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EDT, a tetrahydroacridine derivative inhibits cerebral ischemia and protects rat cortical neurons against glutamate-induced cytotoxicity

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KEY WORDS 9-(4-ethoxycarbonylyphenoxy)-6,7-dimethoxy-1,2,3,4-tetrahydroacridine (EDT); tetrahydroacridines; brain ischemia; cell culture; neurons; glutamic acid; nitric oxide

ABSTRACT

AIM: To study the effects of 9-(4-ethoxycarbonylyphenoxy)-6,7-dimethoxy-1,2,3,4-tetrahydroacridine (EDT) on cerebral ischemia and glutamic acid (Glu) and sodium nitroprusside (SNP)-induced neurocytotoxicity in primary cortical culture. **METHODS:** Focal cerebral ischemia was produced by permanent occlusion of left middle cerebral artery (MCA) in mice. The infarct tissue was measured by 2,3,5-triphenyltetrazolium chloride (TTC) staining technique. The extent of neurological deficits was evaluated. In primary cortical culture, colorimetric MTT assay was used to determine cell survival rate, and leakage of LDH and NO release assay were measured. **RESULTS:** In focal cerebral ischemia, pretreatment with EDT 2.5, 5, and 10 mg/kg and nimodipine 2 mg/kg for 5 d effectively improved the abnormal neurological symptoms and reduced the infarct rate. In primary cortical culture, EDT 0.01-3 μmol/L concentration-dependently attenuated NO release induced by Glu 500 μmol/L and increased the cell survival. It also remarkably reduced the LDH excessive efflux. **CONCLUSION:** EDT possessed protective effects against cerebral ischemia, which may be related to blocking Glu receptor and inhibiting NO formation.

INTRODUCTION

Lots of evidences implicate that endogenous excitatory amino acid (EAA), especially glutamic acid (Glu) plays an important role in the neuronal degeneration associated with some neurological diseases, such as stroke, Alzheimer's disease, Huntington's disease, and Parkinson's disease. In these degenerative disorders, Glu is always excessively released and then activate Glu

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receptor. Overactivation of Glu receptor may induce uncontrolled Ca²⁺ influx resulting in activation of Ca²⁺dependent protease, kinase, and lipase. Also, calcium overload may activate cNOS, resulting in excessive production of NO. All these factors, Glu, Ca²⁺, and NO, can cause serious cell damage^[1]. Many chemicals, such as Glu-receptor antagonists, calcium antagonists, and NOS inhibitors have protective effects on neurons, but their clinical effects are not satisfactory for their serious toxic effects or ambiguous therapeutic effects. In many labs, researches are taken to search for novel drugs with neuroprotective effects as well as lower toxicity^[2].

9-(4-Ethoxycarbonylyphenoxy)-6,7-dimethoxy-1, 2,3,4-tetrahydroacridine (EDT) is a kind of tetrahydro-

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acridine derivative, synthesized by the New Drug Research and Development Center of China Pharmaceutical University guided by tacrine (an cholinesterase inhibitor, used for treating Alzheimer's disease) combined with the structure of Glu-receptor antagonist. The screening results showed that it could inhibit cholinesterase moderately while remarkably reverse the Glu-induced cytotoxicity in PC12 cells^[3]. The former research indicated that EDT, within the range of 0.01-1 µmol/L antagonized the ischemia & anoxia injury in PC12 cells^[4]. Also, EDT 2.5, 5, and 10 mg/kg ig for 5 d improved the memory impairment in mice^[5]. The present study was to determine whether EDT could protect cerebral ischemia *in vivo* and its mechanism.



MATERIALS AND METHODS

Chemicals EDT (mp: 121-123 °C, purity >98 %, light yellow powder), provided by Dr ZHOU Jin-Pei of China Pharmaceutical University, was dissolved in dimethylsulfoxide (Me₂SO). The concentration of Me₂SO in the final culture media was <0.1 % (v/v), which was safe to the culture in the control group. Glu and sodium nitroprusside (SNP) were obtained from Sigma. Nimodipine (Nim) was obtained from Sandong Xinhua Pharmaceutical Factory. Dulbecco's modified Eagle's medium (DMEM) was from Gibco. 2,3,5-Triphenyltetrzolium chloride (TTC) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were from Fluca. The assay kit of lactate dehydrogenase (LDH) was purchased from Nanjing Jiancheng Bioengineering Institute. All other chemicals were of analytical grade.

Middle cerebral artery (MCA) occlusion Kunming mice (20.6 \pm 1.7) g (supplied by the Animal Breeding Center of China Pharmaceutical University, male and female matched, Grade II, Certificate No 98004) were divided into 5 groups in random: ig NS, Nim 2 mg/kg, and EDT 2.5, 5, and 10 mg/kg, respectively for 5 d. One hour after the last administration the mice were anesthetized by sodium pentobarbital 50 mg/kg, ip. The mouse body temperature was maintained at (37 ± 0.5) °C with a heating pad. The MCA was occluded by electrocautery. In sham-operated group the MCA was exposed without ligation. After the temporalis muscle and skin were closed in layers, the mice were returned to cages for the next 24-h period^[6].

