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Synthesis and protective effects of aralkyl alcoholic 2-acetamido-2-deoxy-β-D-pyranosides on hypoglycemia and serum limitation induced apoptosis in PC12 cell

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ABSTRACT

Neuroprotective agents have been in the focus of attention in the treatment of ischemic stroke. Salidroside, a phenylpropanoid glycoside isolated from *Rhodiola rosea* L., possessed a wide range of biological activities, especially neuroprotection. In an attempt to improve neuroprotective effects of new salidroside analogs for ischemic stroke, a series of novel aralkyl alcoholic 2-acetamido-2-deoxy-β-D-pyranosides were synthesized and their protective activities against the hypoglycemia and serum limitation induced cell death in rat pheochromocytoma cells (PC12 cells) were studied. Most compounds showed strong neuroprotective effects, especially for **4g** and **4h**, which exhibited a great potency superior to salidroside. MTT assay, Hoechst 33342 staining, and flow cytometry with annexin V/PI staining collectively showed that pretreatment with **4g** and **4h** attenuated cell viability loss and apoptotic cell death in cultured PC12 cells. Caspase-3 colorimetric assay and Rhodamine 123 staining revealed the changes in expression levels of caspase-3 and mitochondrial membrane potential in PC12 cells on exposure to hypoglycemia and serum limitation with and without **4g** and **4h** pretreatment, respectively. All the results suggested that **4g** and **4h** protects the PC12 cells against hypoglycemia and serum limitation induced apoptosis possibly by modulation of apoptosis-related gene expression and restoration of the mitochondrial membrane potential. Therefore, these novel findings may provided a new framework for the design of new aralkyl alcoholic 2-acetamido-2-deoxy-β-D-pyranosides as neuroprotective agents for treating cerebral ischemic stroke and neurodegenerative diseases.

1. Introduction

Stroke is one of the leading causes of death and permanent disability in adults[1] Most strokes (80%) are ischemic and the disease leads loss of blood flow in a specific cerebral region, which upsets the critical balance between the demand and supply of glucose, oxygen, serum, and nutrient that are indispensable for the energy generation.[2] Morphological manifestations of programmed cell death in the ischemic brain and biochemical evidence have revealed that apoptosis was implicated in neurological diseases including ischemic stroke.[3,4] In general, cerebral ischemia could induce neuronal apoptosis, which is a complex process with multiple mechanisms, such as the release of proapoptotic proteins or stimulation of caspase-3, mitochondrial dysfunction and so on.[5–7] Given that there is no effective chemotherapy for cerebral ischemic stroke and neuroprotective agents could attenuate lots of the clinical problems of ischemic stroke, the development of new neuroprotective agents that inhibit neuronal apoptosis induced by cerebral ischemia will be of great significance.[8]

Salidroside (Fig. 1), one of the most potent compounds in *Rhodiola rosea* L. was well known as an adaptogen in traditional Chinese medicine and has been shown to have diverse pharmacological activities, including anti-oxidation,[9] anti-aging,[10] anti-fatigue[11] and anti-carcinogenic.[12] Many recent reports and reviews have also highlighted that salidroside exhibit potent neuroprotective activity.[13,14] Our previous studies have revealed that salidroside exhibited protective effects on hypoglycemia and serum limitation induced cell apoptosis in cultured PC12 cells.[15] It supposed that salidroside probably possess an ability to protect neurons from ischemic damage.

Both natural and synthetic glucosamine-containing compounds have been reported to possess neuroprotective action,[16,17] immunomodulatory activity, anticoagulation[18] and were used to treat clinically kidney disorders[19] and heart disease.[20] *N*-acetylglucosa-

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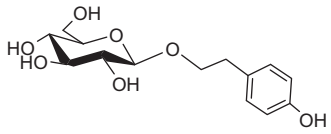

Figure 1. Chemical structure of salidroside (*p*-hydroxyphenethyl-β-D-glucoside).

mine has been shown, therefore, to be an available starting material for physiologically or pharmacologically important products. In prior studies, we described the synthesis and preliminary biological evaluation of some salidroside analogs and found that different aglycons of aralkyl alcohol influenced their activities.[21] In an ongoing study on the discovery and development of new anti-ischemic agents, we hypothesized that novel type of aralkyl alcoholic 2-acetamido-2-deoxy-β-D-pyranosides could have neuroprotective effects on the PC12 cells against the damage caused by hypoglycemia and serum limitation and inhibit hypoglycemia and serum limitation induced cell apoptosis. Therefore, a total of 11 target compounds (**4a–h**, **5i–k**) were designed and synthesized by coupling the *N*-acetylglucosamine with various aralkyl alcohols. Their in vitro neuroprotective activities in the cell injury induced by hypoglycemia and serum limitation, the inhibition of caspase-3 like protease activity, and the preservation of mitochondrial membrane potential were evaluated. Herein, the synthesis and preliminary biological evaluation of these compounds were reported.

2. Results and discussion

2.1. Synthesis of aralkyl alcoholic 2-acetamido-2-deoxy-β-D-pyranosides

The synthesis route for aralkyl alcoholic β-D-*N*-acetylglucopyransides **4** and **5** was shown in Fig. 2. 2-acetamido-3, 4, 6-tri-*O*-acetyl-2-deoxy-α-D-glucopyranosyl chloride **2** was prepared from 2-acetamido-2-deoxy-D-glucose by esterification using a well-known procedure.[22] The intermediates **3a–k** were obtained in moderate yields at room temperature by the glycosidation of compound **2** in dry dichloromethane with different substituted aromatic alcohol using zinc chloride as a catalyst and 4,4′-dimethoxytrityl chloride(DMT-Cl) as a cocatalyst.[23] Further, 3a–k were deacetylated with CH_3ONa/CH_3OH and afforded the corresponding aralkyl alcoholic β-D-*N*-acetylglucopyransides **4a–k** with high yields. Finally, **4i–k** on catalytic hydrogenation, using ammonium formate and 5% Pd/C in anhydrous methanol, gave **5i–k**. The structures of the compounds **4a–h**, **5i–k** were confirmed by IR, MS, ^1H NMR, and elemental analysis. All the glucosidic bonds of compounds were identified as β configurations by the coupling constants (*J*) of the protons between C1 and C2 in their ^1H NMR spectra. The proton at C1 gave the signal as a doublet at δ 4.39–4.54 ppm (J = 8.4–8.5 Hz).

