Complexes, 1 and 4, in KK-A^y Mice KK-A^y mice (4 weeks old : CREA Japan Inc., Tokyo, Japan) were kept in the laboratory for 4 weeks. The 8 weeks old KK-A^y mice with a type 2 DM model received daily intraperitoneal (i.p.) injections (5 mice in a group) of **1** and **4** dissolved in 5% acacia vehicle at about 10 : 30 a.m. after the determination of their blood glucose levels for 14 d. The blood sample for the analysis of glucose levels was obtained from the tail vein of each mouse and measured with Glucocard. Body weights of KK-A^y mice who were allowed free access to solid food (CREA Japan Inc.) and tap water were measured daily during the administration of **1** and **4**. In addition, intakes of solid food and drinking water in each mouse were checked daily throughout the experiments. The dose of **1** and **4** were 4.0 mg Zn (61.2 μmol for **1** and **4**)/kg body weight. The blood samples for the analyses of BUN, GOT, GPT, TCHO, and FFA were withdrawn from the cavernous sinus with capillary under anesthesia with ether.

Oral Glucose Tolerance Test (OGTT) After daily i.p. injection of **1** and **4** for 14 d, the mice were fasted for 14 h and the blood glucose levels (0, 30, 60, 90, 120 min) were measured after gastric gavage of 1 g glucose/kg body weight for each mouse. The blood samples obtained from a tail vein were measured with Glucocard.

Results and Discussion

***In Vitro* Insulinomimetic Activity of Zn(II) Complexes; 1—4** *In vitro* insulinomimetic activities of four Zn(II) complexes were examined with regard to inhibition of FFA release from isolated rat adipocytes treated with epinephrine. Complexes **1**—**4** were confirmed to act dose-dependently in the concentration of 10^{-4}, 5×10^{-4} and 10^{-3} M of the Zn(II) complexes (Fig. 1). The apparent IC_{50} values, the 50% inhibitory concentration of the Zn(II) complexes on the FFA release, were 0.70, 0.71, 0.95, and 0.97 mM for **1**, **2**, **3**, and **4**, respectively (Table 1). These Zn(II) complexes showed higher insulinomimetic activities than the standards $VOSO_4$ and $ZnSO_4$. The IC_{50} values of Zn(II) complexes with different types of counter anion (Cl^- and ClO_4^- for Zn(pa-a)$_3^{2+}$, Cl^- and SO_4^{2-} for Zn(6mpa-ma)$_2^{2+}$) were not significant. From these results, the effect of counter anions of Zn(II)

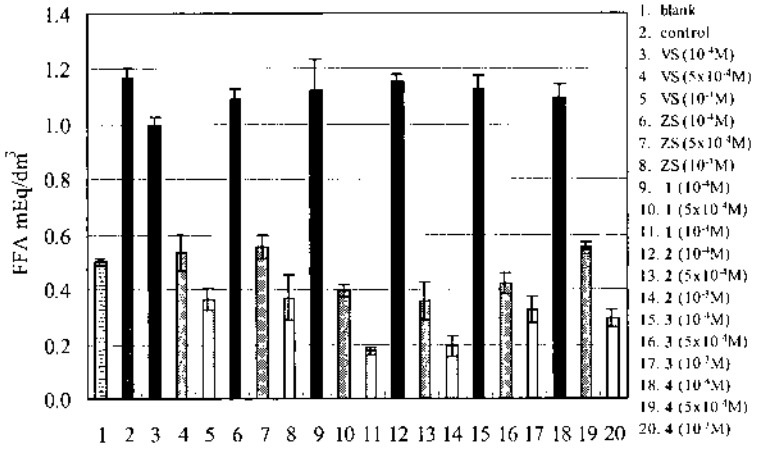

Fig. 1. Inhibitory Effects of $VOSO_4$ (VS), $ZnSO_4$ (ZS) and Zn(II) Complexes (**1**—**4**) on FFA Release from Isolated Rat Adipocytes Treated with Epinephrine (EP)

Rat adipocytes were prepared as reported ref. 26. Each column is expressed as the mean±S.D. for 3 experiments. Blank: cells only; control: cells plus 10^{-5} M EP. In each system, adipocytes (1.0 ± 10^6 cells/ml) were treated with 10^{-4}, 5×10^{-4}, 10^{-3} M (3—17 column) of the compound in each numerical order, respectively, for 30 min and then incubated with 10^{-5} M EP for 3 h at 37 °C.

Table 1. Estimated IC_{50} Values of Zn(II) Complexes (**1**—**4**)

Complex	IC_{50} (mM)
$VOSO_4$	1.00±0.08
$ZnSO_4$	1.58±0.05
1	0.70±0.04**
2	0.71±0.05**
3	0.95±0.05*
4	0.97±0.04*

* Significance at $p<0.01$ *vs.* $ZnSO_4$. ** Significance at $p<0.005$ *vs.* $ZnSO_4$.

