Characterization of a Synthetic Steroid 24-keto-cholest-5-en-3 β , 19-diol as a Neuroprotectant

Min Yan,¹ Ai-Ling Liu,¹ Shu-Jia Zhou,² Li-Peng Tang,¹ Yan-Qiu Ou,³ Wei Yin,¹ Xin-Ying Chen,² Xing-Wen Su,¹ Peng-Xin Qiu,¹ Yi-Jun Huang,¹ Jing-Xia Zhang,² Guang-Mei Yan¹ & Tian-Dong Leng¹

¹ Department of Pharmacology, Zhongshan Medical College, Sun Yat-Sen University, Guangzhou, China

² School of pharmaceutical sciences, Sun Yat-Sen University, Guangzhou, China

³ Department of Cardiovascular Epidemiology, Guangdong General Hospital, Guangzhou, China

Keywords

Neuroprotection; NMDA; Steroid; Stroke.

Correspondence

J. Zhang, School of pharmaceutical sciences, Sun Yat-sen University, 510006, China.

Tel.: +86-20-87331480;

Fax: +86-20-87333323;

E-mail: jingxiazhang@163.com

and

T. Leng, Department of Pharmacology, Zhongshan Medical College, Sun Yat-sen University, Guangzhou, GD 510080, China.

Tel.: +86-20-87333258;

Fax: +86-20-87330578;

E-mail: lengtiandong@sina.com

Received 11 September 2014; revision 11

December 2014; accepted 12 December 2014

SUMMARY

Background: Neuroactive steroids represent promising candidates for the treatment of neurological disorders. Our previous studies identified an endogenous steroid cholestane-3 β , 5 α , 6 β -triol (Triol) as a novel neuroprotectant. **Aim:** We aimed to identify a potent candidate for stroke treatment through a screening of Triol analogs. **Methods:** Hypoxia- and glutamate-induced neuronal injury models *in vitro*, middle cerebral artery occlusion (MCAO)-induced cerebral ischemia model *in vivo*, fluorescein diacetate (FDA) for alive and propidium iodide (PI) for dead staining, LDH assay, and calcium imaging techniques were used. **Results:** 24-keto-cholest-5-en-3 β , 19-diol (Diol) showed the most potent neuroprotective effect among the screened structurally related compounds. FDA and PI staining showed that Diol concentration dependently increased the survival rate of cerebellar granule neurons (CGNs) challenged with glutamate or hypoxia, with an effective threshold concentration of 2.5 μ M. Consistently, the quantitative LDH release assay showed the same concentration-dependent protection in both models. Diol, at 10 μ M, potently decreased glutamate- and hypoxia-induced LDH release from 51.6 to 18.2% and 62.1 to 21.7%, respectively, which values are close to the normal LDH release (~16–18%). Moreover, we found Diol effectively decreased MCAO-induced infarction volume in mice from ~23% to 7%, at a dose of 6 mg/kg. We further explored the underlying mechanism and found that Diol attenuated NMDA-induced intracellular calcium ([Ca²⁺]_i) increase in cortical neurons, suggesting a negative modulatory effect on NMDA receptor. **Conclusion:** Taken together, we identified Diol as a potent neuroprotectant. It may represent a novel and promising neuroprotectant for stroke intervention.

doi: 10.1111/cns.12378

The first two authors contributed equally to this work.

Introduction

Glutamate is a major excitatory neurotransmitter that plays important physiological roles in the central nervous system (CNS). However, excess release of glutamate and subsequent activation of NMDA subtype glutamate receptors induce an overwhelming calcium influx into central neurons, resulting in neuronal injury/death. This process is associated with several neurological disorders including stroke, amyotrophic lateral sclerosis (ALS), Parkinson disease, and Alzheimer disease [1–3]. In this regard, NMDA receptors are attractive therapeutic targets and NMDA receptor antagonists should provide therapeutic benefits for those neurological disorders where excessive activation of NMDA receptors is involved. For instance, two NMDA receptor antagonists memantine and amantadine have been successfully

approved by FDA for treatment of Alzheimer and Parkinson diseases, respectively [4–6].

Neurosteroids and neuroactive steroids have various effects on the nervous system. In particular, the rapid modulation of neuronal excitability through interactions with ligand-gated ion channels and other cell surface receptors attracts academic and pharmaceutical interests recently. Neurosteroids are promising candidates for the treatment of neurological disorders including epilepsy, traumatic brain injury, and stroke [7,8]. In a previous study, we identified an endogenous steroid cholestane-3 β , 5 α , 6 β -triol (Triol), a metabolite of cholesterol, which exhibited significant neuroprotective effects in multiple primary cultures *in vitro* and cerebral ischemia *in vivo* via negative modulation of NMDA receptors [9]. Triol would serve as a promising lead compound for the development of novel and

potent neuroprotectants for stroke and other NMDA receptor-related neurological disorders.