2.2. Effects of target compounds 4a–h, 5i–k against hypoglycemia and serum limitation induced cytotoxicity in differentiated PC12 cells

The aim of the research was to assess the neuroprotective effects of aralkyl alcoholic 2-acetamido-2-deoxy-β-D-pyranosides on hypoglycemia and serum limitation-induced cell injury to partially model the pathological process of cerebral ischemia.[2] PC12 cells were chosen for in vitro studies because they constitute a widely used neuronal model system.[24] First, we investigated the effects of target compounds on preventing decreased cell viability in PC12 cells induced by hypoglycemia and serum limitation. As the results shown in Fig. 3, hypoglycemia and serum limitation led to significant reduction of cell viability to 45.08 ± 3.45%, compared to control. When cells pretreated with different concentrations (8, 40, 200 μM) of compounds **4a–h** and **5i–k** for 24 h followed by hypoglycemia and serum limitation treatment, most compounds showed protective effects. Especially, compounds **4g** and **4h** exhibited the most significant protective effects at the concentration of 40 μM, which increased cell viability to 86.62 ± 3.99% and 80.41 ± 3.99%, respectively, whereas **4d**, **4e**, **5i**, and **5k** restored cell survival to 60.65 ± 2.34%, 65.90 ± 2.45%, 65.36 ± 3.21%, and 62.38 ± 3.45%, which was approximate to salidroside group (65.62 ± 3.87%). At the same time, **4c**, **4f**, and **5j** (40 μM) were found to be moderately active, **4a** and **4b** (40 μM) didn't exert obvious protective effects.

Compounds **4g** and **4h** which superior to salidroside were further researched by Hoechst 33342 staining, flow cytometry with annexin V/PI staining, caspase-3 colorimetric assay and Rhodamine 123 staining.

2.3. Anti-apoptotic effects of the compounds 4g and 4h by Hoechst 33342 staining and flow cytometry with annexin V/PI staining

Apoptosis after cerebral ischemia is one of the major pathways that leads to the process of cell death.[25] Therefore, we investigated the anti-apoptotic effects of the compounds **4g** and **4h** by Hoechst 33342 staining assay. In the control group, the nuclei of PC12 cells were round and homogeneously stained. After hypoglycemia and

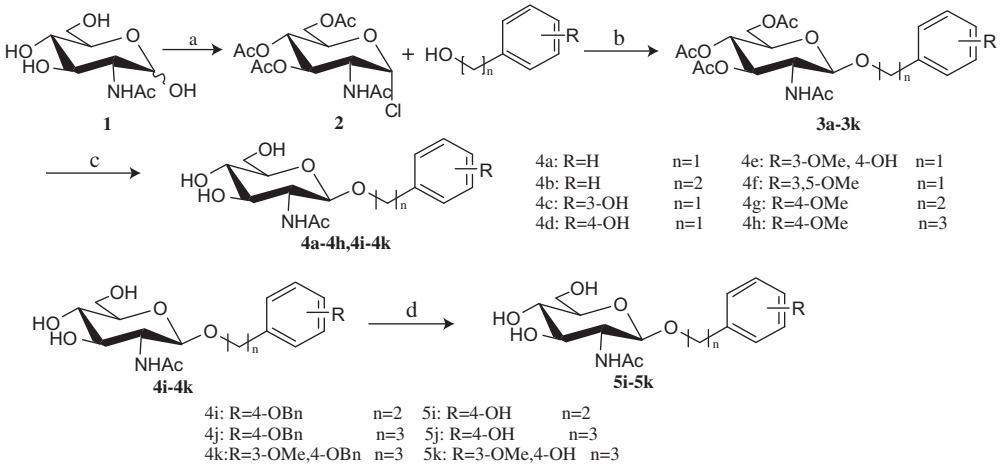

Figure 2. Synthetic route for aralkyl alcoholic 2-acetamido-2-deoxy-β-D-pyranosides. Reagents and conditions: (a) CH_3COCl; (b) $ZnCl_2$, DMT-Cl, CH_2Cl_2; (c)CH_3ONa, CH_3OH; (d) $HCOONH_4$, 5% Pd/C, CH_3OH, reflux.

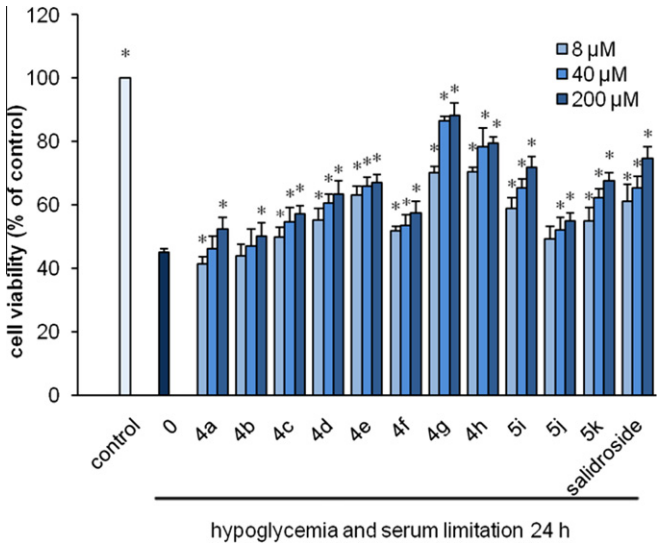

Figure 3. Protective effect of aralkyl alcoholic β-D-*N*-acetylglucopyransides on hypoglycemia and serum limitation induced apoptosis in PC12 cells. PC12 cells were pretreated with test compounds or salidroside on different concentrations (8, 40, 200 μM) for 24 h, then exposed to hypoglycemia and serum limitation for an additional 24 h with respective original concentrations of compounds maintained. Viability was calculated as the percentage of living cells in treated cultures compared to those in control cultures by using MTT assay. $^{*}P$ <0.05 versus exposure to hypoglycemia and serum limitation in alone. The data were presented as mean ± SD of three independent experiments (each in triplicate).

serum limitation insult for 24 h, 13.93% of cultured cells showed typical characteristics of apoptosis, including the condensation of chromatin, the shrinkage of nuclear and the appearance of a few apoptotic bodies. However, under pretreatments of 40 μM **4g** or **4h**, the apoptotic percentages were significantly reduced to 5.90% or 7.12%, respectively (Fig. 4A and B). These apoptotic percentages were lower than that of salidroside (8.31%).

