

Insulinomimetic Zinc(II) Complexes with Natural Products: *In Vitro* Evaluation and Blood Glucose Lowering Effect in KK-A^y Mice with Type 2 Diabetes Mellitus

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***In vitro* insulinomimetic activities of Zn(II) complexes with three natural products, betaine, L-lactic acid, and D-(−)-quinic acid (qui), were found in rat adipocytes treated with epinephrine in terms of the inhibition of free fatty acid release. Based on the results, the blood glucose lowering effect in KK-A^y mice with type 2 diabetes mellitus was observed by daily i.p. injections of a monomeric zinc(II) complex, Zn(qui)₂, for 13 d.**

Key words Zinc(II) complex; natural product; insulinomimetic activity; KK-A^y mice; blood glucose lowering effect

Diabetes mellitus (DM) is a disease associated with absolute or relative insulin deficiency. Several types of medicines for the treatment of non-insulin-dependent type 2 DM have been developed all over the world. However, such therapeutic medicines have some problems involving side effects.¹⁾ In 1980, Coulston and Dandona reported the first insulinomimetic activity²⁾ of Zn(II) ion, which is one of the essential trace elements and exists in more than 300 metalloproteins and active sites of various metalloenzymes.³⁾ Following this finding, several research groups attempted to confirm the insulinomimetic activity of Zn(II) ion.^{4–6)} For example, Shisheva *et al.* reported that Zn(II) stimulates glucose uptake and lipid synthesis in rat adipocytes and oral administration of a high dose of ZnCl₂ (210 mg/kg body weight) to STZ-induced model diabetic rats with insulin-dependent type 1 diabetes reduces the blood glucose concentration as much as 50%.⁵⁾ On the other hand, Chen *et al.* found the hypoglycemic effect of ZnCl₂ (20 mM) in type 2 diabetic *ob/ob* mice.⁶⁾ However, Zn(II) complexes have never been examined.

It was reported that bis(maltolato)Zn(II) complex, Zn(Mal)₂, increases the absorption in erythrocytes more than free Zn(II) ion.⁷⁾ Recently, we found that Zn(Mal)₂ with a Zn(O₄) coordination mode has a higher insulinomimetic activity than free Zn(II) ions as estimated in *in vitro* experiments.⁸⁾ In addition, we have reported that Zn(II) complexes with Zn(O₄), Zn(N₂O₂), Zn(N₂S₂), Zn(O₂S₂), and Zn(S₄) coordination modes have high insulinomimetic activities with regard to *in vitro* experiments and blood glucose lowering effects.^{9–17)} In this paper, we report the insulinomimetic activities of Zn(II) complexes with natural products (betaine (bet), L-lactic acid (lac), and D-(−)-quinic acid (qui)) with a Zn(O_n) coordination mode and the blood glucose lowering effects of Zn(lac)₂ and Zn(qui)₂.

Experimental

Materials Zinc sulfate (ZnSO₄·7H₂O), barium hydroxide (Ba(OH)₂·8H₂O), bet, qui, and acasia were purchased from Wako Pure Chemicals (Osaka, Japan). L-Lactic acid was obtained from Tokyo Kasei Inc. (Tokyo, Japan). D-(+)-Glucose and LiOH·H₂O were obtained from Nakalai Tesque, Inc. (Kyoto, Japan). (±)Epinephrine hydrochloride, collagenase, and bovine serum albumin (BSA) were purchased from Sigma Chemi-

cal Co. (St. Louis, U.S.A.). All other reagents were of analytical reagent quality. Purity of ZnSO₄·7H₂O was determined by chelatometry using Cu-Pan (Cu-1-(2-pyridyl-azo-2-naphthol) complex, Dojindo (Kumamoto, Japan).

Instruments and Analytical Methods Elemental analyses were carried out on a Perkin-Elmer 240C Elemental Analyzer (MA, U.S.A.). FT-IR spectra were recorded on a Jasco FT/IR-420 spectrophotometer (Tokyo, Japan). Melting points were taken with a Yanako MP-J3 Micro Point Aparatus (Kyoto, Japan). Hemoglobin A_{1c} (HbA_{1c}) levels was measured with an immunoassay method (DCA2000 System, Bayer-Sankyo Co. Ltd., Tokyo, Japan) on the final administration (13 d, 10:30). Blood glucose levels were measured with a Glucocard (Arkray Co. Ltd., Kyoto, Japan). Blood samples for analyses of the triglyceride (TG), blood urea nitrogen (BUN), glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT), total cholesterol (TCHO), and free fatty acid (FFA) were obtained from orbital exsanguination under anesthesia with ether. The serum concentrations of TG, BUN, GOT, GPT, TCHO and FFA were determined by Fuji Dry Chem (Fuji Medical Co. Ltd., Tokyo, Japan).