Based on Triol structure, we performed a preliminary screening of structurally related compounds. We identified Diol as a more potent neuroprotectant. Diol showed potent protective effect against glutamate- and/or hypoxia-induced neuronal injury *in vitro* and middle cerebral artery occlusion-induced ischemic brain injury *in vivo*. We further demonstrated that Diol inhibited NMDA-induced intracellular calcium ($[Ca^{2+}]_i$) increase, suggesting a negative modulatory effect on NMDA receptor. In summary, our findings identify Diol as a potent neuroprotectant and a promising candidate for stroke treatment.

Materials and Methods

Synthesis of 24-keto-cholest-5-en-3 β , 19-diol (Diol)

Diol was synthesized starting from 3 β -Acetoxy-cholest-5-en-24-one, through several steps of reactions (Figure 2), using a similar method as described previously [10]. All chemicals and solvents were analytical grade and purchased from the Guangzhou Chemical Reagents Company (Guangzhou, Guangdong, China). Melting points were determined on an $\times 6$ melting point apparatus and were uncorrected. IR spectra were recorded as KBr pellets on an EQUINOX 55 FT spectrophotometer (Bruker, Karlsruhe, Germany). 1H NMR and ^{13}C NMR spectra were recorded on a Varian Unity Inova 500 NB (500 MHz) spectrometer (Palo Alto, CA, USA). Chemical shifts were reported in values relative to TMS as the internal standard. MS data were measured on a DSQ and LCQ mass spectrometer (Suwanee, GA, USA). Flash column chromatography was carried out with silica gel (200–300 mesh).

Neuronal Cultures and Glutamate Treatment

Primary cultures of cerebellar granule neurons (CGNs) and cortical neurons were prepared as described previously [11,12]. CGNs were isolated and cultured from 7-day-old Sprague–Dawley rat pups (15–19 g), and cortical neurons were isolated from brains of fetuses (embryonic day 16). Neurons were used at 8–10 days for CGNs and at 10–14 days for cortical neurons. Diol and MK-801 (Sigma, St. Louis, MO, USA) were dissolved in DMSO as stock. For glutamate treatment, CGNs were rinsed twice with Locke's buffer (134 mM NaCl, 25 mM KCl, 4 mM $NaHCO_3$, 2.3 mM $CaCl_2$, 5 mM D-glucose, 5 mM HEPES, pH 7.4) and then incubated in Locke's buffer with or without DMSO vehicle ($<0.1\%$), MK-801, and different concentrations of Diol for 5 min. After that, 200 μM glutamate and 10 μM glycine were added to vehicle, Diol, and MK-801-treated cells and incubated for 40 min. Subsequently, the cells were switched back to the culture medium for 24 h until FDA and PI staining, and LDH measurements were taken.

NMDA Treatment

Cortical neurons at 10–14 days were used. Briefly, cortical neurons were rinsed twice with normal extracellular fluid (ECF composition, in mM: NaCl, 140; KCl, 5.4; $MgCl_2$, 1; $CaCl_2$, 2;

HEPES, 20; glucose, 10, pH 7.4) and then challenged with Mg^{2+} -free ECF containing 200 μM NMDA plus 10 μM glycine for 45 min, in the presence of nimodipine (5 μM ; Sigma), CNQX (10 μM ; Sigma), and TTX (0.3 μM ; Alomone labs, Jerusalem, Israel). DMSO vehicle ($<0.1\%$), Diol, and MK-801 were applied with NMDA (co-application) or applied after NMDA treatment (postapplication). LDH measurements were taken at 24 h.

Hypoxia/reoxygenation (H/R) Treatment

At day 8–10 *in vitro*, CGNs were switched to glucose and serum-free culture medium. 5 min before hypoxia exposure, the cultures were treated with different concentrations of Diol (2.5, 5, 10 μM), 10 μM MK-801, and same amount of DMSO vehicle. Then, the cultures were incubated in a hypoxic atmosphere. Hypoxic conditions (95% N_2 , 5% CO_2 , and $<0.1\%$ O_2) were achieved using an anaerobic jar equipped with a new type AnaeroPack and an indicator to monitor oxygen depletion as described previously [13]. After incubation in a hypoxic environment for 3 h at 37°C, cultures were returned to a normoxic atmosphere at 37°C for 24 h (reoxygenation). Normal cultures were maintained under standard incubation conditions.

Fluorescein Diacetate (FDA) Alive and Propidium Iodide (PI) Dead Staining

At 24 h following glutamate or hypoxia treatment, the viability of CGNs was assessed by PI and FDA staining as described previously [11]. Briefly, CGNs were incubated in standard extracellular solution with addition of 2 μM PI and 5 μM FDA for 15 min followed by wash with standard extracellular solution. PI-positive and FDA-positive cells were viewed on a microscope (Zeiss) equipped with epifluorescence at 580/630 nm excitation/emission for PI and 500/550 nm for FDA. The numbers of neurons from 4–5 random fields were counted by a blinded observer and normalized to normal control for FDA staining, and glutamate or hypoxia treatment for PI staining.