To provide further evidence that **4g** and **4h** pretreatment can prevent hypoglycemia and serum limitation induced apoptosis in cultured PC12 cells, flow cytometer analysis with annexin V and PI double-staining was investigated. We found that a significant induction of PC12 cells apoptosis by hypoglycemia and serum limitation, increasing early and late apoptotic percent, and the total apoptotic rate reached to about 41.94%. While PC12 cells were pre-cultured with 40 μM of **4g**, **4h** or salidroside prior to hypoglycemia and serum limitation, early stage of apoptosis were prevented, and the apoptotic rate decreased to 11.21%, 15.51% and 17.56% respectively. The percentage of late stage of apoptotic cells, however, displayed no significant difference. (Fig. 4C). Therefore, we believed that **4g** and **4h** prevented the increased apoptosis rate and nuclear morphologic changes induced by hypoglycemia and serum limitation. In addition, the result revealed that the protective effects of **4g** and **4h** pretreatment manifested itself mainly in an inhibition of early cell apoptosis.

2.4. Inhibition of caspase-3-like protease activity in PC12 cells

Caspases are cysteine dependent enzymes that play important role in the induction, transduction and amplification of intracellular apoptotic signals. Among them, caspase-3 is a frequently activated death protease, catalyzing the specific cleavage of many key cellular proteins.[26] Therefore, we measured the effects of **4g** and **4h** (40 μM) on the activity of caspase-3 by colorimetric assay. As shown in Fig. 5, after exposure to hypoglycemia and serum limitation for 24 h, the activity of caspase-3 was enhanced by 4.2-fold, as compared to control group. However, pretreatment with **4g**, **4h** or salidroside significantly attenuated these increase of caspase-3 activity induced by hypoglycemia and serum limitation, causing the decrease in caspase-3 activity back to 1.55, 1.69, or 1.97-fold, compared to control. The obtained data demonstrated that the two compounds suppressed hypoglycemia and serum limitation induced apoptosis in PC12 cells.

2.5. Restoration of mitochondrial membrane potential in PC12 cells

Increasing evidence suggests that mitochondrial dysfunction participates in the induction of apoptosis and even be central to the apoptotic pathway. Indeed, depolarization of the transmembrane potential, release of apoptogenic factors and loss of oxidative phosphorylation could be induced by opening the mitochondrial permeability transition pore.[27] The loss of mitochondrial membrane potential may be a consequence of the apoptotic-signaling pathway.[28] Therefore, we investigated the changes of the mitochondrial membrane potential (MMP) by Rhodamine fluorescence. After exposure to hypoglycemia and serum limitation for 24 h alone, the MMP of PC12 cells was significantly decreased to 42.12% compared with that of control. However, the MMP decrease were markedly recovered after being pretreated with **4g**, **4h** or salidroside (40 μM), up to 82.86%, 79.05% or 71.43%, respectively. (Fig. 6).

2.6. Preliminary analysis of structure activity relationship

From structure activity relationship (SAR) studies, the protective effects of target compounds were influenced by the molecular structure of the chemicals. It was indicated that the neuroprotective activities of the aralkyl alcoholic 2-acetamido-2-deoxy-β-D-pyranosides against hypoglycemia and serum limitation-induced cell injury were markedly influenced by the aromatic substituents. Among the synthesized compounds, **4g** and **4h** containing *para*-methoxy group of phenyl ring generally showed stronger protective activities than their analogs **5i** and **5j** with hydroxy at the same position. Also, protective activities of **4g** and **4h** were higher than those compounds with methoxy group at the *meta*-position such as **4e**, **4f**, **5k**. The enhancement of activity might be related to the increment of hydrophobicity, which means that compounds with higher lipophilicity would be easier to penetrate cell membrances.[29] From the data of **4c** and **4d**, it seemed that *para*-hydroxy of phenyl ring might be favorable for protective efficacy in comparison with *meta*-hydroxy. There was unapparent correlation between the neuroprotective activities and the length of alkyl chain connecting benzene ring with *N*-acetylglucosamine. Compound **5i** containing alkyl chain with two carbons exhibited better activity than that of one or three carbons. The space length of the alkyl chain of two carbons may be appropriate for small molecules binding with correlative acceptors. In addition, compounds **4a** and **4b** without aromatic substituents displayed poor protective effects and this may be oxygen atom of substituent on the aromatic ring could form a hydrogen bond with protein. Additional study is necessary to elucidate their mechanism and structure activity relationships.

3. Conclusion

In summary, we synthesized a series of novel aralkyl alcoholic 2-acetamido-2-deoxy-β-D-pyranosides and studied their protective activities against the hypoglycemia and serum limitation-induced cell death in differentiated PC12 cells. Most target compounds displayed strong protective effects on the cell viability against the damage caused by hypoglycemia and serum limitation, especially

