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Antihyperglycemic and neuroprotective effects of one novel Cu–Zn SOD mimetic

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ABSTRACT

Increasing evidence supports that OS plays important roles in diabetes mellitus and cerebral ischemia. This suggests that recovering an impaired endogenous superoxide dismutase (SOD) enzyme system induced by OS with a mimetic would be beneficial and protective for these diseases. In present study, one nonpeptidyl small molecular weight compound (D34) was synthesized. Its SOD mimetic activity and the potential therapeutic actions were also evaluated both in vivo and in vitro. The in vitro nitro blue tetrazolium (NBT) assay indicated that D34 presents an SOD mimetic activity. D34 (20 μmol/kg) exhibited significant antihyperglycemic activity in alloxan-diabetic mice. D34 could also ameliorate the cerebral neuronal death in hippocampus of global cerebral ischemia mice. Furthermore, the D34 treatment significantly decreased malondialdehyde (MDA) contents and increased SOD activities in brains or livers of diabetes mice or cerebral ischemic mice. In conclusion, these preliminary findings support that D34 exhibits SOD mimetic activity and possesses significant antihyperglycemic and neuroprotective effects.

Oxidative stress (OS) is characterized by over-produced cellular oxidants (e.g., superoxide and hydrogen peroxide) and/or decreased antioxidants and antioxidant enzymes [e.g., vitamin E and superoxide dismutase, (SOD)]. Excessive OS can directly lead to DNA and protein modification and lipid peroxidation,[1–3] decreases insulin mRNA, cytosolic ATP, calcium influx into cytosol and mitochondria, and causes apoptosis.[4,5]

Increasing evidence in studies of both rodents and human has suggested that OS is associated with numerous pathological conditions, including diabetes mellitus and cerebral ischemia. In diabetes mellitus, OS participates not only in β-cell dysfunction and insulin resistance but also in the genesis of other late complications of diabetes.[6–8] Cerebral ischemia is another one pathological condition correlated closely with OS. Temporary or permanent hypoperfusion could induce depletion of SOD, and then production of OS,[9–11] ultimately leading to neuronal death.[12]

Over expression of SOD or supplements of antioxidants including SOD mimetics, targeted to overcome OS, reduce reactive oxygen species and increase antioxidant enzymes, has been shown to prevent diabetes mellitus[13–17] and reduce neuronal damage in cerebral ischemia.[18–23] Recently, the nonpeptidyl low molecular weight compounds have been reported, which possess significant antioxidant activity to that of the native SOD enzymes.[24–29] The use of these compounds suggests to provide a better therapeutic approach in diseases mediated by OS.

In present study, we investigated the SOD mimetic activity, antihyperglycemic and neuroprotective effects of D34, a novel, low molecular weight, nonpeptidyl compound. The compound (Fig. 1, structure formula: $C_{21}H_{24}N_2O_4Cl_2CuZn$; molecular weight: 568.23) was synthesized in the laboratory of the Department of Pharmacy, College of Chemistry and Chemical Engineering, Liaoning Normal University, China, and is currently pending for Chinese and international patent.

D34 is a liposoluble binuclear schiff base Cu(II)–Zn(II) complex with good solubility in DMSO or methanol D34 exerts good stability in aqueous environment without releasing of Copper or Zinc ions due to its poor water-solubility.

The SOD mimetic activity of D34 was measured by nitro blue tetrazolium (NBT) method in vitro.[29,30] Superoxide anions were produced from the riboflavin/methionine system. The indicator utilized in this case is NBT, which reacts with O_2^- to form blue formazane. Reaction system containing 3.3×10^{-6} M riboflavin, 0.01 M methionine, 4.6×10^{-5} M NBT, and 0.05 M phosphate buffer pH 7.4, and 0–1.5 μg/ml D34 or 2×10^{-9}–2×10^{-8} M native Cu–Zn SOD, were illuminated under fluorescent lamps. The absorbance at 560 nm increased linearly with time of illumination. The reduction of NBT was measured in terms of increased absorbance at 560 nm on a Shimadzu UV-240 spectrophotometer. All photo-induced reactions were performed at 25 °C. Each test was performed in triplicate.