Detection of Chromatin Condensation

Chromatin condensation was detected by nucleus staining with Hoechst33258 as described previously [12]. Briefly, at 24 h after glutamate or hypoxia treatment, the cells plated onto poly-L-lysine-coated 6-well chamber slides were fixed with 4% paraformaldehyde in PBS for 20 min at 4°C. Then, the cells were washed twice with PBS and incubated with Hoechst33258 (5 mg/L) for 10 min at room temperature in the dark. Stained nuclei were visualized with a Zeiss Axiophot fluorescence microscope at a 640 magnification with an excitation wavelength of 355–366 nm and an emission wavelength of 465–480 nm. In this way, apoptotic CGNs would be stained into bright blue because of their chromatin condensation.

LDH Assay

Neuronal injury was quantified by measurement of LDH release in the culture medium using a cytotoxicity detection kit (Roche Diagnostics, Indianapolis, IN, USA) as described previously [14].

Supernatant (50 μ L) was transferred from the culture wells to a 96-well plate. Cells were then lysed in 0.5% Triton X-100 for 30 min at 37°C. Then, 50 μ L supernatants were transferred from each well for maximal LDH measurement. 50 μ L assay reagent from cytotoxicity detection kit (Roche Diagnostics) was added to each sample and mixed. 30 min later, the absorbance at 492 and 620 nm was examined by spectrometer (SpectraMax Plus, Molecular devices), and the values of the absorbance at 492 nm were subtracted by those at 620 nm to yield the value of LDH release. The LDH release is presented as the ratio of supernatant LDH (minus background absorbance) versus the maximal LDH release (minus background absorbance).

Focal Ischemia

Transient focal ischemia was induced by suture occlusion of the middle cerebral artery (MCAO) for 1 h as described previously [15]. Ischemia was induced by introducing a coated filament from the external carotid artery into the internal carotid artery to the branching point of the left MCA. Rectal temperature was maintained at 37°C $\pm$ 0.5°C with a thermostatically controlled heating pad and lamp. Cerebral blood flow was monitored by transcranial LASER doppler. Animals with blood flow not reduced below 15% were excluded. Reperfusion was accomplished by withdrawing the suture after 1 h of ischemia. Diol (6 mg/kg) was administrated through caudal vein injection 15 min prior MCAO. Animals were killed with 10% chloral hydrate 24 h after ischemia. Brains were rapidly removed, coronally sectioned at 1-mm intervals starting 2 mm posterior to the frontal pole to yield five coronal slices, stained by immersion, incubated in 1.0% 2,3,5-triphenyltetrazolium chloride (TTC) at 37°C for 30 min, and fixed in 4.0% paraformaldehyde solution. Infarct volume was calculated by summing infarction areas of all sections and multiplying by slice thickness. All manipulations and analyses were performed by individuals blinded to treatment groups. All experimental procedures on live mice were performed according to the guidelines for the care and use of laboratory animals as published by Sun Yat-sen University (Guangzhou, China).

Measurement of Intracellular Calcium

The intracellular calcium level in cortical neurons was measured using a Ca^{2+} -sensitive fluorescent dye, fura-2-acetoxymethyl ester. Briefly, Cortical neurons at 10–14 days grown on 25 $\times$ 25 mm glass coverslips were washed three times with normal extracellular fluid (ECF) (ECF composition, in mM: NaCl, 140; KCl, 5.4; MgCl_2 , 1; CaCl_2 , 2; HEPES, 20; glucose, 10, pH 7.4). Then, the cells were incubated in ECF with 5 μ M fura-2-acetoxymethyl ester for 30 min in the dark, followed by three times of wash with ECF and kept in the dark for additional 30 min until Ca^{2+} measurement. Ca^{2+} measurement was performed in the presence of nimodipine (5 μ M; Sigma), CNQX (10 μ M; Sigma), and TTX (0.3 μ M; Alomone labs). The fluorescence signal was monitored and recorded by a cooled CCD camera (Sensys KAF 1401, Photometrics). Digital images were acquired and analyzed by Axon Imaging Workbench software 6.0 (Axon Instruments, Sunnyvale, CA, USA). The shutter and filter wheel (Lambda 10–2) were controlled by the software to allow timed illumination of

cells at 340 or 380 nm excitation wavelengths. Fura-2 fluorescence was detected at emission wavelength of 510 nm. Ratio images (340/380) were analyzed by averaging pixel ratio values in circumscribed regions of cells in the field of view. Sigmaplot 8.0 was used for further analysis.

Statistical Analysis

Results were expressed as mean $\pm$ SEM. Significant differences between groups were determined by Student's *t*-test and one-way analysis of variance (ANOVA). Statistical significance was set at **P* < 0.05 and ***P* < 0.01.