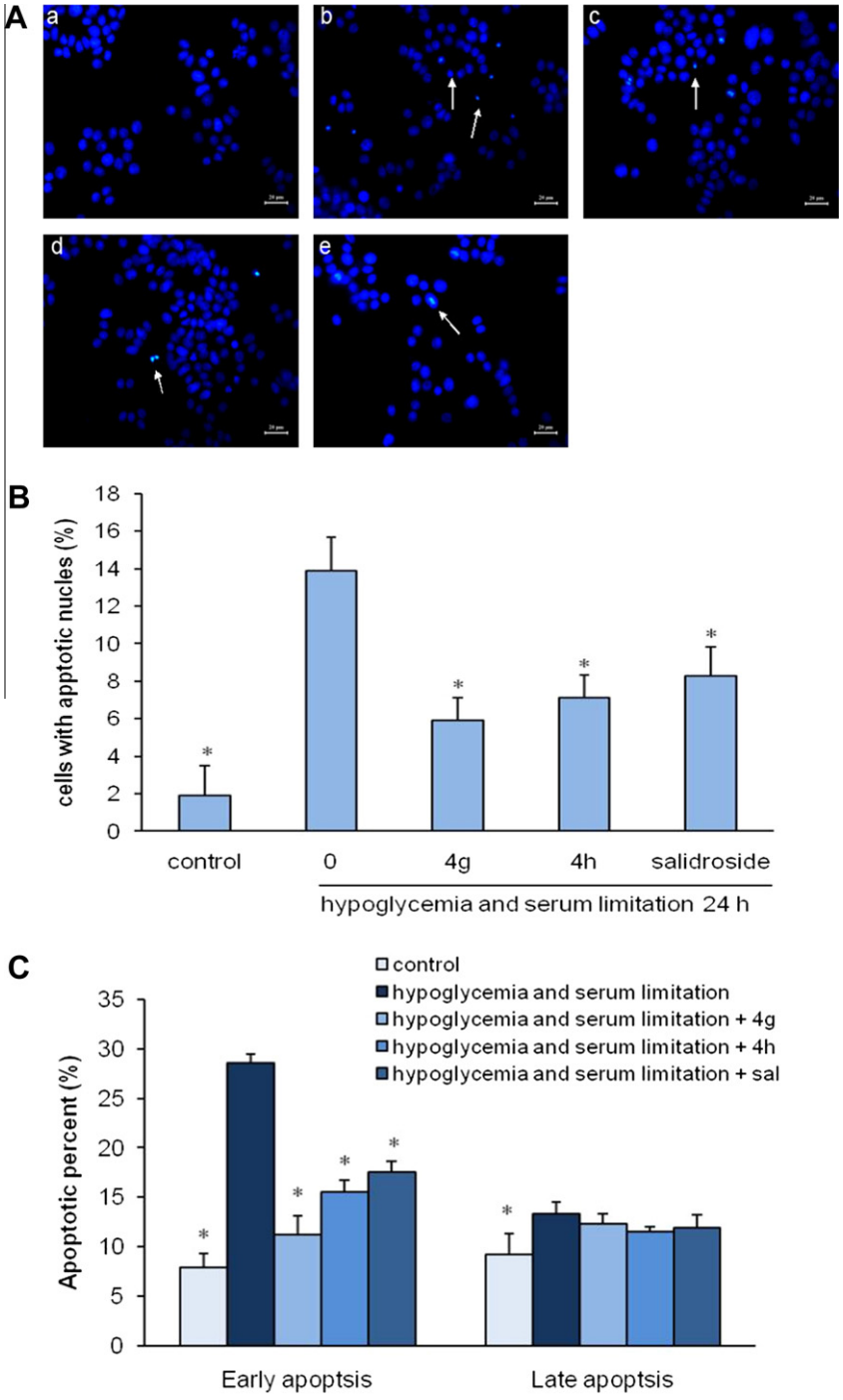

Figure 4. Protective effect of 4 g and 4 h on hypoglycemia and serum limitation-induced apoptosis in PC12 cells. (A) Representative fluorescence micrographs of Hoechst 33342 staining, which showed morphological apoptosis for PC12 cells in control (a), exposure to hypoglycemia and serum limitation alone (b), 40 μM 4 g pretreatment plus hypoglycemia and serum limitation (c), 40 μM 4 h pretreatment plus hypoglycemia and serum limitation (d), and 40 μM salidroside pretreatment plus hypoglycemia and serum limitation (e), respectively. Original magnification ×600. (B) The percentage of nuclear condensation in total cell population was counted after different treatments of PC12 cells. $^{*}P$ <0.01 versus exposure to hypoglycemia and serum limitation alone. (C) Flow cytometry with annexin V/PI staining for PC12 cells in the above five treatments and the percentage of apoptotic and necrotic cells in total cell population were shown after different treatments. $^{*}P$ <0.01 versus exposure to hypoglycemia and serum limitation alone. The data were expressed as mean ± SD of three independent experiments (each in triplicate).

for **4g** and **4h**, which had a great potency superior to salidroside and efficiently inhibited hypoglycemia and serum limitation induced cell apoptosis through the inhibition of caspase-3 activation and restoration of mitochondrial membrane potential. Although a detailed molecular mechanism that links all these events should be further investigated, the present findings provided a preliminary pharmacological basis to exploit preventive or therapeutic strategies for stroke and other neurological insults.

4. Experiment

Melting points were determined on an X-6 melting point apparatus and were uncorrected. ^{1}H NMR spectra were recorded using TMS as an internal standard on a Bruker AC 400 instrument at 500 MHz or 300 MHz with D_2O or CD_3OD as solvent. IR was recorded on AVATAR-370 FT-IR Thermo Nicolet with KBr disk. Elemental analysis(C, N and H) were performed on Elementar VarioEL

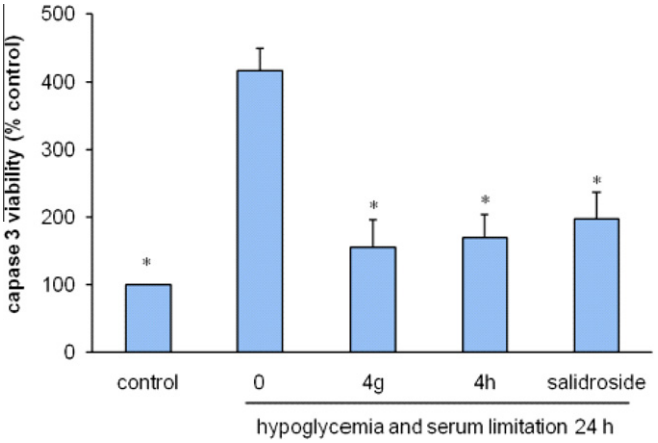

Figure 5. Effects of 4 g and 4 h on activities of caspase-3 induced by hypoglycemia and serum limitation in PC12 cells. PC12 cells were pre-incubated with 4 g, 4 h and salidroside (40 μM) respectively for 24 h then exposed to hypoglycemia and serum limitation for 24 h. The activity of caspase 3 in PC12 was measured using colorimetric assay and expressed as the percentage of the activity of control cells. The values presented were mean ± SD. $^{*}P$ <0.05 versus exposure to hypoglycemia and serum limitation alone group.

III analyzer (German). Optical rotations were measured with a JASCO P-1020 polarimeter (cell length, 100 mm). Flash chromatography was performed on Silica gel (200–300 mesh). Commercial reagents were used without further purification unless otherwise stated. Solutions after reactions and extractions were concentrated using a rotary evaporator operating at a reduced pressure of ca. 20 Torr.

4.1. Synthesis

4.1.1. General procedure for preparation of compounds 4a–k

Compound **2** (2.1 g, 5.7 mmol) and substituted aromatic alcohol (3.8 mmol) were added to a suspension of zinc chloride (0.51 g, 3.8 mmol) and 4,4′-dimethoxytrityl chloride (1.28 g, 3.8 mmol) in dry dichloromethane (15 ml), then stirred overnight at room temperature. After diluting with dichloromethane, the mixture was washed with saturated aqueous sodium hydrogen carbonate. The solvent was removed in vacuum and the residue was purified by column chromatography (petroleum ether–EtOAc 2:1, v/v) to yield the glycosides **3a–k** (54–71%). To compounds **3a–k** (3.8 mmol) in MeOH (15 ml) was added NaOMe (0.2 g, 3.8 mmol) and the mixture was allowed to stir for 2 h at room temperature. On completion of the reaction as indicated by TLC (methanol/chloroform 1/5–1/3, v/v), the solution was neutralized with Amberlite IRA-120 (H^+) resin to pH 7, then filtered and evaporated to dryness under reduced pressure afford **4a–k**.