To further investigate potential therapeutic effects of D34 for diabetes and cerebral ischemia in vivo, alloxan-diabetic model and bilateral common carotid artery occlusion (BCCAO) model

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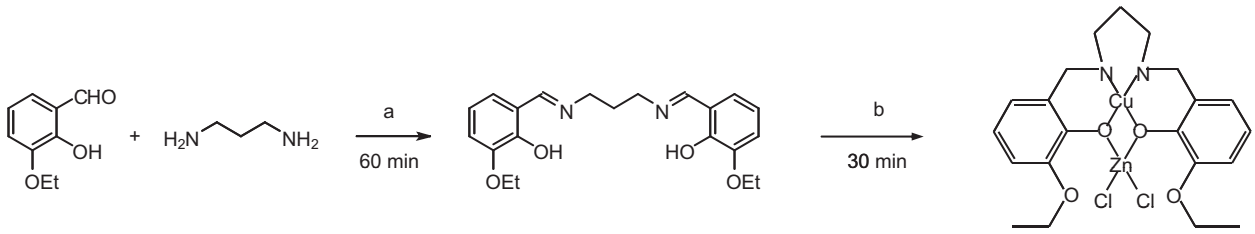

Figure 1. Synthesis of ligand and D34, a novel nonpeptidyl copper(II)–zinc(II) superoxide dismutase mimetic. Reagents and conditions: (a) 3-ethoxysalicylaldehyde, 1,3-diaminopropane, methanol, 92% yield; (b) $Cu(CH_3COO)_2{\cdot}H_2O$ and $ZnCl_2$, methanol 50% yield.

were established in mice. For evaluating the hypoglycemic effects of D34 on healthy mice with normal blood glucose level, mice were administrated intraperitoneally (ip 0.2 ml/day) for 3 days with D34 (20 μmol/kg body weight) or vehicle following an overnight fasting. Blood samples were collected by tail nipping before the first administration and 1 h after the last administration. Blood glucose levels were determined using a commercially available quantification kit based on glucose oxidase method. For evaluating the antihyperglycemic effect, the alloxan-diabetic animal model was established as described previously.[31] Briefly, Mice were injected with alloxan (150 mg/kg body weight, ip) after overnight fasting for 12 h. Seventy-two hours after alloxan injection, mice with blood glucose levels above 11.1 mmol/l were included in the study, and then treated daily with D34 or saline for 2 days. Blood samples were collected by tail nipping for the following assessment for blood glucose. Finally, the mice were sacrificed, and the livers and brains were removed and weighed. The tissues were rapidly homogenized for bioassay or stored at −80 °C until required.

For global cerebral ischemia, mice were anesthetized with Chloral Hydrate (400 mg/kg, ip) and then subjected to transient cerebral hypoperfusion, as previously described with minor modification.[32] In brief, transient cerebral hypoperfusion was induced by BCCAO with aneurysm clips for 20 min, and circulation was restored by removing the clips. During the surgical procedure, rectal temperature was maintained at 36 ± 0.5 °C with heating pad. After reperfusion, the animals were placed in a warm incubator (32–33 °C). Hippocampal neuronal damage were evaluated 3 days after BCCAO surgery since in our preliminary study significant neuronal cell death was found in the CA1, CA3 and dentate gyrus (DG) subregions of hippocampus at this time point. Briefly, mice were anesthetized with Chloral Hydrate (400 mg/kg, ip). After decapitation, brains were rapidly removed and homogenized for bioassay, or frozen quickly on dry-ice powder and stored at −80 °C for the histological examination. For the assessment of neural damage, coronal sections (10 μm) were prepared and then stained with 0.1% (w/v) cresyl violet. Morphologically abnormal neurons were quantified at 400× magnifications. Damaged neurons in each brain subregion were semi-quantitatively scored as: (0) no ischemic neurons; (1) 1–30% ischemic neurons; (2) 31–65% ischemic neurons; (3) 66–100% ischemic neurons. The utility of this approach has been demonstrated in a previous study with MF1 and C57Bl/6J mice.[33,34]

Collected tissues were thawed, weighed and homogenized with Tris–HCl (5 mmol/l containing 2 mmol/l EDTA, pH 7.4). Homogenates were centrifuged (1000*g*, 15 min, 4 °C) and the supernatant was used immediately for the assays of SOD activities and MDA levels in liver or brain homogenates following the commercial kits instructions.