Results

Effects of a Series of Triol Analogs on Glutamate-Induced Neuronal Injury

Based on cholestane-3 β , 5 α , 6 β -triol (Triol) structure, we screened a series of structurally related compounds (Figure 1) in a glutamate-induced neuronal injury model *in vitro* aimed at identifying a potent neuroprotectant. As Triol exerted significant neuroprotective effect at 10 μ M, we compared all the analogs at the same concentration. As shown in Table 1, some compounds including E and L slightly increased glutamate-induced LDH release due to a potential toxicity effect (>54.6%). In contrast, other compounds including Triol, G-K, significantly decreased LDH release, indicating neuroprotective effects. Among all compounds, Diol showed the most potent neuroprotective effect. It decreased glutamate-induced LDH release from 53.4 to 19.0%.

Synthesis of 24-keto-cholest-5-en-3 β , 19-diol (Diol)

Diol is designed and synthesized starting from 3 β -Acetoxy-cholest-5-en-24-one via multiple steps of reactions (Figure 2). The final product was purified by flash column chromatography to afford colorless needles. The structural identity of Diol was established on the basis of spectroscopic analysis as follows. m.p. 154–155°C. IR (KBr, cm^{-1}): 3394, 2935, 1709, 1640, 1467, 1446, 1380, 1048; ^1H NMR (CDCl_3 , 400 MHz) δ : 0.73 (s, 3H, 18- CH_3), 0.93 (d, 3H, *J* = 6.5 Hz, 21- CH_3), 1.10 (d, 6H, *J* = 7.0 Hz, 26- CH_3 , 27- CH_3), 2.60 (m, 1H, 25-CH), 3.56 (m, 1H, 3-CHa), 3.60 (d, 1H, *J* = 11.5 Hz, 19-CHa), 3.82 (1H, d, *J* = 11.5 Hz, 19-CHb), 5.74 (1H, t, *J* = 3.0 Hz, 6-CH); ^{13}C NMR (CDCl_3 , 400 MHz): 12.2 (CH_3), 18.3 (CH_3), 18.4 (CH_3), 18.5 (CH_3), 21.8 (CH_2), 24.1 (CH_2), 28.2 (CH_2), 29.9 (CH_2), 31.2 (CH_2), 32.0 (CH_2), 33.4 (CH_2), 35.4 (CH), 37.3 (CH_2), 40.1 (CH_2), 40.8 (CH), 41.5 (C), 42.3 (CH_2), 42.6 (C), 50.4 (CH), 55.9 (CH), 57.6 (CH), 62.8 (CH_2), 71.4 (CH), 127.3 (CH), 135.6 (C), 215.5 (C); EIMS *m/z*: 417 (*M* + 1) $^+$, 416(*M*) $^+$. Analysis calculated for $\text{C}_{27}\text{H}_{44}\text{O}_3$: C, 77.8; H, 10.7. Found: C, 75.5; H, 10.3.

Neuroprotective Effect of Diol Against Glutamate-Induced Neuronal Injury *in vitro*

We focused on Diol and performed a detailed investigation of its neuroprotective effect in glutamate (Glu)-induced neuronal

Figure 1 The structure of the tested steroidal analogs.

Table 1 Effects of a series of steroid analogs on glutamate-induced LDH release in CGNs

Entry Compound	LDH Release (%)
A Cholesterol	54.6 ± 3.68
B Cholesterol 5 α ,6 α -epoxide	49.6 ± 4.52
C Cholesterol 5 β ,6 β -epoxide	48.7 ± 3.86
D 24-Ketocholesterol	49.1 ± 5.09
E 27-Hydroxycholesterol	60.4 ± 4.67
F Cholestane-3 β ,5 α , 6 β -triol (Triol)	26.8 ± 2.32**
G 24-Keto-cholest-3 β ,5 α ,6 β -triol	24.3 ± 1.98**
H 25(R)-spirost-3 β ,5 α ,6 β -triol	27.9 ± 3.46**
I 24-Keto-cholest-3 β ,6 β -diol	34.6 ± 4.79*
J 24-Keto-cholest-5-en-3 β ,19-diol (Diol)	18.9 ± 2.07**
K 24-Keto-cholest-3 β , 19-diol	29.7 ± 3.15*
L 3 β -Acetoxy-cholest-5-en-24-one	61.6 ± 4.87
M 3 β , 19-Diacetoxy-cholest-5-en-24-one	50.4 ± 5.43
N 3 β -Hydroxy-cholestan-24-one	56.9 ± 3.79

