Protection against ischemic injury in primary cultured astrocytes of mouse cerebral cortex by *bis*(7)-tacrine, a novel anti-Alzheimer's agent

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Abstract

The effects of *bis*(7)-tacrine, a novel acetylcholinesterase inhibitor, on ischemia-induced cell death and apoptosis were investigated in primary cerebral cortical astrocytes of mice. Following a 6 h in vitro ischemic incubation of the cultures, a marked decrease in the percentage of viable cells was observed by lactate dehydrogenase (LDH) release assay. Furthermore, using bisbenzimide staining, we determined that approximately 65% of the cells underwent apoptosis. Treatment with *bis*(7)-tacrine (1–10 nM) during ischemic incubation effectively inhibited the ischemia-induced apoptosis, as demonstrated by morphological and biochemical tests. Our results demonstrated that *bis*(7)-tacrine could protect astrocytes against ischemia-induced cell injury, indicating that the drug might be beneficial for the treatment of vascular dementia, in addition to Alzheimer's disease. © 2000 Elsevier Science Ireland Ltd. All rights reserved.

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Acetylcholine (ACh) replacement therapy has been used in the treatment of Alzheimer's disease (AD) [5] because increasing neurochemical and pharmacological evidence has linked AD to cholinergic deficiency [1]. Several approaches to replacing ACh have been proposed, but only the inhibition of acetylcholinesterase (AChE) has produced encouraging results in clinical practice [1]. Tacrine, the first AChE inhibitor for AD therapeutics approved by the FDA, has been widely used in clinics. However, peripheral side effects and hepatotoxicity of tacrine have severely hampered its clinical usefulness. *Bis*(7)-tacrine (heptylene-linked *bis*-(9-amino-1,2,3,4-tetrahydroacridine)) is a dimeric analog of tacrine designed and developed in our laboratories [3,4,10,11]. Compared with tacrine, *bis*(7)-tacrine exhibits superior AChE inhibition efficacy, higher oral bioavailability and fewer peripheral side effects in vivo [3,4,10]. We have also demonstrated that *bis*(7)-tacrine is more potent than tacrine in ameliorating learning and memory impairment in the Morris Water maze induced by scopolamine [11] and AF64A [3], respectively. The anti-dementia efficacy of *bis*(7)-tacrine has been ascribed to the reversal of the cholinergic deficit in the central nervous system (CNS) via AChE inhibition.

Recent reports have shown that several AChE inhibitors can protect the brain against ischemia-induced cell loss, and accelerate recovery from neurological deficits and cerebral disorders induced by ischemia [9,12]. Such drugs might therefore be concurrently beneficial for the treatment of vascular dementia and AD. In this study, we investigated whether *bis*(7)-tacrine could also exert similar neuroprotective effects in an in vitro ischemic model that induces cell apoptosis in primary cultured cerebral cortical astrocytes [13]. The model mimics physiological ischemia, through severe hypoxia, substrate deprivation and accumulation of toxic metabolites [13].

A primary culture of cerebral cortical astrocytes was prepared from newborn ICR mice as described by Yu et al. [13] with minor modifications. The whole brain of newborn ICR mice was removed and suspended in DMEM (Gibco BRL, NY). The cerebral cortex was cut into small cubes (<1 mm^3) and mechanically dissociated

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by vortexing for 90 s. The cells were then cultured in DMEM containing 10% fetal bovine serum. Cultures reached confluence at around 2 weeks in vitro and then were used in the experiments when they were 4 weeks old.

An anaerobic chamber was used for inducing ischemia [13]. Inside the chamber, the astrocytic cultures were washed with the anoxic substrate-deprived DMEM three times and then 800 μl/dish of the same medium containing different concentrations of *bis*(7)-tacrine were added. The cultures were kept in the 37°C incubator within the anaerobic chamber for 6 h. The atmosphere of the entire unit was saturated with 85%/10%/5% N_2/H_2/CO_2 (v/v/v) and residual oxygen was removed by palladium.

Cell injury was examined by the measurement of lactate dehydrogenase (LDH) released into the supernatant medium. LDH activities in the medium and cell lysate were determined spectrophotometrically using a standard 340 nm kinetic LDH assay kit (Sigma, MO). The percentage of leakage of LDH was defined as the ratio of LDH activity in the culture medium to the total activity per culture.