All values are reported as mean ± SEM. Statistical analysis was performed with SPSS software (for windows, version 13.0). Overall significance within and between groups was determined using analysis of variance (ANOVA). Dunnett's post hoc test was applied when significant differences were found using analysis of variance. A Student's *t*-test was used to determine significant differences of individual responses between groups. $p < 0.05$ was determined to be significant.

The results of NBT tests in vitro were summarized in Figure 2. The results showed a considerable antioxidant activity of D34. While the IC_{50} value of native Cu–Zn SOD is 0.015 μM, the IC_{50} value of D34 is 1.31 μM (0.76 μg/ml, Table 1).

The effect of the D34 on blood glucose was assessed in fasting normal mice. The average levels of blood glucose in each group are shown in Figure 3A. D34 treated mice did not exhibit any significant alteration in their blood glucose levels after 3 days treatment. The antihyperglycemic effect of the D34 on the blood glucose levels of fasting diabetic mice is shown in Figure 3B. Intraperitoneal injection of alloxan monohydrate (150 mg/kg) led a significant elevation of blood glucose level ($p < 0.01$). Blood glucose levels were 1.8-fold higher in diabetic mice than control group. Treatment of the D34 for 2 days led to a significant fall in blood glucose levels ($p < 0.05$). The blood glucose level of D34 was about 26.7% lower than those of the diabetes group.

Ischemic neuronal damage in 20 min BCCAO mice was significantly increased in hippocampus CA1, CA3, and dentate gyrus (DG) subregions as compared with control mice (Fig. 4). Pre-treatment with the D34 30 min before BCCAO significantly ameliorated neuronal damage.

Figures 5 and 6 shows the MDA level and SOD activity in liver or brain homogenates of normal and experimental animals. MDA was significantly increased in livers of diabetic mice and in brains of BCCAO mice whereas SOD activities were significantly decreased

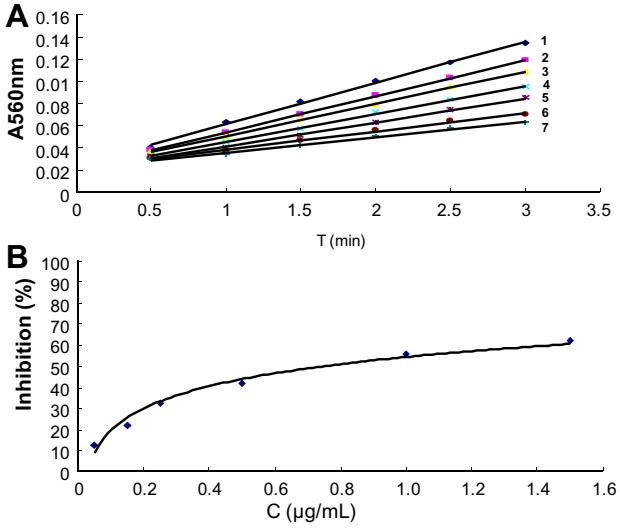

Figure 2. Antioxidant activity of D34 in vitro. (A) Absorbance values of D34 in NBT assay in vitro [complex/M: (1) 0 (in absence of the D34); (2) 0.05 μg/ml; (3) 0.15 μg/ml; (4) 0.25 μg/ml; (5) 0.5 μg/ml; (6) 1.0 μg/ml and (7) 1.5 μg/ml. (B) Inhibition percentage of NBT reduction with an increase in the concentration of the complex.

