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Antidiabetic copper(II)-picolinate: Impact of the first transition metal in the metallopicolinate complexes

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Abstract—In order to examine the effect of metallopicolinate complexes with first transition metals and develop complexes that are more active than an insulinomimetic leading compound such as oxovanadium(IV)-picolinate complex, $VO(pa)_2$, 10 metallopicolinate complexes were prepared, and their in vitro insulinomimetic and in vivo antidiabetic activities were evaluated. The in vitro activity was estimated by determining the inhibitory effects of these complexes on free fatty acid release from isolated rat adipocytes treated with epinephrine. Among the complexes, $Cu(pa)_2$, and $Mn(pa)_3$ exhibited higher activity than their respective metal ions and better activity than $VO(pa)_2$. Since $Cu(pa)_2$ was non-toxic in the cultured rat hepatic M cells, this complex was given streptozotocin (STZ)-induced type 1-like diabetic mice by single intraperitoneal injection, and found that this complex exhibited a higher hypoglycemic effect than the $VO(pa)_2$ complex. Based on these results, we propose that $Cu(pa)_2$ may be a potent alternative antidiabetic agent.

1. Introduction

The number of people suffering from diabetes mellitus (DM) has been showing an annual increase. The most recent study estimates that the prevalence of DM in adults will be 5.4% of the total number of all diseases, and in 2025, the number of adults with DM will increase to approximately 300 million worldwide.[1] The WHO has classified DM into two types, namely, type 1 and type 2 DM.[2] The patients with type 1 DM require daily insulin injections, which is both a physical and mental burden. Furthermore, DM leads to serious life-threatening complications causing severe damage to several organs such as the heart, eyes, kidneys, blood vessels, nerves, gums, teeth, feet, and legs.[3–5] Thus, there is an urgent need to establish a treatment regime that can replace painful insulin injections.

Since 1980 many researches have attempted to identify alternative antidiabetic compounds and have reported that metal ions such as vanadium,[6,7] zinc,[7,8] manganese,[9] copper,[10] chromium,[11] and tungsten[12] exhibit in vitro insulinomimetic activity and in vivo antidiabetic ability in experimental animals. Among these metal ions, we have focused on vanadium and zinc and developed oxovanadium(IV) and zinc(II) complexes to treat both types of DM.[7,13,14] In fact, oxovanadium(IV) complexes exert insulinomimetic and hypoglycemic effects both in vitro and in vivo.[15–19] In 1990, the first orally active hypoglycemic oxovanadium(IV) complexes such as bis(methyl cysteinato)oxovanadium(IV) $[VO(cysm)_2]$ were discovered.[20] Subsequently, in 1995, we found that a new orally active bis(picolinato)oxovanadium(IV) $(VO(pa)_2)$ complex with high insulinomimetic activity exhibited a hypoglycemic effect in streptozotocin (STZ)-induced type 1 diabetic animals.[21] The use of picolinic acid (pa) as a ligand to metals was advantageous because (1) pa is a metabolite of tryptophan and is, therefore, less toxic to mammals, and (2) oral administration of pa promotes the absorption of several metals via the small intestine.[22–24] In this study, we have attempted to find alternative metallopicolinate complexes that are more effective than the $VO(pa)_2$ complex by replacing the central oxovanadium(IV) moiety with other first transition metals. Here, we report on the effect of first transition metal complexes with pa that mimic insulin action in vitro and exhibit hypoglycemic effect

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in vivo, and propose that the bis(picolinato)copper(II) complex is a better alternative than the $VO(pa)_2$ complex.

2. Results and discussion

2.1. Preparation of metallopicolinates with the first transition metals

Ten metallopicolinate complexes were prepared, and previously described methods were used in their preparations[25–30]; however, novel methods were devised to prepare $Fe(pa)_2$, $Fe(pa)_3$, and $Ni(pa)_2$. The physicochemical features of all complexes used in the present study are summarized in Table 1.