Chromosomal condensation was determined with chromatin dye bisbenzimide (Hoechst 33342) staining. Cells were fixed for 30 min in pre-chilled phosphate-buffered saline (PBS) containing 4% paraformaldehyde with gentle agitation. After fixation, the cells were washed with PBS three times, and then exposed to 2 μg/ml Hoechst 33342 in PBS for 5 min at room temperature and washed again as before. After drying in air, coverslips were mounted with 50% glycerol. Cells were viewed under fluorescence microscope (Axiophot, Zeiss). For determination of the percentage of apoptotic cells, the condensed and the total number of nuclei in 12 random fields were counted in each culture.

In this study, an in situ TdT-mediated dUTP nick end labeling (TUNEL) and Hoechst 33342 double labeling method was employed, allowing the simultaneous identification of condensed nuclei and DNA fragmentation in the astrocytes. The ischemic cultures were fixed, permeabilized and then incubated in the TUNEL reaction mixture (Boehringer Mannheim, Germany) in a moist chamber for 75 min at 37°C. The samples were then exposed to Hoechst 33342 in PBS for 5 min. After washing three times, the cells were examined under a fluorescence microscope.

Our data are drawn from three independent experiments with 4–6 cultures per experiment. Statistical significance of results was analyzed by one-way ANOVA followed by Duncan's multiple-range test.

Fig. 1 shows that cultures exposed to ischemic incubation for 6 h produced substantial LDH release (approximately 55% of total LDH) into the medium, nearly five times more than the control (non-ischemic cultures). Treatment of these primary cultures with 10 nM *bis*(7)-tacrine during ischemic incubation significantly decreased the LDH release from 55 to 27% ($P < 0.01$). Approximately 70% protection (from 55 to 17%) was observed in 1 nM *bis*(7)-tacrine treated cultures ($P < 0.01$). The lowest (0.3 nM) and the highest (100 nM) concentrations of *bis*(7)-tacrine tested in this study had no
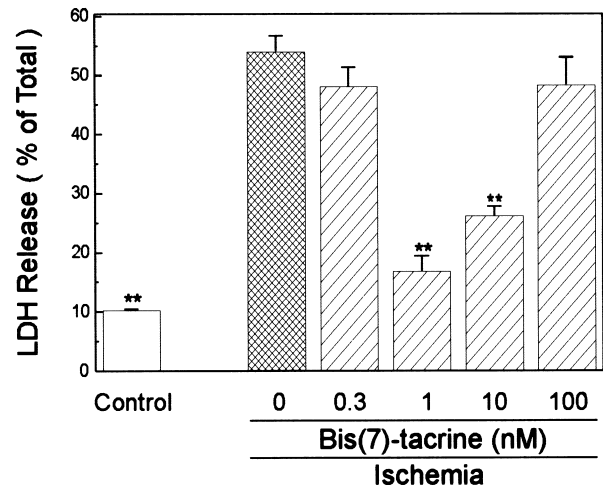

Fig. 1. Protection of *bis*(7)-tacrine against ischemia-induced cell death in primary cultured astrocytes assessed by LDH assay. Cell death was quantified by the percentage of LDH release. Results are expressed as means ± SEM of three independent experiments performed in triplicate. **$P < 0.01$ compared with drug-free ischemic culture (one-way ANOVA followed by Duncan's multiple-range test).

significant effect on ischemia-induced cell injury, as indicated by the LDH release.

Visual inspection by phase-contrast microscopy demonstrates that those normal primary cultured astrocytes were flat polygons grown in a monolayer (Fig. 2A). After ischemic induction for 6 h, astrocytes displayed obvious cell body shrinkage and nuclear condensation (Fig. 2B). Treatment with *bis*(7)-tacrine (1 and 10 nM) during ischemic incubation dramatically prevented cell morphological deterioration. Most of the astrocytes showed normal cell morphology with normal nuclear size and integrity (Fig. 2C).

The specific DNA stain Hoechst 33342 was used to assess changes in DNA and nuclear structure following different treatment. As shown in Fig. 2D, nuclei in control astrocytes were larger and exhibited diffuse Hoechst 33342 staining of the chromatin. In contrast, nuclei in ischemic astrocytes were quite highly condensed (Fig. 2E), a nuclear morphology typical of apoptotic cells. In the presence of 1 nM *bis*(7)-tacrine during ischemic incubation, a significant reduction in number of the condensed nuclei in the cultures was observed. Most of these nuclei maintained their normal shape and size (Fig. 2F).