4.1.1.1. Benzyl-2-acetamido-2-deoxy-β-D-pyranoside (4a). White solid; Yield: 96%; mp 194–196 °C; $[\alpha]_D$: −39.0 (*c* 0.16, CH_3OH); IR (KBr, *ν*, cm^{-1}): 3347, 2941, 2834, 1649, 1543, 1431, 1379, 1231, 1124, 1057, 726; MS: *m*/*z* 312 $[M+H]^+$; ^1H NMR (D_2O, 300 MHz) *δ* (ppm): 7.46–7.37 (5H, m, Ar-H), 4.89 (1H, d, *J* = 12 Hz, $ArCH_2$), 4.68 (1H, d, *J* = 12 Hz, $ArCH_2$) 4.54 (1H, d, *J* = 8.4 Hz, H-1′), 3.95 (1H, d, *J* = 12 Hz, H-6′a), 3.76 (1H, d, *J* = 12.5 Hz, H-6′b), 3.70–3.46 (4H, m, H-2′,H-3′,H-4′, H-5′), 1.94 (3H, s, NAc); Anal. Calcd for $C_{15}H_{21}NO_6$: C, 57.87; H, 6.80; N, 4.50. Found: C, 57.82; H, 6.87; N, 4.40.

4.1.1.2. Phenethyl-2-acetamido-2-deoxy-β-D-pyranoside (4b). White solid; Yield: 95%; mp 207–208 °C; $[\alpha]_D$: −22.0 (*c* 0.18, CH_3OH); IR (KBr, *ν*, cm^{-1}): 3337, 2935, 2842, 1658, 1541, 1420, 1370, 1242, 1125, 1055, 710; MS: *m*/*z* 326 $[M+H]^+$; ^1H NMR (D_2O, 500 MHz) *δ* (ppm): 7.38 (2H, t, *J* = 7.55 Hz, Ar-H), 7.29 (3H, d, *J* = 7.35 Hz, Ar - H), 4.47 (1H, d, *J* = 8.5 Hz, H-1′), 4.19 (1H, dd, *J* = 10.0 and *J* = 5.5 Hz, OCH_2), 3.92 (1H, d, *J* = 12.05 Hz, H-6′a), 3.84 (1H, dd, *J* = 8.65 and *J* = 5.1 Hz, OCH_2), 3.75 (1H, d, *J* = 12.05 Hz, H-6′b), 3.62–3.40 (4H, m, H-2′, H-3′, H-4′, H-5′), 2.93–2.83 (2H, m, $ArCH_2$), 1.83 (3H, s, NAc); Anal. Calcd for $C_{16}H_{23}NO_6$: C, 59.06; H, 7.13; N, 4.31. Found: C, 59.03; H, 7.20; N, 4.25.

4.1.1.3. 1-(3-Hydroxyphenyl)methyl-2-acetamido-2-deoxy-β-D-pyranoside (4c). White solid; Yield: 90%; mp 166–168 °C; $[\alpha]_D$ −48.4 (*c* 0.09, CH_3OH); IR (KBr, *ν*, cm^{-1}): 3336, 2935, 2841, 1635, 1591, 1459, 1376, 1251, 1153, 1038, 883, 761; MS: *m*/*z* 328 $[M+H]^+$; ^1H NMR (D_2O, 300 MHz) *δ* (ppm): 7.33 (1H, t, *J* = 7.77 Hz, Ar-H), 6.94–6.87 (3H, m, Ar-H), 4.88 (1H, d, *J* = 12.25 Hz, $ArCH_2$), 4.62 (1H, d, *J* = 12.3 Hz, $ArCH_2$), 4.53 (1H, d, *J* = 8.4 Hz, H-1′), 3.84–3.45 (6H, m, H-2′,H-3′,H-4′, H-5′, H-6′), 2.0 (3H, s, NAc); Anal. Calcd for $C_{15}H_{21}NO_7$: C, 55.04; H, 6.47; N, 4.28. Found: C, 55.13; H, 6.55; N, 4.26.

4.1.1.4. 1-(4-Hydroxyphenyl)methyl-2-acetamido-2-deoxy-β-D-pyranoside (4d). White solid; Yield: 98%; mp 169–172 °C; $[\alpha]_D$ −37.0 (*c* 0.14, CH_3OH); IR (KBr, *ν*, cm^{-1}): 3322, 2947, 2869, 1667, 1547, 1449, 1375, 1261, 1151, 1033, 820; MS: *m*/*z* 328 $[M+H]^+$; ^1H NMR (D_2O, 300 MHz) *δ* (ppm): 7.27 (2H, d, *J* = 8.28 Hz, Ar-H), 6.93 (2H, d, *J* = 8.4 Hz, Ar- H), 4.89 (1H, d, *J* = 12.21 Hz, $ArCH_2$), 4.59 (1H, d, *J* = 11.9 Hz, $ArCH_2$), 4.52 (1H, d, *J* = 8.46 Hz, H-1′), 3.95 (1H, d *J* = 12.09 Hz, H-6′a), 3.76 (1H, d, *J* = 12.42 Hz, H-6′b), 3.70–3.45 (4H, m, H-2′, H-3′, H-4′, H-5′), 1.91 (3H, s, NAc); Anal. Calcd for $C_{15}H_{21}NO_7$: C, 55.04; H, 6.47; N, 4.28. Found: C, 55.04; H, 6.55; N, 4.29.