CGNs were rinsed and treated with 10 μ M different compounds for 5 min in Locke solution. Then, the neurons were exposed to 200 μ M glutamate in the presence of 10 μ M glycine for 40 min. Subsequently, the neurons were rinsed and switched back to the normal culture medium. LDH release assay was performed 24 h later. Values are expressed as mean $\pm$ SEM, * P < 0.05, and ** P < 0.01 versus the vehicle group (54.6 $\pm$ 4.85%). Three independent experiments were performed. The normal release (baseline) in the untreated cultures was 15.9 $\pm$ 3.57%. One-way analysis of variance (ANOVA) was used to calculate statistical significance.

injury in CGNs. Exposure of cultured CGNs to glutamate results in morphological changes, including shrunken cell bodies and broken dendrites (Figure 3A). Dead staining with propidium iodide (PI, red, Figure 3B) and alive staining with fluorescein diacetate (FDA, green, Figure 3C) show an increased dead and decreased living cell number, respectively, after glutamate treatment. Moreover, Hoechst33258 staining showed that the nuclei of some CGNs treated were condensed and stained into brightly blue after glutamate challenge, suggesting apoptosis occurred (Figure 3D). Diol significantly decreased the apoptotic cell numbers. Pretreatment of CGNs with Diol for 5 min prior to glutamate exposure concentration dependently decreased the number of dead cell (% PI-positive cells) and increased the number of survival cell (% FDA-positive cells) of CGNs (Figure 4E). Quantitative LDH release assay further confirmed the neuroprotective effect of Diol (Figure 4F). Diol, at 10 μ M, decreased glutamate-induced LDH release to a level close to the normal release. MK-801 was used as a positive control.

Neuroprotective Effect of Diol Against Hypoxia/reoxygenation (H/R)-Induced Neuronal injury *in vitro*

To mimic the ischemic conditions in stroke, we used a hypoxia model to further evaluate the neuroprotective effect of Diol in CGNs. Consistent with those findings observed in the glutamate-induced excitotoxicity model, Diol exerted a similar and potent neuroprotective effect against hypoxia-induced neuronal injury. Exposure of cultured CGNs to 3 h hypoxia results in morphological changes, including shrunken cell bodies and loss of dendrites

(Figure 4A), increased cell death (PI staining, Figure 4B), and decreased cell viability (FDA staining, Figure 4C). In addition, as shown in Figure 4D, the nuclei of most CGNs treated with H/R were condensed, which demonstrated that hypoxia induced apoptosis in CGNs. Diol exerted potent neuroprotective effects against H/R-induced neuronal death/apoptosis (Figure 4B–E). It decreased H/R-induced LDH release in a concentration-dependent manner (Figure 4F).

Neuroprotective Effect of Diol against Cerebral Ischemia Injury *in vivo*

We used the transient middle cerebral artery occlusion (MCAO) model in mice to further evaluate the neuroprotective effect of Diol in cerebral ischemia. As shown in Figure 5A, transient MCAO leads to a large infarct volume in the ipsilateral hemisphere in mice. The infarct volume induced by MCAO was 24.1 $\pm$ 1.71% for the vehicle-treated group (Figure 5B). IV administration of 6 mg/kg Diol significantly reduced the infarction volume to 6.81 $\pm$ 1.08% (Figure 5B). Together, these data demonstrated that neuronal injury induced by MCAO was markedly reduced by Diol.

Diol Inhibits NMDA-Induced [Ca²⁺]_i Increase in Cortical Neurons

Increased extracellular glutamate level leads to the activation of Ca²⁺ permeable NMDA receptors and subsequent calcium overload, which is a predominant mechanism for glutamate-mediated excitotoxicity. As Diol potentially protected against glutamate-mediated neuronal toxicity, we asked whether Diol could decrease NMDA receptor-mediated calcium overload. As shown in Figure 6A, bath application of 200 μ M NMDA in cultures induced a remarkable and sustained [Ca²⁺]_i increase. Co-application of 10 μ M Diol significantly reduced the increased [Ca²⁺]_i level (Figure 6A–B).

Neuroprotective Effect of Diol against NMDA-Induced Neuronal Injury *in vitro*

To provide more evidence on the mechanism involved in the neuroprotective effect of Diol, we examined the effect of Diol on NMDA-induced neuronal injury. Exposure of the cultured cortical neurons to 200 μ M NMDA for 45 min resulted in an increased LDH release significantly (Figure 7). Co-application of 10 μ M Diol with NMDA or 10 μ M MK-801 with NMDA significantly decreased NMDA-induced LDH release. However, no significant neuroprotective effects were observed, when Diol and MK-801 were applied after NMDA treatment (Figure 7).