Double labeling of parallel fields with TUNEL showed that normal nuclei did not appear to be labeled with TUNEL (Fig. 2G). However, most abnormal nuclei, which appeared highly condensed using Hoechst 33342 staining, were positively labeled with the TUNEL technique (Fig. 2H). This indicates that chromatin condensation and DNA fragmentation have occurred in these nuclei. Nuclei that may be in a transition stage were also apparent. These nuclei that appeared only partially condensed using Hoechst 33342 staining displayed lower levels of TUNEL labeling,
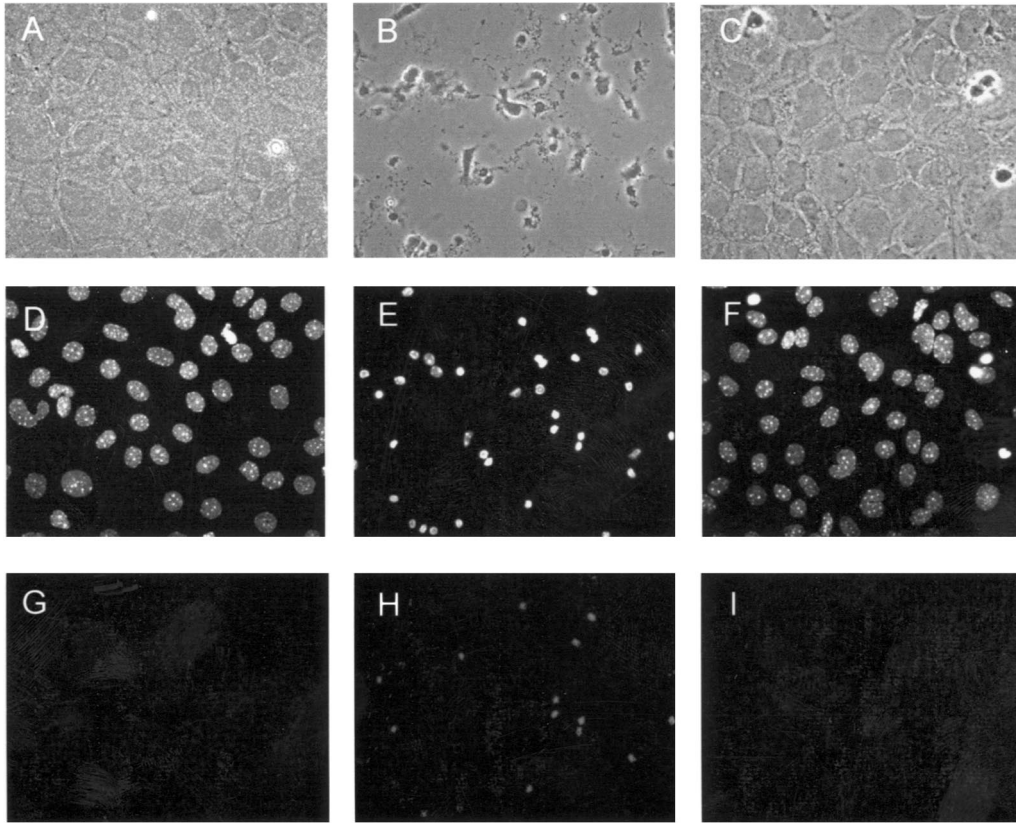
Fig. 2. Micrographs of the primary cultured astrocytes observed with three different methods after 6 h of ischemic incubation. Cortical astrocytes were exposed to (A) no-ischemic incubation, (B) ischemic treatment for 6 h, or (C) ischemic incubation plus 1 nM bis(7)-tacrine added during the incubation period. (D–F) The nuclei morphology of the same field as A–C, respectively, were observed with Hoechst 33342 staining. (G–I) Astrocytes were observed with the TUNEL staining using the same field as A–C, respectively.

suggesting that DNA fragmentation in these nuclei might be at an early stage and thus incomplete. These results also suggested that chromatin condensation may precede DNA fragmentation in ischemic cultures. In the 1 nM bis(7)-tacrine treated astrocytes (Fig. 2I), no TUNEL positive cells were detected, despite the presence of highly condensed nuclei revealed by Hoechst 33342 staining (Fig. 2F). It is possible, therefore, that bis(7)-tacrine delayed internucleosomal DNA cleavage associated with the ischemia-induced astrocytic apoptosis.