4.1.1.5. 1-(3-Methoxy-4-hydroxyphenyl)methyl-2-acetamido-2-deoxy-β-D-pyranoside (4e). White solid; Yield: 91%; mp 170–174 °C; $[\alpha]_D$ −34.6 (*c* 0.10, CH_3OH); IR (KBr, *ν*, cm^{-1}): 3266, 2962, 2849, 1655, 1558, 1457, 1353, 1268, 1161, 1058, 801,628; MS: *m*/*z* 358 $[M+H]^+$; ^1H NMR (D_2O, 500 MHz) *δ* (ppm): 7.03 (1H, s, Ar-H), 6.94 (1H, d, *J* = 7.95 Hz, Ar-H), 6.89 (1H, d, *J* = 7.95 Hz, Ar-H), 4.89 (1H, d, *J* = 11.50 Hz, $ArCH_2$), 4.68 (1H, d, *J* = 11.43 Hz, $ArCH_2$), 4.53 (1H, d, *J* = 8.4 Hz, H-1′), 3.90 (1H, d, *J* = 12.25 Hz, H-6′a), 3.89 (3H, s, OCH_3), 3.77 (1H, d, *J* = 9.35 Hz, H-6′b), 3.67–3.45 (4H, m, H-2′, H-3′, H-4′, H-5′), 1.89 (3H, s, NAc); Anal. Calcd for $C_{16}H_{23}NO_8$: C, 53.78; H, 6.49; N, 3.92. Found: C, 53.76; H, 6.58; N, 3.90.

4.1.1.6. 1-(3, 5-Dimethoxyphenyl)methyl-2-acetamido-2-deoxy-β-D-pyranoside (4f). White solid; Yield: 95%; mp 220–223 °C; $[\alpha]_D$ −31.4 (c 0.11, CH_3OH); IR (KBr, *ν*, cm^{-1}): 3345, 2950, 2836, 1628, 1545, 1473, 1375, 1212, 1164, 1056, 830; MS: *m*/*z* 372 $[M+H]^+$; ^1H NMR (D_2O, 500 MHz) *δ* (ppm): 6.62 (2H, s, Ar-H), 6.60 (1H, s, Ar-H), 4.84 (1H, d, *J* = 12 Hz, $ArCH_2$), 4.62 (1H, d, *J* = 10 Hz, $ArCH_2$), 4.54 (1H, d, *J* = 8.5 Hz, H-1′), 3.95 (1H, d, *J* = 12.5 Hz, H-6′a), 3.85 (6H, s, 2× OCH_3), 3.77 (1H, d, *J* = 10 Hz, H-6′b), 3.70–3.47 (4H, m, H-2′, H-3′, H-4′, H-5′), 1.93 (3H, s, NAc); Anal. Calcd for $C_{17}H_{25}NO_8$: C, 54.98; H, 6.79; N, 3.77. Found: C, 54.93; H, 6.83; N, 3.75.

4.1.1.7. 2-(4-Methoxyphenyl)ethyl-2-acetamido-2-deoxy-β-D-pyranoside (4g). White solid; Yield: 93%; mp 190–191 °C; $[\alpha]_D$ −21.1 (*c* 0.15, CH_3OH); IR (KBr, *ν*, cm^{-1}): 3469, 2934, 2853, 1654, 1571, 1485, 1379, 1253, 1161, 1031, 825; MS: *m*/*z* 356 $[M+H]^+$; ^1H NMR (D_2O, 500 MHz) *δ* (ppm): 7.24 (2H, d, *J* = 8.5 Hz, Ar-H), 6.98 (2H, d, *J* = 8.5 Hz, Ar- H), 4.46 (1H, d, *J* = 8.5 Hz, H-1′), 4.17 (1H, dd, *J* = 10.0 and *J* = 5.0 Hz, OCH_2), 3.93 (1H, d, *J* = 12 Hz, H-6′a), 3.84 (3H, s, OCH_3), 3.82–3.73 (2H, m, OCH_2, H-6′b), 3.62–3.42 (4H, m, H-2′, H-3′, H-4′, H-5′), 2.88–2.80 (2H, m, $ArCH_2$),