Neurological deficit score and infarct area determination The neurological deficit score of mice was evaluated at 4 h and 24 h after ischemia. The total score of 10 was evaluated as follows: (1) When mice were suspended by the tail, the right forelimb was flexed, scored 1-4 according to the severity. (2) Mice circled to the right side, scored 1-2. (3) When mice were placed on a smooth plane, the lateral push resistance toward the right side decreased, scored 1-2. (4) The mice were pulled gently backward by the tail, the right forelimb showed decreased strength, scored 1-2. After scored, the mice were decapitated and the whole brains were removed. The brain was cut coronally into 5 sections, which were then immersed into TTC solution (1 % normal saline solution) and were stained at 37 °C for 30 min in dark. The infarct tissue (unstained) was removed causally and weighed precisely. The infarct rate was expressed as the weight percentage of total forebrain^[7,8].

Cell culture The newborn Wistar rats of 1-3 d old were collected into phosphate buffer solution (PBS) after disinfected. The hemispheres were dissected out into cold D-Hanks' solution. After removal of meninges and blood vessels, the tissues were minced and incubated in 0.25 % trypsin at 37 °C for 30 min. DMEM medium with serum was added to terminate digestion. The whole solution was filtered through nylon mesh (200 mesh, hole width 95 µm). The filtrate was centrifuged at $3000 \times g$ for 10 min, then the sediment was resuspended by DMEM containing 10 % fetal bovine serum, benzylpenicillin 100 kU/L and streptomycin 100 mg/L. The cell density was about 1.5×10^9 /L. The cells were grown on a 24-well plate, which was previously coated with poly-L-lysine 10 mg/L for 24 h, at 37 °C in 95 % air/ 5 % CO2. Arabinosylcytosin 10 mg/L was added at 72 h to prevent the growth of non-neuronal cells. After 48 h, the medium was changed to the normal medium and changed every 2-3 d^[9].

Glu- and SNP-induced neuronal cytotoxicity The 12-14-d culture was used for experiment. After pretreatment with EDT of different concentration for 30 min, Glu or SNP 500 µmol/L was added and incubated with cells for 20 min and 5 min respectively. Then the medium was separated from the wells and the cells were washed twice with PBS and DMEM was appended to each well. After the culture was incubated at 37 °C for 20 h, MTT 0.5 g/L was added to the medium. After additional 4 h, the medium was separated from the well and the formazan crystals were dissolved in 1 mL Me₂SO. The absorbance at 570 nm was measured by Elisa plate reader (Huadong Electronic Company, Nanjing, China). LDH activity in the medium was measured by LDH assay kit at 440 nm^[9-11]. Nitrite (NO) release from neuronal cells was measured by Griess assay. Griess reagent (sulfanilamide 1 %, naphthylethylene diamine 0.01 %, H₃PO₄ 3 %) 2 mL was added to 0.5 mL separated medium. The reaction was preceded at 20 °C for 20 min, and then the absorbance at 550 nm was measured. The concentration of nitrite [NO²⁻], calculated by sodium nitrite, was determined with reference to a standard curve of sodium nitrite^[12].

Statistics Results were expressed as mean±SD. Statistical analysis was performed using unpaired *t* test.

RESULTS

EDT on focal cerebral ischemia in mice The forelimb of model group exhibited obvious abnormal activity. The neurological deficit score was 7.5 at 4 h and 6.9 at 24 h. EDT 2.5, 5, and 10 mg/kg and Nim 2 mg/kg decreased neurological deficit score. Also, from the TTC staining results, Nim 2 mg/kg and EDT 2.5, 5, and 10 mg/kg reduced the infarct rate. Compared with model group, the infarct rate was reduced by 31.0 %, 14.3 %, 35.1 %, and 36.8 %, respectively (Tab 1).

EDT on Glu-induced cytotoxicity and NO release in neuronal cells The exposure of neuronal cells to Glu 500 µmol/L for 20 min followed by incubation with serum-free DMEM for 24 h produced an obvious decrease in cell survival as measured by MTT and the NO release was markedly increased (Tab 2). The formation of nitrite was nearly 2 times than that of control group. When cultures were pretreated with EDT for 30 min, the cell damage was greatly attenuated and NO efflux was attenuated too (Tab 2).

EDT on SNP -induced cytotoxicity in neuronal cells After exposure to SNP for 5 min, the cell survival was markedly decreased while the extra cellular LDH was greatly increased. EDT remarkably antagonized the LDH excessive efflux and increased the cell survival rate (Tab 3).

Tab 1. Effect of EDT on neurological deficit score and infarct rate of focal cerebral ischemia mice. n=10-13 mice. Mean±SD. ^b P<0.05, ^cP<0.01 vs model group.