Discussion

Neurosteroids or neuroactive steroids are endogenous steroids derived from cholesterol or steroidal precursors that can rapidly alter neuronal excitability through a direct modulation of the functions of ion channels or membrane receptors [7,16]. Accumulating evidence proves that steroids have nongenomic effects; for example, several neurosteroids could positively or neg-

Figure 2 Synthesis of 24-keto-cholest-5-en-3 β , 19-diol (Diol). Diol was designed and synthesized starting from 3 β -Acetoxy-cholest-5-en-24-one via several steps of reactions.

actively modulate GABA_A and NMDA receptors [9,17–20]. These rapid and non-genomic effects have attracted much interest in the past few decades, which make steroids promising candidates for the treatment of neurological disorders implicated with ion channel dysfunction [21,22]. Our previous study identified Triol, a metabolite of cholesterol, as an endogenous neuroprotectant via negative modulation of NMDA receptors. Triol not only would serve as a pharmacological tool for the investigation of NMDA receptor functions, but also provides an important lead compound for the development of novel neuroprotectant.

The polyhydroxy structure seems critical for the neuroprotective effect of Triol, because the precursors of Triol, including cholesterol, cholesterol 5 α ,6 α -epoxide and cholesterol 5 β ,6 β -epoxide, exerted no neuroprotective effect under the same conditions. Other cholesterol metabolites including 27-hydroxycholesterol and 24-ketocholesterol also showed no neuroprotective effect, which further confirmed the importance of polyhydroxy group for Diol's neuroprotective effect. The slight structure modification at 24-carbon on the side chain results in compound G did not alter the neuroprotective effect, whereas the replacement of the side chain with a bulky group as in compound H caused a significant decrease in activity. Interestingly, the introduction of a hydroxyl group at 19-C, for example, in 24-keto-cholest-5-en-3 β , 19-diol (Diol), significantly enhanced neuroprotective potency. A large-scale SAR study would help to design and develop a simplified analog, while retaining the efficacy. Diol is a polyhydroxylated sterol, with a terminal keto group on the side chain. We synthesized Diol starting from 3 β -

Acetoxy-cholest-5-en-24-one and confirmed its structure with multiple spectroscopic data. For example, the appearance of ¹³C NMR peak at 71.41 (3-CH) and 62.75 (19-CH₂) indicated the formation of 3 β , 19-Diol, and the spectra of ¹³C NMR peak at 215.49 (24-C) and IR at 1709 cm⁻¹ supported the existence of a terminal keto group.

Excessive activation of NMDA receptors results in intracellular calcium overload. Calcium plays a central role in several pathological processes, particularly in NMDA-type glutamate receptor-mediated neuronal injury [23]. In the current study, we found Diol decreased NMDA-induced [Ca²⁺]_i increase to a moderate level, which suggests that inhibition of NMDA receptor might be one of the underlying mechanisms. We further confirmed this mechanism using a NMDA-mediated neuronal injury model in the presence of a combination of channel inhibitors including CNQX (non-NMDA glutamate receptor inhibitor), TTX (voltage-gated sodium channel inhibitor), and nimodipine (L-type voltage-gated calcium channel inhibitor). We found both Diol and MK-801 protected cortical neurons when they were co-applied with NMDA, whereas no significant neuroprotective effects were observed when they were applied after NMDA treatment. This finding in combination with the calcium imaging result suggests that NMDA receptor might be a critical target of Diol.

The NMDA receptor is strongly implicated in the acute neurotoxicity in stroke, and NMDA receptor antagonists show significant neuroprotective effect in multiple preclinical models. Unfortunately, the clinical tests of NMDA antagonists for neuroprotection failed in clinical trials for stroke treatment [24].

Figure 3 Protective effect of Diol against glutamate-induced neuronal toxicity in CGNs. **(A)** Representative images show the neuroprotective effects of 10 μ M Diol evaluated at 24 h following glutamate treatment. Control, neurons without glutamate treatment; Glu+Vehicle, neurons treated with 200 μ M glutamate plus DMSO vehicle (<0.1%); Glu+MK-801 and Glu+Diol groups, neurons pretreated with glutamate in the presence of MK-801 or Diol. MK-801 and Diol were added 5 min prior to glutamate treatment. **(B and C)** Representative PI and FDA staining of dead and alive neurons, respectively. **(D)** Representative Hoechst 33258 staining of the condensed chromatin. **(E)** Summary data show different concentrations of Diol and 10 μ M MK-801 effectively decrease death rate and increase survival rate of CGNs, as counted by PI and FDA staining. * P < 0.05 and ** P < 0.01 compared with vehicle group. 4–5 fields were counted and averaged for each coverslip. **(F)** Inhibition of glutamate-induced LDH release in CGNs by Diol in a concentration-dependent manner. Data represent mean $\pm$ SEM, * P < 0.05, and ** P < 0.01 compared with vehicle group (for FDA and LDH assays); # P < 0.05 and ## P < 0.01 compared with vehicle group (for PI assay). Three to four independent experiments were performed. One-way analysis of variance (ANOVA) was used to calculate statistical significance.