2.2. In vitro insulinomimetic activity of metallopicolinates

The insulinomimetic activity of the metallopicolinate complexes was estimated in terms of inhibition of free fatty acid (FFA) release from isolated rat adipocytes treated with epinephrine (adrenaline); this is a simple and convenient method as compared to the use of radioisotope reagents.[31] Previously, we reported that oxovanadium(IV) compounds inhibited FFA release from rat adipocytes treated with epinephrine in a concentration-dependent manner.[7,16,20,31] Since all metallopicolinate complexes similarly inhibited FFA release from the adipocytes in a concentration-dependent manner, the effect of the complexes could be compared with that of metal ions and $VO(pa)_2$. The obtained apparent IC_{50} values of the complexes, defined as the concentration of a complex at which 50% inhibition of the FFA release from the adipocytes occurs, are summarized in Table 2. The apparent IC_{50} values of $Mn(pa)_2$ and $Cu(pa)_2$ were estimated to be 0.22 ± 0.02 mM and 0.33 ± 0.02 mM, respectively, whereas that of $VO(pa)_2$ was 0.42 ± 0.06 mM. Both $Mn(pa)_3$ and $Cu(pa)_2$ complexes exhibited significantly higher activity than their respective metal ions and better activity than the $VO(pa)_2$ complex. Other metallopicolinate complexes showed lower activities than that of $VO(pa)_2$ or negligible activities. In addition, the ligand, picolinic acid, exhibited no inhibitory effect on the FFA release.

2.3. Cytotoxicity of metallopicolinates

The cytotoxicities of the selected $VO(pa)_2$, $Mn(pa)_3$, and $Cu(pa)_2$ complexes, which exhibited relatively high insulinomimetic activity, were evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay using cultured rat hepatic M cell lines, as shown in Figure 1. The $Mn(pa)_3$ complex exhibited cytotoxicity at concentrations between 0.05 and 0.4 mM, while both $VO(pa)_2$ and $Cu(pa)_2$ complexes exhibited negligible cytotoxicity between 0 and 0.4 mM. The LD_{50} value, defined as the lethal dose for 50% of the cells, of the $Mn(pa)_3$ complex was approximately 0.35 mM, indicating that it was the most toxic among the three complexes. Hence, it was concluded that the inhibition of FFA release by the $Mn(pa)_3$ complex was a result of the high cytotoxicity on the M cells. Similarly, by performing an MTT assay, we confirmed that tris(acetylacetonato)Mn(III) complex, which is the trivalent form of Mn, also exhibited high cytotoxicity (data not shown). In this assay, MTT is reduced to formazan in the mitochondria of living cells. We previously reported

Table 1. Analytical and physico-chemical features of metallopicolinate complexes

Complex	Elemental analysis calcd/found (%)			FAB-MS *m/z*	IR $\nu_{C=O}$ cm^{-1}	Absorption maximum λ_{max} (ε (M^{-1} cm^{-1}))	$g_{//}$	Yield[c] (%)
	C	H	N					
$VO(pa)_2 \cdot 1.2H_2O$	43.41	3.15	8.42	311 $[M]^+$	1640	738 nm (36), 545 nm (16)[a]	1.98	80
	43.40	3.26	8.09					
$Cr(pa)_3 \cdot 3.3H_2O$	45.25	3.11	8.74	418 $[M]^+$	1680	519 nm (66)[b]	2.07	85
	45.32	3.28	9.28					
$Mn(pa)_2 \cdot 2.3H_2O$	42.32	3.73	8.23	300 $[M+H]^+$	1619	424 nm (560)[b]	2.17	49
	42.26	3.68	7.90					
$Mn(pa)_3 \cdot 2.4H_2O$	46.55	3.65	9.05	421 $[M+H]^+$	1680	524 nm (140), 408 nm (369)[b]	n.d.	56
	46.86	3.07	8.97					
$Fe(pa)_2 \cdot 4.0H_2O$	38.73	4.33	7.53	301 $[M+H]^+$	1630	543 nm (14)[b]	n.d.	71
	38.63	4.36	7.46					
$Fe(pa)_3 \cdot H_2O$	49.12	3.21	9.55	422 $[M]^+$	1645	n.d.	2.48	49
	48.99	3.20	9.59					
$Co(pa)_2 \cdot 4.1H_2O$	38.23	4.33	7.43	303 $[M]^+$	1627	480 nm (19)[a]	n.d.	54
	38.44	4.41	7.22					
$Ni(pa)_2 \cdot 4.0H_2O$	38.44	4.30	7.47	303 $[M]^+$	1635	623 nm (9)[a]	n.d.	73
	38.63	4.31	7.18					
$Cu(pa)_2 \cdot 1.1H_2O$	44.00	3.14	8.55	307 $[M]^+$	1649	638 nm (57)[b]	2.27	74
	43.73	3.12	8.23					
$Zn(pa)_2 \cdot 4.0H_2O$	37.76	4.23	7.34	309 $[M+H]^+$	1625	n.d.	n.d.	48
	37.80	4.19	7.35					