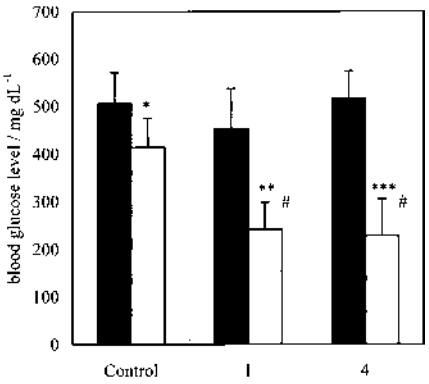

Fig. 2. Changes of Blood Glucose Levels before and after i.p. Injections for 14 d

Hyperglycemic KK-A^y mice received daily i.p. injection of 5% acacia (control) ($n=5$) and Zn(II) complexes of **1** and **4** at a dose of 4.0 mg Zn/kg body weight ($n=5$) for 14 d. Each column is expressed as the mean±S.D. for 5 mice. * Significance at $p<0.05$ *vs.* before the i.p. injections. ** Significance at $p<0.001$ *vs.* before the i.p. injections. *** Significance at $p<0.0001$ *vs.* before the i.p. injections. # Significance at $p<0.001$ *vs.* after the i.p. injections of control.

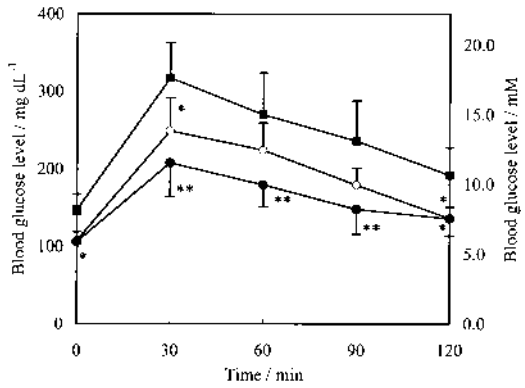

Fig. 3. OGTT for Control KK-A^y Mice (—■—) and KK-A^y Mice Treated with Zn(II) Complex **1** (—○—) and **4** (—●)

After i.p. injections of **1** and **4** for 14 d, the mice were fasted for 14 h and the blood glucose levels (0, 30, 60, 90, 120 min) were measured after gastric gavage of 1 g glucose/kg body weight. The blood samples obtained from a tail vein were measured with Glucocard. Each symbol is expressed as the mean±S.D. for 5 mice. * Significance at $p<0.05$ *vs.* untreated KK-A^y mice. ** Significance at $p<0.01$ *vs.* untreated KK-A^y mice.

Table 2. Hemoglobin A_{1c} (HbA_{1c}) Levels of KK-A^y Mice before and after the i.p. Injections of 5% Acacia (Control) and Zn(II) Complexes **1** and **4** for 14 d

Treatment	HbA_{1c} (%)	
	Before the treatment	After the treatment
Control	7.6±0.5	8.3±0.3
1	7.0±0.8	6.4±0.8*
4	7.4±0.8	6.1±0.5**

Values are mean±S.D. for 5 or 4 mice (**1**; 5 mice and control and **4**; 4 mice). * Significance at $p<0.005$ *vs.* after the i.p. injections of control KK-A^y mice. ** Significance at $p<0.001$ *vs.* after the i.p. injections of control KK-A^y mice.

complexes was not observed in the insulinomimetic activities.

Blood Glucose Normalizing Effects of Zn(II) Complexes with Amides in KK-A^y Mice We tested *in vivo* blood glucose normalizing effects of **1** and **4** in KK-A^y mice receiving daily i.p. injections for 14 d. A complex **1** with Cl^-, which exists in animals and humans, was administered to KK-A^y mice. A complex **4** was injected to KK-A^y mice to compare with **1**. Figure 2 presents the changes of blood glucose levels of KK-A^y mice before and after i.p. administrations. When **1** and **4** were administered at a dose of 4.0 mg (61.2 μmol for **1** and **4**) Zn/kg body weight, the blood glucose levels dropped down to approximately 200 mg/dl (11.1 mM) after 24 h and the same dose was given to the mice to maintain the blood glucose level at around 200 mg/dl. The effectiveness of **1** on the blood glucose level was almost as good as **4**. During the treatment of **1** and **4** for 14 d, the body weights of KK-A^y mice steadily increased from 39.6±1.3 and 40.1±1.5 to 41.9±2.1 and 42.3±2.0 g, respectively. The decrease of body weight associated with toxicity was not observed in KK-A^y mice treated **1** and **4**.