Table 1
SOD-mimetic activities described by IC_{50}

Complex [a]	IC_{50} (μM)	Reference
Native Cu–Zn SOD	0.015	This work
D34	1.31	This work
$ZnCl_2$	127.5	This work
$CuCl_2$	5.12	This work
$[Cu(C_9H_7NO_3)(C_{12}H_8N_2)]$	6.15	29
$[Cu(en)_2](sal)_2$	3.16	
$[Cu(Pu\text{-}6\text{-}MePy)(H_2O)](ClO_4)_2$	2.25	
$SALCuCl_2$	3.9	
$C_{22}H_{24}Cl_2CuN_2O_4Zn$	3.127	

[a] The reaction was conducted in anoxic conditions.

as compared with normal mice. The D34 treatment could reverse these changes.

OS is defined in general as over-production and/or deficient removal of highly reactive molecules, and indirectly initiates lipid peroxidation. Both inhibition of production and enhanced detoxification of reactive oxygen species with pharmacological agents or genetic manipulations have been found to limit the extent of pathological conditions. Thus, administration of native SOD or SOD mimetic could be a promising therapeutic approach,[35–40] although few of them have shown clinical potency.

In present study, the antioxidant and SOD mimetic activity of D34, one nonpeptidyl low molecular weight compound, were evaluated in vitro by NBT method, a convenient and commonly used bio-assay for screening of SOD mimetics.[41] Antioxidant activity of a test complex is usually defined as one unit SOD activity, which is the concentration of a test complex or enzyme, which causes 50% inhibition reduction of NBT. The more efficient a test complex is, the lower the concentration that corresponds to 50% inhibition of NBT or reduction the IC_{50} value. Here, we reported that the NBT reduction was effectively inhibited by D34, though the activity is about 100 times less than that of the native Cu–Zn SOD. However, this complex is still a potent SOD mimic considering the very low molecular weight when compared with that of the native SOD enzyme (molecular weight, 32000 Da), which suggests that the D34 possessed antioxidant activities for ameliorating the OS and have the potential use in diabetes mellitus and cerebral ischemic

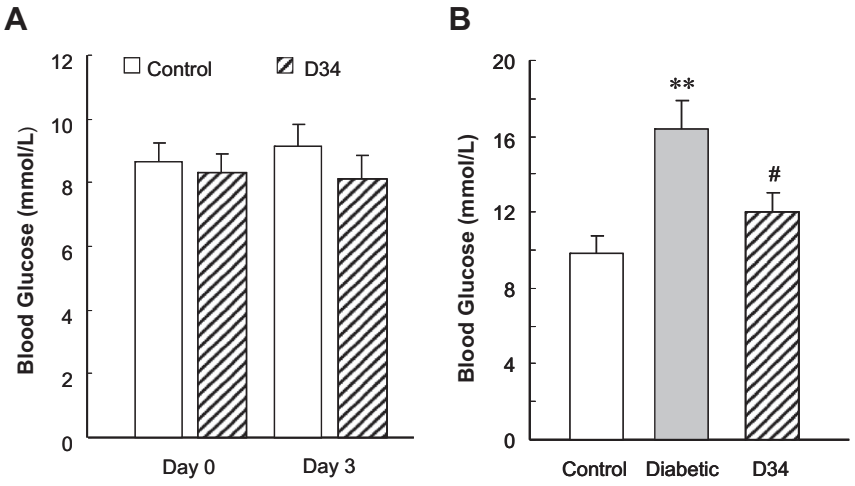

Figure 3. Antiglycemic activity of D34 in normal mice (A) and alloxan-diabetic mice (B). Blood samples were collected from the mice before the first administration and 1 h after the last administration of the D34, and were processed for the analysis of glucose concentrations. $^{**}p$ <0.01 when compared with control group; $^{\#}p$ <0.05 when compared with diabetic group (n = 8–10).