Preparation of Zn(bet)₃(ClO₄)₂ and Zn(lac)₂ Zn(bet)₃(ClO₄)₂ and Zn(lac)₂ were prepared according to the methods in ref. 18 and 19, respectively.

Preparation of [Zn(qui)₂]_n To an aqueous solution of qui (12.0 mmol) and Ba(OH)₂ (6.0 mmol), an aqueous solution of ZnSO₄·7H₂O (6.0 mmol) was added and stirred for 4 h at room temperature. After filtration of the precipitation, water was evaporated. The complex was obtained as the residue, and recrystallized from hot water. Yield: 98%. *Anal.* Found (%): C, 37.24; H, 4.84. *Calcd* (%): Zn(C₇H₁₁O₆)₂·0.2H₂O; C, 37.26; H, 5.00. IR (KBr): 1638.2 cm⁻¹ for ν_{C=O}. [α]_D: −152.4° (H₂O). Mp: 272–282 °C (dec.).

Molecular Weight Measurements Molecular weights of Zn(bet)₃(ClO₄)₂, Zn(lac)₂, and [Zn(qui)₂]_n in aqueous solution were determined by using a vapor pressure osmometer, according to the method described previously.^{20,21)} Urea was used as a standard compound to calibrate the apparatus. A complex, Zn(bet)₃(ClO₄)₂, was reprecipitated from water/ethanol before use. The two Zn(II) complexes (Zn(lac)₂, and [Zn(qui)₂]_n) and urea were recrystallized from water before use. The measurements were performed in an aqueous solution at 30.0±0.05 °C *in vacuo*.

Inhibitory Effects of Zn(II) Complexes on FFA Release from Isolated Rat Adipocytes Treated with Epinephrine Isolated male rat adipocytes (1.0×10⁶ cells/ml) prepared as described in ref. 22 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 CaCl₂, 1.2 mM MgSO₄, 4.75 mM KCl, 1.2 mM KH₂PO₄, 24 mM NaHCO₃, 5 mM glucose; pH 7.4) containing 2% BSA (Sigma Chemical Co.). A 10⁻⁵ M epinephrine was then added to the incubation mixtures and the resulting solutions were incubated at 37 °C for 180 min. The reaction was stopped by soaking in ice water and the mixture was centrifuged at 3000 rpm for 10 min. For the outer solutions of the cells, FFA levels were determined with an FFA kit (Wako Pure Chemicals).

Blood Glucose Lowering Effects of Zn(II) Complexes, [Zn(lac)₂]₂ and

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Zn(qui)₂, in KK-A^y Mice KK-A^y mice (4 weeks old: CREA Japan Inc., Tokyo) were kept in the laboratory for 4 weeks acclimatization period. The 8 weeks old KK-A^y mice with type 2 diabetes mellitus received daily intraperitoneal (i.p.) injections (5 mice in each group) of [Zn(lac)₂]₂ and Zn(qui)₂, dissolved in 5% acacia vehicle at about 10:30 a.m. after the determination of their blood glucose levels for 13 d. The blood sample for the analyses of glucose levels was obtained from the tail vein of each mouse and the blood glucose level was measured with a Glucocard. Body weights of KK-A^y mice which were allowed free access to solid food (CREA Japan Inc.) and tap water were measured daily during the administration of [Zn(lac)₂]₂ and Zn(qui)₂. In addition, intakes of solid food and drinking water in each mouse were checked daily throughout the experiment.

Results and Discussion

Determination of Average Dissociation Number of Zn(II) Complexes in Aqueous Solution The average dissociation numbers of the three Zn(II) complexes prepared in an aqueous solution were determined by means of a vapor pressure osmometer *in vacuo*.

Firstly, the correlation between the bridge potential difference (ΔE (mV)) and the solute concentration ([urea] (mol kg⁻¹)) was examined, in which ΔE is proportional to the temperature difference between a pure solvent and a solution. A good linear relationship between ΔE and [urea] persists up to 0.080 mol kg⁻¹. The least-squares treatment showed the equation, $\Delta E = 3.39[\text{urea}] \pm 0.01$. On the other hand, the observed ΔE values for the complexes at 0.06 mol kg⁻¹ were found as follows: ΔE (mV) = 0.53, 0.09, and 0.18 for Zn(bet)₃(ClO₄)₂, Zn(lac)₂, and [Zn(qui)₂]_n, respectively.