n.d. means not determined.
[a] Solvent is H_2O.
[b] Solvent is DMSO.
[c] On the basis of the ligand.

Table 2. IC_{50} values for metallopicolinate complexes for inhibitory activity of FFA release from rat adipocytes treated with epinephrine

Ionic	IC_{50} value (mM)	Complex	IC_{50} value (mM)
$VOSO_4 \cdot 1.6H_2O$	0.74 ± 0.04	$VO(pa)_2$	$0.42 \pm 0.06^{\#}$
$Cr_2(SO_4)_3 \cdot H_2O$	None	$Cr(pa)_3$	None
$MnSO_4 \cdot 5H_2O$	None	$Mn(pa)_2$	None
$MnPO_3 \cdot H_2O$	None	$Mn(pa)_3$	$0.22 \pm 0.02^{*,\dagger}$
$FeSO_4 \cdot 7H_2O$	None	$Fe(pa)_2$	0.46 ± 0.11
$Fe_2(SO_4)_3 \cdot 6.5H_2O$	None	$Fe(pa)_3$	$0.77 \pm 0.02^{*}$
$CoSO_4 \cdot 7H_2O$	0.85 ± 0.04	$Co(pa)_2$	None
$NiSO_4 \cdot 6H_2O$	None	$Ni(pa)_2$	None
$CuSO_4 \cdot 5H_2O$	$0.35 \pm 0.05^{\#\#}$	$Cu(pa)_2$	$0.33 \pm 0.02^{*}$
$ZnSO_4 \cdot 7H_2O$	$0.55 \pm 0.08^{\#}$	$Zn(pa)_2$	$0.92 \pm 0.08^{**,\ \$}$
Picolinic acid (pa)	None		

Significance at $^{*}p < 0.05$ and $^{**}p < 0.01$ vs $VO(pa)_2$, $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ vs $VOSO_4$, $^{\dagger}p < 0.05$ vs $VOSO_4$, and $^{\$}p < 0.05$ vs $ZnSO_4$.

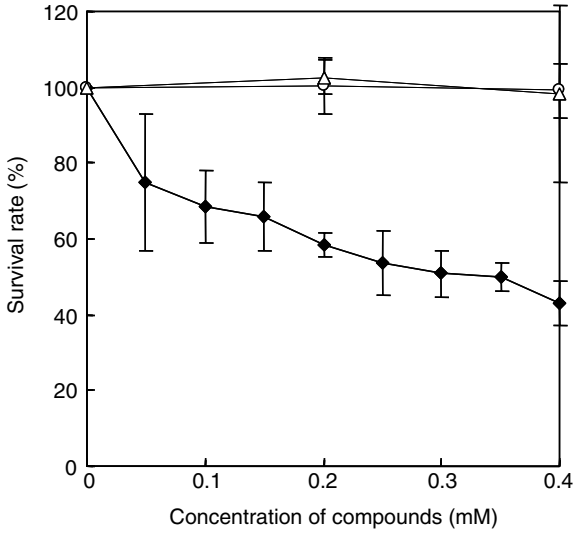

Figure 1. Viability of M cells treated with $VO(pa)_2$ (○), $Cu(pa)_2$ (△), and $Mn(pa)_3$ (♦) complexes as determined by MTT assay. Each point is expressed as the mean ± SD for three experiments.