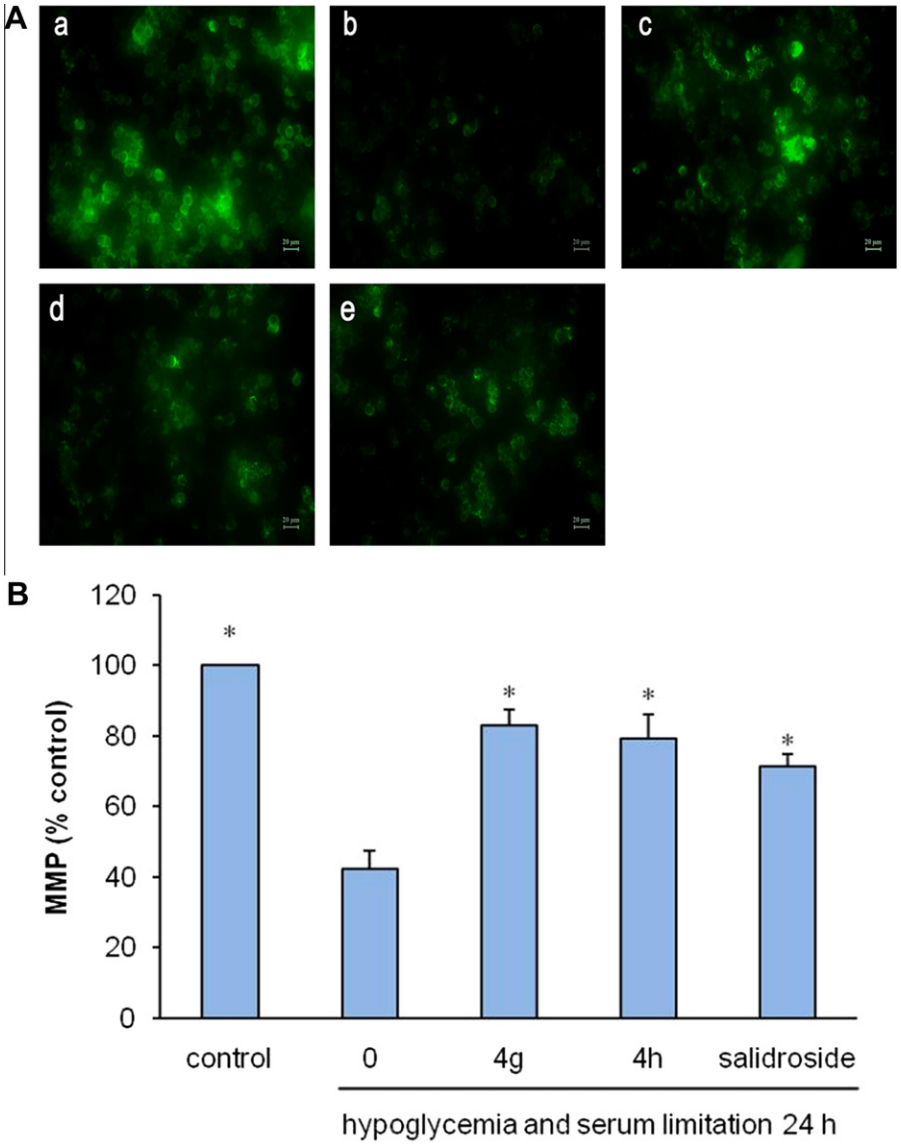

Figure 6. Effects of **4g** and **4h** on decrease of MMP induced by hypoglycemia and serum limitation in PC12 cells. (A) Representative fluorescent micrographs of Rhodamine 123 staining for comparing the mitochondrial membrane potential of PC12 cells in control (a), exposure to hypoglycemia and serum limitation alone (b), 40 μM 4 g pretreatment plus hypoglycemia and serum limitation (c), 40 μM 4 h pretreatment plus hypoglycemia and serum limitation (d), and 40 μM salidroside plus hypoglycemia and serum limitation (e), respectively. Original magnification ×400. (B) the percentage of fluorescent intensity of Rhodamine 123 in treated cultures compared to those in control cultures was also shown. $^{*}P$ <0.05 versus exposure to hypoglycemia and serum limitation alone. The data were expressed as mean ± SD of three independent experiments (each in triplicate).

1.82 (3H, s, NAc); Anal. Calcd for $C_{17}H_{25}NO_7$: C, 57.45; H, 7.09; N, 3.94. Found: C, 57.41; H, 7.15; N, 3.89.

4.1.1.8. 3-(4-Methoxyphenyl)propyl-2-acetamido-2-deoxy-β-D-pyranoside (4h). White solid; Yield: 93%; mp 201–203 °C; $[\alpha]_D$ −10.9 (*c* 0.13, CH_3OH); IR (KBr, *v*, cm^{-1}): 3464, 2928, 2866, 1653, 1552, 1514, 1482, 1383, 1243, 1168, 1051, 820; MS: *m*/*z* 370 $[M+H]^+$; ^1H NMR (CD_3OD, 300 MHz) δ (ppm): 7.09 (2H, d, *J* = 8.7 Hz, Ar-H), 6.81 (2H, d, *J* = 7.2 Hz, Ar-H), 4.39 (1H, d, *J* = 8.4 Hz, H-1′), 3.91–3.84 (2H, m, OCH_2, H-6′a), 3.75 ((3H, s, OCH_3), 3.71–3.62 (2H, m, OCH_2, H-6′b), 3.50–3.41 (4H, m, H-2′, H-3′, H-4′, H-5′), 2.6 (2H, t, *J* = 6.9 Hz, $ArCH_2$), 1.99 (3H, s, NAc), 1.8 (2H, t, *J* = 6.9 Hz, $BnCH_2$); Anal. Calcd for $C_{18}H_{27}NO_7$: C, 58.52; H, 7.37; N, 3.79. Found: C, 58.65; H, 7.32; N, 3.81.

4.1.1.9. 2-(4-Benzyloxyphenyl)ethyl-2-acetamido-2-deoxy-β-D-pyranoside (4i). White solid; Yield: 93%. The product could be used in subsequent reactions without further purification.

4.1.1.10. 3-(4-Benzyloxyphenyl)propyl-2-acetamido-2-deoxy-β-D-pyranoside (4j). White solid; Yield: 95%. The product could be used in subsequent reactions without further purification.

4.1.1.11. 3-(3-methoxy-4-benzyloxyphenyl)propyl-2-acetamido-2-deoxy-β-D-pyranoside (4k). White solid; Yield: 91%. The product could be used in subsequent reactions without further purification.

4.1.2. General procedure for preparation of compounds 5i–k

To a stirred suspension of **4i–k** (2 mmol) in methanol (10 mL), 5% Pd/C (90 mg) and ammonium formate (650 mg, 10 mmol) were added, then the mixture was heated to reflux for 6 h. After reaction the mixture was filtered and concentrated, then resorted to column chromatography using 8:1 $CHCl_3$–MeOH to afford compounds **5i–k**.

4.1.2.1. 2-(4-Hydroxyphenyl)ethyl-2-acetamido-2-deoxy-β-D-pyranoside (5i). White solid; Yield: 95%; mp 175–178 °C; $[\alpha]_D$

−31.4 (c 0.18, CH_3OH); IR (KBr, ν, cm^{-1}): 3346, 2928, 2845, 1658, 1519, 1452, 1384, 1274, 1129, 1081, 1007,825; MS: m/z 342 $[M+H]^+$; ^1H NMR (D_2O, 500 MHz) δ (ppm): 7.16 (2H, d, J = 7.5 Hz, Ar-H), 6.86 (2H, d, J = 8.0 Hz, Ar-H), 4.45 (1H, d, J = 8.5 Hz, H-1′), 4.16 (1H, d, J = 9.0 Hz, OCH_2), 3.92 (1H, d, J = 12.5 Hz, H-6′a), 3.76–3.73 (2H, m, OCH_2, H-6′a), 3.61–3.42 (4H, m, H-2′, H-3′, H-4′, H-5′), 2.86–2.78 (2H, m, $ArCH_2$), 1.81 (3H, s, NAc); Anal. Calcd for $C_{16}H_{23}NO_7$: C, 56.30; H, 6.79; N, 4.10; Found: C, 56.20; H, 6.85; N, 4.11.

4.1.2.2. 3-(4-Hydroxyphenyl)propyl-2-acetamido-2-deoxy-β-D-pyranoside (5j). White solid; $[\alpha]_D$ −15.7 (c 0.17, CH_3OH); IR (KBr, ν, cm^{-1}): 3308, 2934, 2866, 1615, 1557, 1517, 1449, 1370, 1248, 1166, 1072, 820; MS: m/z 356 $[M+H]^+$; ^1H NMR (D_2O, 300 MHz) δ (ppm): 7.16 (2H, d, J = 8.4 Hz, Ar - H), 6.87 (2H, d, J = 8.3 Hz, Ar-H), 4.5 (2H, d, J = 8.5 Hz, H-1′), 3.94–3.85 (2H, m, OCH_2, H-6′a), 3.78–3.68 (2H, m, OCH_2, H-6′b), 3.60–3.44 (4H, m, H-2′, H-3′, H-4′, H-5′), 2.59 (2H, t, J = 7 Hz, $ArCH_2$), 2.04 (3H, s, NAc), 1.83 (2H, t, J = 6.66 Hz, $BnCH_2$); Anal. Calcd for $C_{17}H_{25}NO_7$: C, 57.45; H, 7.09; N, 3.94. Found: C, 57.44; H, 7.20; N, 3.99.