Figure 4 Protective effect of Diol against hypoxia/reoxygenation (H/R)-induced neuronal injury in CGNs. **(A)** Representative images show the neuroprotective effects of 10 μ M Diol and MK-801. Images were taken at 24 h following hypoxia treatment. Control, neurons without hypoxia treatment; H/R+Vehicle, neurons challenged with 3 h hypoxia and 24 h reoxygenation in the presence of DMSO vehicle (<0.1%); H/R+Diol and H/R+MK-801 groups, neurons challenged with H/R in the presence of MK-801 or Diol. MK-801 and Diol were added 5 min prior to H/R challenge. **(B and C)** Representative PI and FDA staining of dead and alive neurons, respectively. **(D)** Representative Hoechst 33258 staining of the condensed chromatin. **(E)** Summary data show different concentrations of Diol and 10 μ M MK-801 effectively decrease the death rate of CGNs, as counted by PI staining. * P < 0.05 and ** P < 0.01 compared with vehicle group. 4–5 fields were counted and averaged for each coverslip. **(F)** Inhibition of H/R-induced LDH release in CGNs by Diol in a concentration-dependent manner. Data represent mean $\pm$ SEM, * P < 0.05, and ** P < 0.01 compared with the vehicle group, n = 4. Three to four independent experiments were performed. One-way analysis of variance (ANOVA) was used to calculate statistical significance.

Figure 5 Neuroprotective effect of Diol against MCAO-induced cerebral ischemia injury in mice. (A) Representative coronal brain sections stained with TTC show the infarction (pale region). Treatment with Diol (6 mg/kg, *i.v.*) significantly reduced the infarction area compared with vehicle-treated group. (B) Summary data show infarction volumes (% infarction volume from the total hemisphere) in male mice treated with vehicle ($n = 11$) or Diol ($n = 11$). All values are mean $\pm$ SEM. * $P < 0.05$ compared with the vehicle DMSO group. Data were from five independent experiments. Student's *t*-test was used to calculate statistical significance.

Figure 7 Inhibition of NMDA-induced LDH release in cortical neurons by Diol. Control, neurons without NMDA treatment; vehicle, neurons were treated DMSO vehicle ($<0.1\%$); Diol (10 μ M) and MK-801 (10 μ M), neurons were treated 10 μ M Diol and MK-801, respectively. All the NMDA treatments were combined with 10 μ M glycine, 5 μ M nimodipine, 10 μ M CNQX, and 0.3 μ M TTX. Diol and MK-801 were applied with NMDA (co-application) or applied immediately after NMDA treatment (postapplication). LDH measurements were performed at 24 h. Data represent mean $\pm$ SEM and ** $P < 0.01$ compared with vehicle group. Four independent experiments were performed. One-way analysis of variance (ANOVA) was used to calculate statistical significance.

Figure 6 Attenuation of NMDA-induced $[Ca^{2+}]_i$ elevation by Diol in cortical neurons. (A) Diol (10 μ M) prevented NMDA-induced $[Ca^{2+}]_i$ increase in cultured cortical neurons. NMDA was applied at 50 S as shown by the arrow. (B) Summary results show the effects of Diol on NMDA-induced $[Ca^{2+}]_i$ elevation at 200 S. ** $P < 0.01$ compared with vehicle group ($n = 220$ –257 cells). Four independent experiments were performed. Student's *t*-test was used to calculate statistical significance.

The failure is mainly attributable to the intolerable side effects such as disruption of cognition, leading to psychotomimetic effects, and limited short therapeutic time window. NMDA receptor subtype may mediate beneficial or deleterious effects depending on the subtypes and the distribution in different subcellular compartments. For example, deleterious effects can be mediated by over activation of extrasynaptic receptors containing GluN2B subunits [24], and thus, NR2B represents a promising target for stroke. It is needed to explore the subunit selectivity of Diol on NMDA receptors in the future. Moreover, the neuroprotective effect of Diol in MCAO model was achieved by pretreatment in the current study, which remains to be examined in a wide range of time window. Particularly, the therapeutic animal studies are required to test the effects of poststroke administration of Diol.

In summary, we have provided evidence supporting that Diol, a synthetic steroid, functions as a novel neuroprotectant through inhibition of NMDA receptor. We speculate that the neuroprotect-

tive effect of Diol will not only be limited to brain ischemia, but could also be extended to other neurological disorders where overstimulation of NMDA receptors plays an etiological role.

Acknowledgments

This work was supported by the Project of Natural Science Foundation of Investigation Team, Guangdong Province of China (No. 039191), the National High Technology Research and Development Program of China (No. 2006AA090502), China Science Foundation (No. 20080440806).

Conflict of Interest

The authors declare competing commercial interests. They are named inventors on the patents WO2007076656 A1.