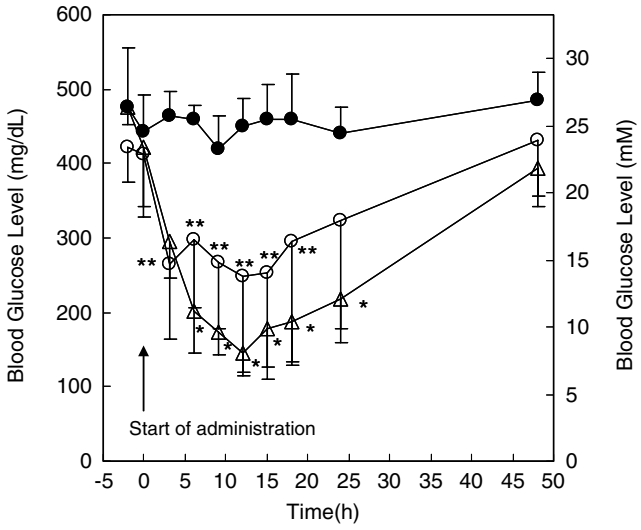

Figure 2. Changes in the blood glucose level in STZ-induced type 1 diabetic mice after a single ip injection of vehicle (control; ●), $VO(pa)_2$ (10 mg V/kg body weight (0.20 mmol); ○), and $Cu(pa)_2$ (3 mg Cu/kg body weight (0.047 mmol); △). Each value is expressed as the mean ± SD for 4–5 animals. Significance at $^{**}p < 0.05$ and $^{*}p < 0.01$ vs control.

that the Mn(III) content in the liver mitochondria in rats was higher than that in other subcellular fractions.[32] In addition, it was reported in 1988 that Mn(III) inhibited the activity of mitochondrial aconitase.[33,34] The occurrence of Mn(III) in the mitochondria may be associated with the high cytotoxicity of the $Mn(pa)_3$ complex.

2.4. In vivo hypoglycemic effect of metallopicolinates

The in vivo hypoglycemic effect of the selected $VO(pa)_2$ and $Cu(pa)_2$ complexes with high insulinomimetic activity and low cytotoxicity was examined using STZ-induced type 1 diabetic mice (STZ-mice). The STZ-mice were treated with individual complexes by a single intraperitoneal (ip) injection at a dose of 10 (0.20 mmol for $VO(pa)_2$ complex) or 3 (0.047 mmol for $Cu(pa)_2$ complex) mg metal/kg body weight (BW). These doses were selected because the acute LD_{50} value of $CuSO_4$ was reported to be 5.6 mg/kg BW.[35] A single administration of $VO(pa)_2$ and $Cu(pa)_2$ complexes caused similar hypoglycemic effects. However, the hypoglycemic effect after 6 h of $Cu(pa)_2$ complex injection was significantly faster, and the effect persisted for 24 h (Fig. 2). Previous studies have examined the hypoglycemic effects of bis and tris(maltolato)metal complexes involving Cu(II), Co(II), VO(IV), Zn(II), Cr(III), and MoO_2(IV) in STZ-rats after a single dose of 0.60 mmol/kg BW by oral gavage; these studies have reported that bis(maltolato)copper(II) complex showed little hypoglycemic activity and *cis*-bis(maltolato)(dioxo)molybdenum(IV) and bis(maltolato)oxovanadium(IV) complexes exhibited significant hypoglycemic effect.[36] From these results, it was concluded that the VO(IV) complex was more effective than the Cu(II) and MoO_2(IV) complexes. In addition, a Cu(II) acetate imidazole complex ($Cu(OAc)_2(Im)_4$) was reported to exhibit hypoglycemic effect in STZ-rats following an intramuscular injection at a dose of 7.5 mg Cu/kg BW (0.12 mmol),[37] and bis(3-hydroxypyridine-2-carboxylato)copper(II) complex, which was prepared in 2005, was shown to possess high in vitro insulinomimetic activity as compared to VO(IV), Mn(II), Fe(II), Co(II), and Zn(II) complexes.[38] Our present finding and previously reported observations suggest that both copper(II) and oxovanadium(IV) are specific metals that exert a hypoglycemic effect in experimental diabetic animals. Since 1995, we have considered that the choice of

ligands and administration routes are important factors affecting the pharmacological efficacy of metal complexes such as insulinomimetic agents.[7,13,39–41] In the present study, we discovered that among all the metallopicolinate complexes formed with first transition metals, $Cu(pa)_2$ was the most effective hypoglycemic complex, and it will be alternative potent complex following the $VO(pa)_2$.