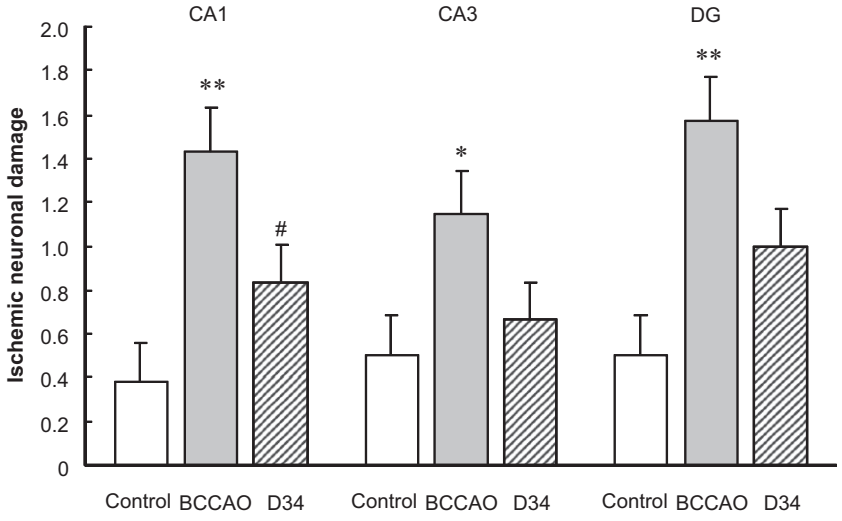

Figure 4. Neuronal damage in CA1, CA3, and DG subregions of hippocampus in BCCAO ischemia mice. Coronal sections (10 μm) were stained with cresyl violet. Ischemic neurons in each brain region were scored semi-quantitatively. $^{*}p$ <0.01 when compared with control group.

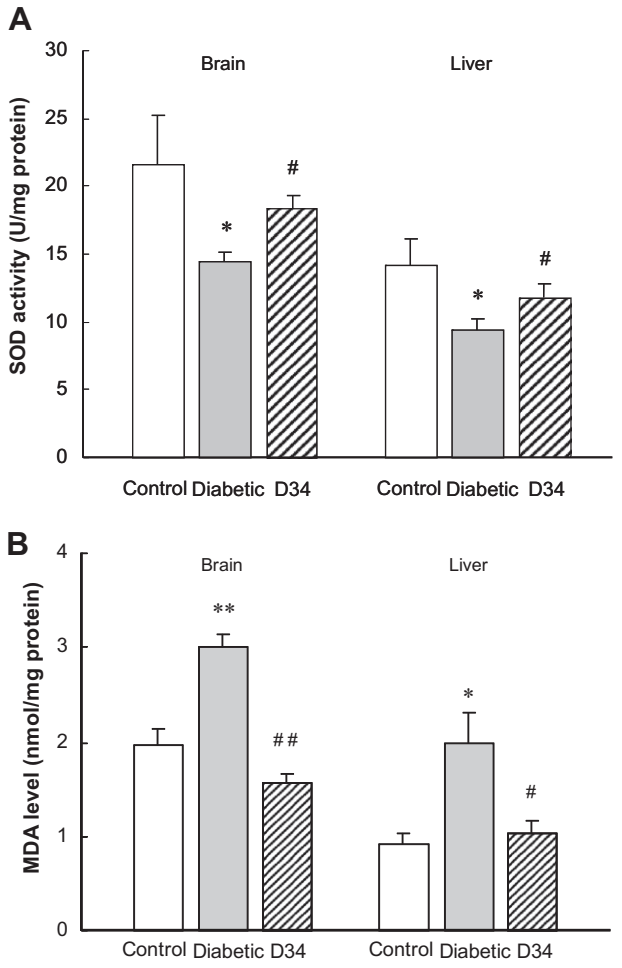

Figure 5. SOD activities and MDA levels in liver or brain of alloxan-diabetic mice. $^{*}p$ <0.05 when compared with control group; $^{\#}p$ <0.05 when compared with diabetic group. $^{**}p$ <0.01 when compared with control group; $^{\#\#}p$ <0.01 when compared with diabetic group (n = 8–10).

diseases. Moreover, IC_{50} value of D34 is higher than Cu^{2+} and Zn^{2+} indicating that the antioxidant activity of D34 might not be due to released ions.