References

- Dingledine R, Borges K, Bowie D, Traynelis SF. The glutamate receptor ion channels. *Pharmacol Rev* 1999;**51**:7–61.
- Kalia LV, Kalia SK, Salter MW. NMDA receptors in clinical neurology: Excitatory times ahead. *Lancet Neurol* 2008;**7**:742–755.
- Kornhuber J, Weller M. Psychogenicity and N-methyl-D-aspartate receptor antagonism: Implications for neuroprotective pharmacotherapy. *Biol Psychiatry* 1997;**41**:135–144.
- Lipton SA. Paradigm shift in neuroprotection by NMDA receptor blockade: Memantine and beyond. *Nat Rev Drug Discov* 2006;**5**:160–170.
- Olivares D, Deshpande VK, Shi Y, et al. N-methyl D-aspartate (NMDA) receptor antagonists and memantine treatment for Alzheimer's disease, vascular dementia and Parkinson's disease. *Curr Alzheimer Res* 2012;**9**:746–758.
- Stieg PE, Sathi S, Warach S, Le DA, Lipton SA. Neuroprotection by the NMDA receptor-associated open-channel blocker memantine in a photothrombotic model of cerebral focal ischemia in neonatal rat. *Eur J Pharmacol* 1999;**375**:115–120.
- Baulieu EE. Neurosteroids: Of the nervous system, by the nervous system, for the nervous system. *Recent Prog Horm Res* 1997;**52**:1–32.
- Compagnone NA, Mellon SH. Neurosteroids: Biosynthesis and function of these novel neuromodulators. *Front Neuroendocrinol* 2000;**21**:1–56.
- Hu H, Zhou Y, Leng T, et al. The major cholesterol metabolite cholestane-3beta,5alpha,6beta-triol functions as an endogenous neuroprotectant. *J Neurosci* 2014;**34**:11426–11438.
- Leng T, Zhang J, Xie J, et al. Synthesis and anti-glioma activity of 25(R)-spirostan-3beta,5alpha,6beta,19-tetrol. *Steroids* 2010;**75**:224–229.
- Leng TD, Lin J, Sun HW, et al. Local Anesthetic Lidocaine Inhibits TRPM7 Current and TRPM7-Mediated Zinc Toxicity. *CNS Neurosci Ther* 2015;**21**:32–39.
- Yan GM, Irwin RP, Lin SZ, Weller M, Wood KA, Paul SM. Diphenylhydantoin induces apoptotic cell death of cultured rat cerebellar granule neurons. *J Pharmacol Exp Ther* 1995;**274**:983–990.
- Seko Y, Tobe K, Ueki K, Kadowaki T, Yazaki Y. Hypoxia and hypoxia/reoxygenation activate Raf-1, mitogen-activated protein kinase kinase, mitogen-activated protein kinases, and S6 kinase in cultured rat cardiac myocytes. *Circ Res* 1996;**78**:82–90.
- Sun H, Leng T, Zeng Z, Gao X, Inoue K, Xiong ZG. Role of TRPM7 channels in hyperglycemia-mediated injury of vascular endothelial cells. *PLoS One* 2013;**8**:e79540.
- Xiong ZG, Zhu XM, Chu XP, et al. Neuroprotection in ischemia: Blocking calcium-permeable acid-sensing ion channels. *Cell* 2004;**118**:687–698.
- Schumacher M, Weill-Engerer S, Liere P, et al. Steroid hormones and neurosteroids in normal and pathological aging of the nervous system. *Prog Neurobiol* 2003;**71**:3–29.
- Weaver CE Jr, Marek P, Park-Chung M, Tam SW, Farb DH. Neuroprotective activity of a new class of steroidal inhibitors of the N-methyl-D-aspartate receptor. *Proc Natl Acad Sci USA* 1997;**94**:10450–10454.
- Vallee M, Mayo W, Le Moal M. Role of pregnenolone, dehydroepiandrosterone and their sulfate esters on learning and memory in cognitive aging. *Brain Res Brain Res Rev* 2001;**37**:301–312.
- Adamusova E, Cais O, Vyklicky V, et al. Pregnenolone sulfate activates NMDA receptor channels. *Physiol Res* 2013;**62**:731–736.
- Gong QH, Smith SS. Characterization of neurosteroid effects on hyperpolarizing current at alpha4beta2delta GABAA receptors. *Psychopharmacology* 2014;**231**:3525–3535.
- Jiang N, Chopp M, Stein D, Feit H. Progesterone is neuroprotective after transient middle cerebral artery occlusion in male rats. *Brain Res* 1996;**735**:101–107.
- Chen J, Chopp M, Li Y. Neuroprotective effects of progesterone after transient middle cerebral artery occlusion in rat. *J Neurol* 1999;**171**:24–30.
- Wojda U, Salinska E, Kuznicki J. Calcium ions in neuronal degeneration. *IUBMB Life* 2008;**60**:575–590.
- Traynelis SF, Wollmuth LP, McBain CJ, et al. Glutamate receptor ion channels: Structure, regulation, and function. *Pharmacol Rev* 2010;**62**:405–496.