Vanadium compounds have been known to bind with protein tyrosine phosphatase 1B (PTP1B) and inhibit the phosphatase activity. Therefore, tyrosine phosphorylations of insulin receptor β-subunits and insulin receptor substrates (IRS) are enhanced. This results in the activation of the phosphoinositol-3-kinase (PI3-K) and Akt/PKB, which are downstream targets of the insulin receptor, and subsequently the translocation of glucose transporter 4 (GLUT 4) to the cell membranes.[42–44] The $VO(pa)_2$ complex thus exhibits its hypoglycemic effect by inhibiting PTP1B in part. On the other hand, Cu is reported to activate the PI3-K/Akt pathway leading to GLUT 4 translocation.[45,46] These observations indicate that the $Cu(pa)_2$ complex presumably improves hyperglycemia by activating PI3-K/Akt in STZ-mice. Further studies on the molecular mechanism of $Cu(pa)_2$ are required.

In conclusion, the purpose of our present study was to compare a range of metallopicolinate complexes in terms of their in vitro insulinomimetic activity and in vivo hypoglycemic effect. The $Cu(pa)_2$ complex with low cytotoxicity and high insulinomimetic and hypoglycemic effects was discovered to be a potent alternative antidiabetic compound to the $VO(pa)_2$ complex.

3. Experimental

3.1. Materials

The following materials were obtained from Wako Pure Chemical Co. (Osaka, Japan): Oxovanadium(IV) sulfate ($VOSO_4{\cdot}nH_2O$), chromium(III) sulfate ($Cr_2(SO_4)_3{\cdot}H_2O$), manganese(II) sulfate ($MnSO_4{\cdot}5H_2O$), manganese(III) phosphate ($MnPO_4{\cdot}H_2O$), iron(II) sulfate ($FeSO_4{\cdot}7H_2O$), iron(III) sulfate ($Fe_2(SO_4)_3{\cdot}nH_2O$), cobalt(II) sulfate ($CoSO_4{\cdot}7H_2O$), nickel(II) sulfate ($NiSO_4{\cdot}6H_2O$), copper(II) sulfate ($CuSO_4{\cdot}5H_2O$), zinc(II) sulfate ($ZnSO_4{\cdot}7H_2O$), picolinic acid (pa), D-(+)-glucose, and arabic gum (acacia). MTT was purchased from Dojindo Laboratories (Kumamoto, Japan). $VOSO_4$ and $Fe_2(SO_4)_3$ were complexometrically standardized with EDTA (Dojindo Laboratories, Kumamoto, Japan) and determined as $VOSO_4{\cdot}1.6H_2O$ and $Fe_2(SO_4)_3{\cdot}6.5H_2O$, respectively. Collagenase (type II), STZ, (±)-epinephrine hydrochloride, and bovine serum albumin (BSA) were purchased from Sigma Chemical Co. (St. Louis, USA). All reagents were of analytical or reagent grade and were used without purification.

3.2. Preparation of metallopicolinate complexes

Bis(picolinato)VO(II) ($VO(pa)_2$), tris(picolinato)Cr(III) ($Cr(pa)_3$), bis(picolinato)Mn(II) ($Mn(pa)_2$), tris(picolinato)Mn(III) ($Mn(pa)_3$), bis(picolinato)Co(II) ($Co(pa)_2$), bis(picolinato)Cu(II) ($Cu(pa)_2$), and bis(picolinato)Zn(II) ($Zn(pa)_2$) complexes were prepared according to previously published methods.[21,25–30] Bis(picolinato)Fe(II) ($Fe(pa)_2$), tris(picolinato)Fe(III) ($Fe(pa)_3$), and bis(picolinato)Ni(II) ($Ni(pa)_2$) complexes were novel preparations. In brief, an aqueous solution of the metal ion (2 mmol) was added to an aqueous solution of picolinic acid (4 mmol) containing $Ba(OH)_2{\cdot}8H_2O$ (2 mmol), and stirred for 3 h at room temperature. After filtration of the precipitated $BaSO_4$ and removal of the solvent, the precipitate was washed with a small quantity of hot water. The physical parameters of these complexes were confirmed by elemental analyses (EA; Perkin-Elmer, 240C elemental analyzer), infrared (IR; Shimadzu, FT-IR 8100A), electron spin resonance (ESR; JEOL, RE1X spectrometer), and fast atom bombardment-mass (FAB-MS; JEOL, JMS-SX 102AQQ) spectrometry. The physicochemical properties of all prepared complexes used in the present study are summarized in Table 1.