It is known that urea is one of the most suitable compounds as a standard solute when a vapor pressure osmometer is utilized in an aqueous solution, because urea is neither a dissociable nor an associate compound at relatively low concentration ranges in aqueous solution. Namely, urea exists as a monomeric species in aqueous solution. In general, the average dissociation number, n , can be defined as: $n = (\Delta E \text{ value of the complex}) / (\Delta E \text{ value of urea})$ at a given concentration of the solute. The resulted n values were $n = 2.65$, 0.47, and 0.92 for Zn(bet)₃(ClO₄)₂, Zn(lac)₂, and Zn(qui)₂ at 0.060 mol kg⁻¹, respectively.

Because the average dissociation number in aqueous solution of [Zn(qui)₂]_n²³⁾ was almost $n = 1$, it was concluded that this complex forms a monomeric molecular species, Zn(qui)₂, in aqueous solution. In the case of Zn(bet)₃(ClO₄)₂, n was approximately 3 indicating that this complex probably dissociates into ions as follows: Zn(bet)₃(ClO₄)₂ · [Zn(bet)₃]²⁺ + 2ClO₄⁻. On the other hand, n value for Zn(lac)₂, which is a monomeric complex in the solid state, was almost $n = 1/2$ in aqueous solution. It is reasonably deduced that this complex is not monomeric, but exists as a dimer, [Zn(lac)₂]₂, in aqueous solution.

Thus, Zn(bet)₃(ClO₄)₂ and Zn(lac)₂ have monomeric tetrahedral and octahedral structures in solid state,^{18,19)} respectively, but they are present in the monomeric ion, Zn(bet)₃²⁺, and dimer, [Zn(lac)₂]₂, in aqueous solution. The molecular complex, Zn(qui)₂, has a polymeric octahedral structure in solid state,²³⁾ but is a monomer in aqueous solution.

In Vitro Insulinomimetic Activity of Zn(II) Complexes, Zn(bet)₃²⁺, [Zn(lac)₂]₂, and Zn(qui)₂ *In vitro* insulinomimetic activities of three Zn(II) complexes were estimated by inhibition of the release of FFA from isolated rat adipocytes treated with epinephrine,¹⁷⁾ and were confirmed to be dose-dependent in the concentration range of 0.1–1 mM

Table 1. Estimated IC₅₀ (mM) Values for the Free Fatty Acids (FFA) Release from Isolated Rat Adipocytes in the Presence of Glucose of Zinc(II) Complexes

Complex	IC ₅₀ /mM (=S.D. ^{a)})
Zn(bet) ₃ ²⁺	1.06 (0.03)
[Zn(lac) ₂] ₂	0.81 (0.06)*
Zn(qui) ₂	0.98 (0.06)
VOSO ₄	1.00 (0.08)
ZnSO ₄	0.81 (0.10)*

^a) The standard deviation is expressed as the mean ± S.D. for 3 experiments. * Significance at $p < 0.05$ (by Student's *t*-test) vs. VOSO₄.

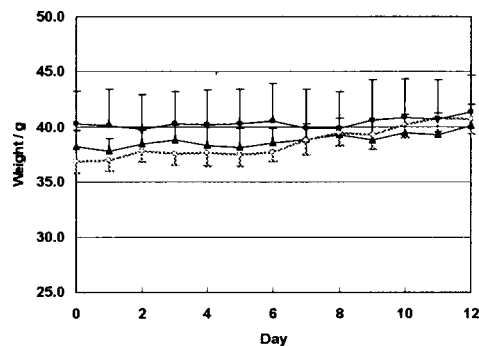


Fig. 1. Changes of Body Weight in Control KK-A^y Mice ($n=4$; ●), KK-A^y Mice Treated with [Zn(lac)₂]₂ ($n=5$; ○), KK-A^y Mice Treated with Zn(qui)₂ ($n=5$; ▲)

Values are means ± S.D. for four or five mice.

of the complex. The apparent IC₅₀ values, a 50% inhibitory concentration of the FFA release, of the three complexes exhibited insulinomimetic activities comparable with those of VOSO₄ and ZnSO₄ as shown in Table 1.

Blood Glucose Lowering Effects of Zn(II) Complexes, [Zn(lac)₂]₂ and Zn(qui)₂, in KK-A^y Mice Two complexes, [Zn(lac)₂]₂ and Zn(qui)₂ (IC₅₀ = 0.81 and 0.98, respectively), were used in this test because they had the top 2 of insulinomimetic activities as shown in Table 1. As shown in Fig. 1, the body weights didn't decrease in all groups. The food intake decreased temporarily when Zn(II) complexes with lactic and quinic acids were administered, but the food intake recovered for two days after their administrations. The drinking water intake didn't change in all groups.