Group	Dose	Infarct rate/%	Abnorma symp	Abnormal nervous symptoms		
	$/mg \cdot kg^{-1}$		4 h	24 h		
Sham	-	-	10	10		
Model		26.0 ± 4.2	7.5±1.0	6.9±1.0		
EDT	2.5	22.2±2.8 ^b	$6.0\pm0.8^{\circ}$	$5.2 \pm 1.0^{\circ}$		
	5	16.9±2.7°	$4.8\pm0.7^{\circ}$	$4.4\pm0.8^{\circ}$		
	10	16±3°	4.7±1.0°	$4.2\pm0.8^{\circ}$		
Nim	2	18±5°	4.8±1.5°	4.5±1.1°		

Tab 2. Protective effects of EDT on Glu 500 μ mol/L-induced cytotoxicity and NO release in cultured rat cortical neurons. n=6 (6 wells in each experiment). Mean±SD. *P>0.05, *P<0.05, *P<0.01 vs Glu group.

Drugs/µmol· L ⁻¹		A 570	Inhibitory rate/%	NO/µmol∙ L ⁻¹
Control Glu Glu +EDT (Glu +EDT (Glu +EDT (Glu +EDT (Glu +EDT 1 Glu +EDT 1).01).03).1).3	$\begin{array}{c} 1.32\pm0.12^{c}\\ 0.73\pm0.10\\ 0.83\pm0.08^{a}\\ 0.92\pm0.07^{c}\\ 1.02\pm0.10^{c}\\ 1.13\pm0.10^{c}\\ 1.20\pm0.11^{c}\\ 0.000\end{array}$	16.8 32.2 49.4 68.3 82.3	1.01±0.11° 1.91±0.23 1.7±0.3 ^a 1.59±0.29 ^b 1.4±0.3° 1.29±0.23° 1.24±0.17°

Tab 3. Protective effects of EDT on SNP 500 μ mol/Linduced cytotoxicity in cultured rat cortical neurons. n=6(6 wells in each experiments). Mean±SD. ^aP>0.05, ^bP<0.05, ^cP<0.01 vs SNP group.

Drugs/µmol· L ⁻¹	A ₅₇₀	Inhibition rate/%	LDH/ kU· L ⁻¹	Inhibitory rate/%
Control SNP SNP +EDT 0.01 SNP +EDT 0.03 SNP +EDT 0.1 SNP +EDT 0.3 SNP +EDT 1 SNP +EDT 3	$\begin{array}{c} 1.65 \pm 0.14^{\circ} \\ 1.04 \pm 0.14 \\ 1.14 \pm 0.13^{a} \\ 1.27 \pm 0.12^{b} \\ 1.31 \pm 0.08^{\circ} \\ 1.42 \pm 0.10^{\circ} \\ 1.43 \pm 0.09^{\circ} \\ 1.57 \pm 0.09^{\circ} \end{array}$	16.4 37.7 44.3 62.3 63.9 86.9	$438\pm21^{\circ}$ 709 \pm 70 590 \pm 65 ^t 562 \pm 58° 530 \pm 42° 532 \pm 42° 496 \pm 35° 452 \pm 19°	43.8 54.2 66.3 65.5 78.6 95.1

DISCUSSION

As is mentioned before, cerebral ischemia is a complicated pathological course. Neurotoxicity of Glu and the following cascade play an important role in the pathogenesis of this disease state. However, the pathogenesis of the disease is not fully understood yet. Although lots of researches have been taken to find new neuroprotective drugs to treat stroke, there is no effective remedy which can cure or reverse the course of it to date^[13]. EDT is a kind of tetrahydroacridine derivative combined with the structure of Glu-receptor antagonist. In in vitro test, EDT inhibited AChE moderately and improved the memory impairment in mice. On the other hand, we found that EDT could antagonize the ischemia & anoxia injury in PC 12 cells, suggesting that this chemical may have combined pharmacological activity. In this paper, we want to detect the effect of EDT on cerebral ischemia. However, from the reference and the preparing experiment, we did not find any effect of tacrine on cerebral ischemia. So we turn to nimodipine, a calcium antagonist, which has been widely used to treat stroke in clinic. The clinical experiments showed that it was effective to prevent cerebral ischemia^[14]. In this report, pretreatment with EDT 2.5, 5, and 10 mg/kg and Nim 2 mg/kg for 5 d effectively improved the abnormal neurological symptoms and reduced the infarct area in focal cerebral ischemia. In primary cortical culture, 24 h after transient exposure of cortical culture to Glu, most neurons progressively degenerated. The MTT colorimetric value was reduced and NO was triggered to release. EDT 0.01-3 µmol/L concentration-dependently increased the cell survival and attenuated NO release. It could also remarkably antagonize the LDH excessive efflux and increase colorimetric absorbance in SNP model. It is known that SNP slowly delivers NO in vivo, so the protective effects of EDT on SNP model may also hint its action on NO. Therefore, we speculated that blockade of Glu receptors and suppression of NO efflux might be related to the neuroprotective effect of EDT.

These results indicated that EDT protected cerebral ischemia and improved learning and memory ability in mice. It may be developed into an effective remedy for cerebral ischemia and dementia. In conclusion, our studies supported that EDT effectively antagonized cerebral ishcemia *in vivo*. The mechanism may be related to blocking Glu receptor and the following cascade, especially overproduction of NO.

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