The number of cells with condensed nuclei was counted in 11 random fields in each culture dish. In general, more than 65% of ischemic cells had condensed nuclei, and the condensation was largely prevented by the treatment of bis(7)-tacrine (Fig. 3). Among the dosages of bis(7)-tacrine studied, the astrocytes treated with 1 nM bis(7)-tacrine showed the lowest percentage of nuclear condensed astrocytes, approximately 25% of that of the drug-free ischemic culture ($P < 0.01$). At 10 nM bis(7)-tacrine, the percentage of astrocytes with condensed nuclei was about 50% of that of the drug-free ischemic culture ($P < 0.01$). However, in the lowest (0.3 nM) and the highest concentrations (100 nM) studied, the percentage of cells with condensed nuclei did
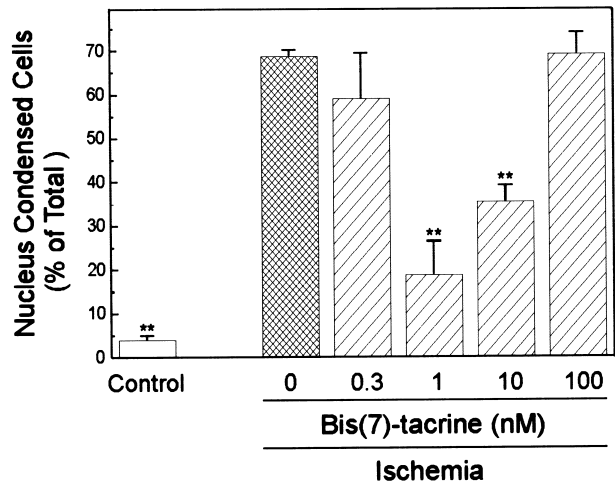

Fig. 3. Protection of bis(7)-tacrine against ischemia-induced apoptosis in primary cultured astrocytes assessed by Hoechst 33342 staining. After 6 h of ischemic incubation, cells were fixed, stained with Hoechst 33342 and then examined by FITC microscopy. The condensed nuclear cells were estimated in 12 random fields and expressed as the percentage of the total. Results are expressed as means ± SEM of three independent experiments performed in triplicate. **$P < 0.01$ compared with drug-free ischemic culture (one-way ANOVA followed by Duncan's multiple-range test).

not differ significantly from the drug-free ischemic culture (Fig. 3).

Cerebral ischemia induces neural cell death in the CNS [8], yet the exact mechanisms involved in ischemia-induced neural damage remain unclear. One general mechanism identified in ischemic neuronal cell injury is glutamate neurotoxicity [8]. It has been demonstrated that the neuronal death induced by excessive amount of glutamate under ischemia might be caused by the dysfunctions of glutamate uptake and glutamine output systems in astrocytes, and that neuronal death can be modulated by the number of surrounding astrocytes in the cultured brain cells [6]. Thus, the integrity and the function of astrocytes are very important in preventing this neurotoxicity. Therefore, agents that could protect astrocytes might be beneficial to lessen the ischemia-induced neurotoxicity.

In this study, ischemic incubation of astrocytes for 6 h was shown to result in severe chromatin condensation, nuclear shrinkage and prominent DNA fragmentation. Similar observations of TUNEL positive astrocytes in transient focal ischemia in rat brains have been reported previously [2]. Treatment of the astrocytes with *bis*(7)-tacrine was shown to significantly ameliorate this ischemia-induced apoptosis. Both biochemical and morphological studies have shown that the protective effects of *bis*(7)-tacrine against cell injury follow a U-shaped dose-response curve, with protective concentrations ranging from 1 to 10 nM. The neuroprotective effect of other AChE inhibitors against ischemia-induced neurotoxicity has been reported previously [9], as has their efficacy to reverse memory deficits in the ischemic animal model [12]. The neuroprotective effects of these AChE inhibitors are believed to be associated with their AChE inhibitory action, via activation of the central cholinergic system, increase of cerebral blood flow or interaction with other neurotransmitter systems [7,12]. We previously showed that the IC_{50} of *bis*(7)-tacrine for inhibiting rat brain AChE is 1.5 nM [10], which is quite comparable with the effective concentration of *bis*(7)-tacrine reported in the present study. Thus, although other mechanisms cannot be excluded, it is possible that the neuroprotective effects of bis(7)-tacrine described in this study might be due in large part to cholinesterase inhibition. Further investigation is necessary to elucidate the relationship between the cholinergic system and neural protection.