3.3. Evaluation of in vitro insulinomimetic activity

The in vitro insulinomimetic activities of the metallopicolinate complexes were determined by the inhibitory effect of FFA release in isolated rat adipocytes treated with epinephrine (adrenaline).[31] Briefly, the isolated adipocytes obtained from male rats weighing 200–210 g were preincubated at 37 °C for 30 min with various concentrations (10^{-4}–10^{-3} M) of metal compounds in Krebs–Ringer bicarbonate buffer (pH 7.4) containing 2% BSA. Epinephrine (10^{-4} M) was then added to the reaction mixtures, and the resulting solutions were incubated at 37 °C for 3 h. The reactions were stopped by incubating the mixtures in ice water, and centrifuging them at 3000 rpm for 10 min. For the outer solution of the cells, the FFA levels were determined by using an FFA kit (Wako Pure Chemical Co., Osaka, Japan). The IC_{50} value, which is the 50% inhibitory concentration of the complex, was determined from the results of the concentration-dependent inhibitory effect of the various metal complexes on the FFA released from isolated rat adipocytes treated with epinephrine.

3.4. MTT assay

M cells (rat hepatocyte cell line; American Type Culture Center, Rockville, MD, USA) were grown in Dulbecco's modified Eagle's medium (DMEM; Nikken Bio Medical Laboratory, Kyoto, Japan) supplemented with 10% fetal bovine serum and penicillin/streptomycin (Invitrogen, Tokyo, Japan) at 37 °C in an atmosphere of 5% CO_2. The MTT assay was performed as described previously.[47] Briefly, a stock solution of MTT was prepared by dissolving 5 g MTT/l in phosphate buffer saline (−) and filtering through a 0.2 μm filter. Essentially, the M cells were grown to sub-confluency on 24-multiwell plates. The supernatant medium was removed and replaced with serum-free DMEM. The cells were incubated in this medium for 24 h. Again, the supernatant medium was removed, and DMEM supplemented with MTT (0.1 mM) and solution of the metal complexes

was added to the cells. The cells were incubated at 37 °C in 5% CO_2 atmosphere. After 3 h, the supernatant was discarded, and the reaction was stopped using 0.04 N HCl in isopropanol. The plates were read on a microplate reader (Bio-Rad Japan Co., Tokyo, Japan) at a wavelength of 570 nm.

3.5. Evaluation of the in vivo hypoglycemic effect in STZ-induced type 1 diabetic mice

STZ was dissolved in 0.1 M sodium citrate buffer, pH 5, and was used within 5 min after preparation. Male ddY mice (8-week-old) that were fasted for 6 h received ip STZ injections (100 mg/kg BW) twice at weekly intervals. A single ip injection in 5% acacia vehicle was used to administer the STZ-induced type 1 diabetic mice with $VO(pa)_2$ and $Cu(pa)_2$ complexes at doses of 10 and 3 mg metal/kg BW, respectively. Blood samples for analysis of blood glucose levels were obtained from the tail vein of the mice, and the blood glucose levels were measured by using the glucose oxidase method (Glucocard; Arkray, Kyoto, Japan). The mice were allowed free access to both food and water.

3.6. Statistical analysis

All experimental results are expressed as the mean values ± standard deviations (SD). Differences were analyzed by either the paired Student's *t* test or a one-way analysis of variance followed by Dunnett's multiple-comparison test.

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