When [Zn(lac)₂]₂ and Zn(qui)₂ complexes were injected daily at a dose of 3 mg (45.9 μmol) Zn/kg of body weight of mice for 13 d, the blood glucose level of the animals given Zn(qui)₂ was lowered to approximately 200 mg/dl (11.1 mM) (Fig. 2). The blood glucose level of the animals given [Zn(lac)₂]₂ was also lowered, but the fall ratio was low compared with that of Zn(qui)₂.

HbA_{1c} and Serum Parameters The levels of HbA_{1c}, which shows the number of glucose molecules attached to the hemoglobin in the erythrocytes over a long period, were measured. In KK-A^y mice untreated and treated with [Zn(lac)₂]₂, the HbA_{1c} levels increased from 8.0 ± 1.1 to 8.6 ± 1.0 (%) and 6.5 ± 1.1 to 6.9 ± 1.0 (%) before and after the administration, respectively. In contrast, the HbA_{1c} level of the KK-A^y mice treated with Zn(qui)₂ decreased from 8.1 ± 1.1 to 5.7 ± 1.7 (%) before and after the administration,

respectively. The result suggested that the blood glucose lowering effect of $\text{Zn}(\text{qui})_2$ complex continued for a long-term.

After KK- A^y mice were administered the complex for 13 d and fasted for 14 h on the final administration day, all parameters were measured. GOT, GPT, BUN, TG, TCHO, and FFA levels in the control KK- A^y mice (untreated) and KK- A^y mice treated with $\text{Zn}(\text{qui})_2$ complex for 13 d were summarized in Table 2.

No serum parameters changed compared with those of the control KK- A^y mice. In short, the administration of $\text{Zn}(\text{qui})_2$ complex by daily i.p. injections exhibited no renal and hepatic disturbances, indicating that $\text{Zn}(\text{II})$ complex with a natural product is safe for KK- A^y mice. In conclusion, the present study revealed that daily i.p. injections of a monomeric complex, $\text{Zn}(\text{qui})_2$, lowered the blood glucose level in KK- A^y mice with type 2 diabetes mellitus. Moreover, the dimeric complex, $[\text{Zn}(\text{lac})_2]_2$, showed a blood glucose lowering effect, however, it was weak. On the bases of the results, we proposed here that the monomeric complex in aqueous solution is a better antidiabetic agent than the dimeric complex.

The result on $\text{Zn}(\text{qui})_2$ shows not only the importance of $\text{Zn}(\text{II})$ monomeric complex with a low toxic natural product in treating experimental diabetic mellitus but also the possi-

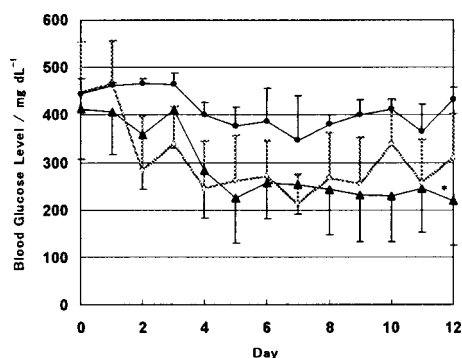


Fig. 2. Changes of Blood Glucose Level in Control KK- A^y Mice (●), KK- A^y Mice Treated with $[\text{Zn}(\text{lac})_2]_2$ (○), KK- A^y Mice Treated with $\text{Zn}(\text{qui})_2$ (▲)

Hyperglycemic KK- A^y mice were received i.p. injections of 5% acacia ($n=4$), $[\text{Zn}(\text{lac})_2]_2$ (3 mg Zn/kg body weights, $n=5$) or $\text{Zn}(\text{qui})_2$ (3 mg Zn/kg body weights, $n=5$). Values are means \pm S.D. * Significance at $p < 0.01$ (by Student's t -test) vs. 0 d of blood glucose level.

Table 2. Values of TG, BUN, GOT, GPT, TCHO, and FFA Levels in the Control (Untreated) KK- A^y Mice ($n=4$) and KK- A^y Mice ($n=5$) Treated with $\text{Zn}(\text{qui})_2$ Complex for 13 d

	GOT (U/l)	GPT (U/l)	BUN (mg/dl)	TG (mg/dl)	TCHO (mg/dl)	FFA (mEq/l)
Control	103 (22)	40 (23)	35.6 (7.8)	141 (6)	152 (12)	1.39 (0.16)
$\text{Zn}(\text{qui})_2$	108 (24)	43 (19)	27.2 (6.4)	178 (41)	140 (14)	1.31 (0.16)

Parentheses mean the standard deviations.

bility to develop a new clinically useful and low toxic $\text{Zn}(\text{II})$ complex.

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