[1] Benzi, G. and Moretti, A., Is there a rationale for the use of acetylcholinesterase inhibitors in the therapy of Alzheimer's disease? Eur. J. Pharmacol., 346 (1998) 1–13.

[2] Chen, J., Jin, K., Chen, M., Pei, W., Kawaguchi, K., Greenberg, D.A. and Simon, R.P., Early detection of DNA strand breaks in the brain after transient focal ischemia: implications for the role of DNA damage in apoptosis and neuronal cell death, J. Neurochem., 69 (1997) 232–245.

[3] Liu, J., Ho, W.L., Lee, N.T.K., Carlier, P.R., Pang, Y.P. and Han, Y.F., *Bis*(7)-tacrine, a novel acetylcholinesterase inhibitor, reverses AF64A-induced deficits in navigational memory in rats, Neurosci. Lett., 282 (2000) 165–168.

[4] Pang, Y.P., Quiram, P., Jelacic, T., Hong, F. and Brimijoin, S., Highly potent, selective, and low cost *bis*-tetrahydroaminoacrine inhibitors of acetylcholinesterase, J. Biol. Chem., 271 (1996) 23646–23649.

[5] Peskind, E.R., Pharmacological approaches to cognitive deficits in Alzheimer's disease, J. Clin. Psychiatry, 59 (Suppl. 9) (1998) 22–27.

[6] Rosenberg, P.A., Accumulation of extracellular glutamate and neuronal death in astrocyte-poor cortical cultures exposed to glutamate, GLIA, 4 (1991) 91–100.

[7] Scremin, O.U., Li, M.G., Scremin, A.M. and Jenden, D.J., Cholinesterase inhibition improves blood flow in the ischemic cerebral cortex, Brain Res. Bull., 42 (1997) 59–70.

[8] Siesjo, B.K., Zhao, Q., Pahlmark, K., Siesjo, P., Katsura, K. and Folbergrova, J., Glutamate, calcium, and free radicals as mediators of ischemic brain damage, Ann. Thorac. Surg., 59 (1995) 1316–1320.

[9] Tanaka, K., Ogawa, N., Mizukawa, K., Asanuma, M., Kondo, Y., Nishibayashi, S. and Mori, A., Acetylcholinesterase inhibitor ENA-713 protects against ischemia-induced decrease in pre- and postsynaptic cholinergic indices in the gerbil brain following transient ischemia, Neurochem. Res., 19 (1994) 117–122.

[10] Wang, H., Carlier, P.R., Ho, W.L., Wu, D.C., Lee, N.T.K., Li, C.P., Pang, Y.P. and Han, Y.F., Effects of *bis*(7)-tacrine, a novel anti-Alzheimer's agent, on rat brain AChE, NeuroReport, 10 (1999) 789–793.

[11] Wang, H., Carlier, P.R., Ho, W.L., Lee, N.T.K., Pang, Y.P. and Han, Y.F., Attenuation of scopolamine-induced deficits in navigational memory performance in rats by *bis*(7)-tacrine, a novel dimeric AChE inhibitor, Acta. Pharmacol. Sin., 20 (1999) 211–217.

[12] Yamamoto, T., Ohno, M., Kitajima, I., Yatsugi, S. and Ueki, S., Ameliorative effects of the centrally active cholinesterase inhibitor. NIK-247, on impairment of working memory in rats, Physiol. Behav., 53 (1993) 5–10.

[13] Yu, A.C.H. and Lau, L.T., Expression of interleukin-1 alpha, tumor necrosis factor alpha and interleukin-6 genes in astrocytes under ischemic injury, Neurochem. Int., 36 (1999) 369–377.