In order to further evaluate the antihyperglycemic and neuroprotective effects in vivo, the alloxan-diabetic model and BCCAO model in mice were established. Diabetes mellitus is a group of metabolic diseases characterized by abnormality of the blood glucose metabolism resulting from altered insulin production or activity. The precise cellular and molecular mechanisms underlie the etiology and progressions of diabetes are still not fully understood. However, OS is thought to play a central role on the development of diabetes and many diabetic complications. In line with this, an increase in lipid peroxidation and deficits in the antioxidant defense systems have been observed in a variety of experimental models of diabetes.[42–53] Among those animal models, alloxan-induced diabetic model has been widely accepted. Alloxan induces type I diabetes mellitus (insulin-dependent diabetes) by inducing ROS formation and resulting in the selective necrosis of beta cells.[54] Thus, compounds that present antioxidant effects are of potential therapeutic interest for the treatment of human and animal diabetics. This postulates that D34 having high antioxidant potential may have a role to prevent the development of diabetes. The present investigation showed that D34 could reverse the hyperglycemia in mice induced by alloxan treatment, without influence on normal blood glucose level of heathy animals. The decreased SOD activity and the enhanced MDA level in both brains and livers of diabetic mice were reversed by D34, suggesting that the antidiabetic activities of D34 might be due to its SOD mimetic property and antioxidant activities.

The present investigation also revealed that D34 exhibited the neuroprotective potential against BCCAO induced OS and neuronal damage. It is observed that D34 attenuated the neuronal death in CA1, CA3, and DG. The activity of D34 appears to work by restoring the altered antioxidants enzymes as well as decrease the production of MDA induced by BCCAO. There is a considerable evidence supports that the role of OS in the pathogenesis of cerebral ischemia.[55,56] Brain reperfusion after ischemia frequently leads to neuronal death, which occurs preferentially in some brain regions, such as hippocampus. Such neuronal death has long been associated with excessive production of reactive oxygen species during OS, which reacts with cellular macromolecules and leads to oxidative damage of the neurons. Therefore, the potent antioxidant properties or LPO inhibiting ability of SOD mimetic compounds protecting the neurons from OS may provide useful therapeutic agent for cerebral ischemia.

In summary, these preliminary experimental findings reveal that D34 possesses SOD mimetic activity, and exhibits antihyper-

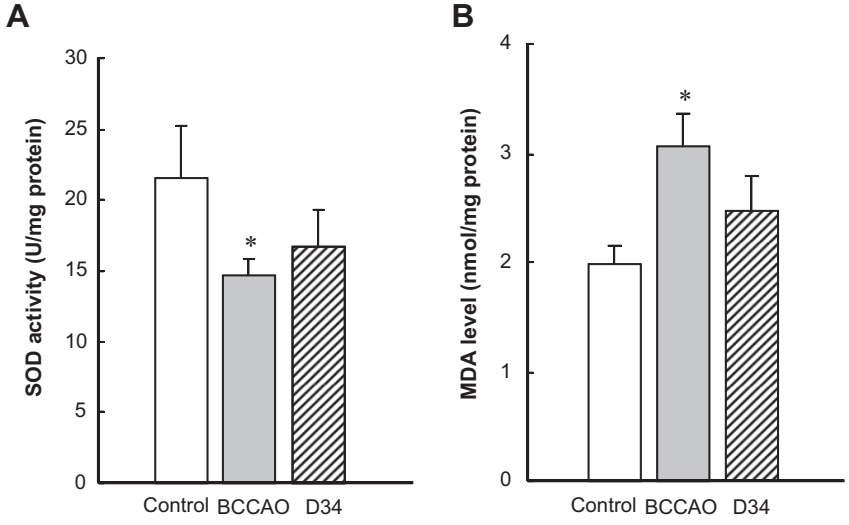

Figure 6. SOD activities and MDA levels in brain homogenates of BCCAO mice. $^{*}p$ <0.05 when compared with control group (n = 8–10).

glycemic and neuroprotective effects in mouse model by potentiating the antioxidant defense system. These results support the efficacy of D34 for diabetes and cerebral ischemia treatment. However, further studies are still necessary for the evaluation of the detailed mechanism of antidiabetic and neuroprotective activities of D34, and clinical trials should be performed to identify the clinical treatment potential for human.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.05.051.

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