4.1.2.3. 3-(3-Methoxy-4-hydroxyphenyl)propyl-2-acetamido-2-deoxy-β-D-pyranoside (5k). White solid; $[\alpha]_D$ −15.6 (c 0.17, CH_3OH); IR (KBr, ν, cm^{-1}): 3367, 2935, 2865, 1651, 1516, 1452, 1375, 1273, 1155, 1034, 810, 641; MS: m/z 385 $[M+H]^+$; ^1H NMR (D_2O, 300 MHz): 6.94 (1H, s, Ar-H), 6.89 (1H, d, J = 8.27 Hz, Ar-H), 6.80 (1H, d, J = 8.07 Hz, Ar-H), 4.51 (1H, d, J = 8.5 Hz, H-1′), 3.95–3.90 (2H, m, OCH_2, H-6′a), 3.87 (3H, s, OCH_3), 3.82–3.60 (2H, m, OCH_2, H-6′b), 3.57–3.46 (4H, m, H-2′, H-3′, H-4′, H-5′), 2.60 (2H, t, J = 7.35 Hz, $ArCH_2$), 2.03 (3H, s, NAc), 1.85 (2H, t, J = 7.2 Hz, $BnCH_2$); Calcd for $C_{18}H_{27}NO_8$: C, 56.09; H, 7.06; N, 3.63. Found: C, 56.18; H, 7.09; N, 3.57.

4.2. Cell culture

PC12 cells, obtained from the American Type Culture Collection (Manassas, VA), were plated and maintained in high-glucose DMEM supplemented with 10% horse serum, 5% fetal bovine serum, 100 U/ml penicillin, and 100 U/ml streptomycin in a humidified atmosphere of 5% CO_2 at 37 °C. The cells were differentiated by treating with nerve growth factor (NGF) (50 ng/ ml) every other day for 6 days.

4.3. Exposure target compounds (4a–h, 5i–k) to differentiated PC12 cells

After being pretreated with 8, 40, and 200 μmol/L salidroside and synthetic aralkyl alcoholic 2-acetamido-2-deoxy-β-D-pyranoside for 24 h, respectively, the cells were subjected to hypoglycemia and serum limitation by changing the culture medium with the glucose-free DMEM supplemented with 1% horse serum and 1% fetal bovine serum, in the presence of compounds at original concentrations for another 24 h incubation. In the aforementioned process, treatment only with culture medium containing high glucose/enough serum was considered control group, meanwhile, only exposure to hypoglycemia and serum limitation as hypoglycemia and serum limitation alone group.

4.4. Cell viability assay

At the end of cell treatments, MTT solution was added to the cell samples to make final concentration of 0.5 mg/ml, then incubate at 37 °C for 4 h followed by the addition of 20% sodium dodecyl sulfide (SDS) to dissolve the resulting formazan. The absorbance (OD) values were measured by spectrophotometry at 570 nm with an EIX-800 Microelisa reader (Bio-Tek Inc., USA).

4.5. Hoechst 33342 staining

At the end of cell treatments, the PC12 cells were fixed with 4.0% paraformaldehyde for 20 min, washed with 0.15 M NaCl for 3 times then stained with 5 μg/ml Hoechst 33342 dye at 37 °C for 10 min. Morphologic changes in apoptotic nuclei were observed under a DMR fluorescence microscope (Leica Microsystems, Wetzlar, Germany) with ultraviolet illumination. The dye was excited at 340 nm, and emission was filtered with a 510 nm barrier filter. In order to quantify the apoptotic process, cells with fragmented or condensed DNA and normal DNA were respectively counted. Data were expressed as the ratio of apoptotic cells to total cells.

4.6. Flow cytometry with annexin V/PI staining

The PC12 cells were harvested by centrifugation after treatment as described above (treated with either **4g**, **4h**, or salidroside plus hypoglycemia and serum limitation). The pellets resuspended in 1 × binding buffer (10 mM HEPES, 140 mmM NaCl, 2.5 mM $CaCl_2$) at a concentration of 1×10^6 cell/ml. In addition to a 100 μl aliquot of the cell suspension, 5 μl of FITC-conjugated annexin V (PharMingen, San Diego, CA) and 5 μl of 50 μg/ml propidium iodide (PI) were added. After 15 min incubation in the dark at room temperature, cells were analyzed for annexin V binding within 1 h with a flow cytometer (BD FACScalibur, BD Bioscience, San Jose, CA). Apoptotic and necrotic cells were quantitated by annexin V binding and PI uptake.

4.7. Caspase-3 colorimetric assay

Caspase-3 activity was determined using the caspase-3 activity kit, which was based on the ability of caspase-3 to change acetyl-Asp-Glu-Val-Asp *p*-nitroanilide (Ac-DEVD-pNA) into the yellow formazan product, *p*-nitroaniline (pNA). According to the manufacturer's instruction, treated cells were lysed, then the supernatant was centrifuged at 12,000 g for 10 min, and protein concentrations were determined by Bradford protein assay. After incubating the mixture composed of 10 μl of cell lysate, 80 μl of reaction buffer and 10 μl of 2 mM Ac-DEVD-pNA in 96-well microtiter plates at 37 °C for 4 h, caspase-3 activity was quantified in the samples with an EIX-800 Microelisa reader (Bio-Tek Inc., USA) at an absorbance of 405 nm. The caspase activities were expressed as percentage compared to control.

4.8. Measurement of mitochondrial membrane potential

At the end of cell treatments, the fluorescent dye Rhodamine 123 was added to the PC12 cells to make a final concentration of 2 μM. After 30 min culture at 37 °C, the cells were fixed with 4.0% paraformaldehyde for 20 min, and washed twice with PBS, followed by visualization under a TCS SP2 confocal microscope (Leica Microsystems, Wetzlar, Germany). The signal of Rhodamine 123 was excited by 488 nm laser light and emission was captured at 530 nm. Each field of cells was photographed (magnification, 400) for calculation of the relative fluorescence intensity.

4.9. Statistical analysis

Data were expressed as means ± SD (n = 3, where n represents the number of independent experiments). Statistical differences between groups were analyzed by one-way ANOVA tests. Differences were considered significant at P <0.05.

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