Oral Glucose Tolerance Test After the daily i.p. administrations for 14 d, we examined the oral glucose tolerance test (OGTT) to examine if the glucose metabolism of KK-A^y mice was improved or not. As shown in Fig. 3, when the KK-A^y mice were given orally 1 g glucose/kg body weight after they were fasted for 14 h, the blood glucose levels of KK-A^y mice treated with 5% acacia (untreated KK-A^y mice) went up to a maximum of 317 mg/dl (17.6 mM) in 30 min and decreased slowly afterwards. The changes of the blood glucose levels of the group treated with **4** for 2 h at 30 min intervals were significantly lower compared with the untreated KK-A^y mice (0 min: at the time of the administration of glucose). It was observed that the glucose metabolism of KK-A^y mice was improved by i.p. administration of Zn(II) complex **4**. In comparison with the blood glucose levels of the group treated with **4**, the group treated with **1** was slightly higher, but the state of DM in both groups treated with **1** and **4** was confirmed to be improved.

HbA_{1c} and the Serum Parameters Furthermore, we measured HbA_{1c}, which shows the number of glucose molecules attached to hemoglobin, in red blood cells and indicated average blood glucose levels over a long period. In the untreated KK-A^y mice, the HbA_{1c} levels increased from 7.6±0.5 to 8.3±0.3 (%) before and after the examination, respectively (Table 2). In contrast, HbA_{1c} levels of the KK-A^y mice treated with **1** and **4** were decreased from 7.0±0.8 and 7.4±0.8 to 6.4±0.8 and 6.1±0.5 (%), respectively, indicating that the blood glucose normalizing effects of Zn(II) complexes are long-term.

The serum parameters, GOT, GPT, TCHO, and BUN levels of KK-A^y mice, untreated and treated with **1** and **4** after 14 d administrations are summarized in Table 3. The GPT and TCHO levels were not altered between the untreated and the treated KK-A^y mice with Zn(II) complexes (Table 3). The GOT levels of KK-A^y mice treated with **1** and **4** were higher

Table 3. Serum Parameters of KK-A^y Mice after the i.p. Injections of 5% Acacia (Control) and Zn(II) Complexes **1** and **4** for 14 d

Treatment	BUN (mg/dl)	GOT (U/l)	GPT (U/l)	TCHO (mg/dl)	FFA (mEq/l)
Control	32.9±2.4	61±15	22±6	181±27	1.75±0.07
1	25.1±4.3	99±28*	24±6	140±37	1.27±0.10**
4	23.9±2.7*	111±36*	25±4	154±19	0.89±0.20**
C57/black	26.5±2.3	86±24	23±5	89±11	—

Values are mean±S.D. for 5 mice. * Significance at $p<0.05$ *vs.* control KK-A^y mice. ** Significance at $p<0.001$ *vs.* control KK-A^y mice.

than that of the untreated KK-A^y mice, because some serum samples of KK-A^y mice treated with **1** and **4** were partially hemolytic. The BUN levels of KK-A^y mice treated with **4** were lower than those of untreated KK-A^y mice. C57/black mice, non-diabetic mice and the same series as KK-A^y mice, received 5% acacia daily i.p. injections for 14 d and blood samples were taken to compare with the serum parameters of KK-A^y mice. The BUN levels of KK-A^y mice were higher than those of C57/black mice. The average BUN level of 10 weeks old C57/black for 3 mice was 26.5±2.3 (mg/dl). The BUN levels of KK-A^y mice treated with **4** were not different from those of C57/black. From these results, we consider that the function of the kidney of KK-A^y mice given Zn(II) complexes are not damaged, suggesting that Zn(II) complexes with amides, **1** and **4**, appear to be essentially non-toxic to the hepatic and renal functions. Furthermore, we measured the FFA levels of KK-A^y mice after 14 d administrations. KK-A^y mice administered Zn(II) complexes exhibited significantly lower FFA levels compared with the control mice. It was reported that increasing in the FFA levels give rise to the insulin resistance,[27] thus, it was suggested that the administrations of Zn(II) complex **1** and **4** improve the insulin resistance of KK-A^y mice.

In conclusion, we found that new Zn(II) complexes with 2-picolinamide and 6-methyl-2-picolinmethylamide show the high insulinomimetic activity *in vitro*, and furthermore the i.p. injections of these Zn(II) complexes normalize the blood glucose levels in KK-A^y mice with type 2 DM with slight body gain and without symptoms of toxicity in hepatic and renal functions. The present results propose that Zn(II) complexes with amide have the beneficial effects on type 2 DM. We continue our investigations focusing on the action mechanism in adipocytes, liver, and muscle, in the future.

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