ONO-1603, a Potential Antidementia Drug, Delays Age-Induced Apoptosis and Suppresses Overexpression of Glyceraldehyde-3-Phosphate Dehydrogenase in Cultured Central Nervous System Neurons

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ABSTRACT
Primary cultures of rat cerebral cortical cells and cerebellar granule cells die by an apoptotic mechanism after more than 2 weeks in cultures in the absence of medium change and glucose supplement, a process termed age-induced apoptosis of cultured neurons. Our preliminary study has shown that age-induced apoptosis of cerebellar granule cells is protected by pretreatment with tetrahydroaminoacridine (THA), an antidementia drug. In this study, we systematically compared the neuroprotective effects of THA with those of (S)-1-[N-(4-chlorobenzyl)succinamoyl]pyrrolidine-2-carbaldehyde (ONO-1603), a novel prolyl endopeptidase inhibitor and potential antidementia drug. Both ONO-1603 and THA effectively delay age-induced apoptosis of cerebral and cerebellar neurons, as demonstrated morphologically with toluidine blue and fluorescein diacetate/propidium iodide staining or biochemically by DNA laddering analysis on agarose gels. ONO-1603 is about 300 times more potent than THA, with a maximal protective effect at 0.03 and 10 μM, respectively. ONO-1603 shows a wide protective range of 0.03 to 1 μM in contrast to a narrow effective range of 3 to 10 μM for THA. Moreover, ONO-1603 is nontoxic to neurons, even at the high concentration of 100 μM, whereas THA elicits severe neurotoxicity at a dose of ≥30 μM. Both ONO-1603 and THA robustly suppress overexpression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH; EC 1.2.1.12) mRNA and accumulation of GAPDH protein in a particulate fraction of cultured neurons undergoing age-induced apoptosis. Because we documented that GAPDH overexpression participates in neuronal apoptosis induced by various insults, we conclude that the neuroprotective actions of ONO-1603 and THA appear to be mediated by suppression of this protein overexpression.

Several studies suggest that there is a prominent loss of cholinergic, noradrenergic, and dopaminergic neurons in the brains of patients with Alzheimer’s disease (AD) (Coyle et al., 1983; Selkoe, 1991). It is generally believed that acetylcholine is the key neurotransmitter involved in memory (Winkler et al., 1995) and that memory enhancement can be achieved by acetylcholinesterase inhibition (Summers et al., 1986). Thus, to develop therapeutic agents for the treatment of AD, most efforts have been on the neurochemical manipulations of the cholinergic signal transduction system. So far, only tetrahydroaminoacridine (THA) and donepezil hydrochloride (E-2020) have been approved for the clinical use of treatment for this illness (Summers et al., 1986; Rogers et al., 1996). The mechanisms of action of both drugs are generally believed to reverse the cholinergic deficits occurring in the central nervous system of Alzheimer’s patients via action as an anticholinesterase. However, other anticholinesterases have resulted in little improvement in AD symptoms (Bartus et al., 1982) and, therefore, other action(s) of THA or E-2020 may contribute to its therapeutic effect on AD.

We attempted to establish a cellular model of neuronal apoptosis to study the actions of potential antidementia drugs. Apoptosis, one form of programmed cell death, is a normal physiological process that occurs during development to maintain a homeostasis of neuronal populations (Oppenheim, 1991). Abnormality of normal apoptosis has been linked to pathogenesis of a number of human diseases including cancer, viral infections, autoimmune diseases, and neu-

ABBREVIATIONS: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ONO-1603, (S)-1-[N-(4-chlorobenzyl)succinamoyl]pyrrolidine-2-carbaldehyde; THA, tetrahydroaminoacridine; AD, Alzheimer’s disease; NMDA, N-methyl-D-aspartate; CCC, cerebrocortical cells; FDA, fluorescein diacetate; PI, propidium iodide; DIV, days in vitro; β-APP, β-amyloid precursor protein.
ONO-1603 Protects Neuronal Apoptosis

Materials and Methods

Neuronal Cell Cultures. Primary cultures of CCC and cerebellar granule cells were prepared from 1- and 8-day-old Sprague-Dawley rat pups (Clea Japan, Inc., Aobada, Tokyo), respectively, as described previously (Ishitani et al., 1996b; Sunaga et al., 1993a).

Briefly, cerebral cortices dissociated from dienecephalic structures and hippocampus were pooled and sliced (0.4 mm in thickness) in two orthogonal directions. Carefully dissected cerebella were also chopped into 0.4-mm cubes. The respective cubes were mechanically dispersed in the presence of trypsin (0.025%) and DNase (0.008%), and were then plated onto poly-l-lysine-coated 35-mm culture dishes. Cells were seeded at a density of 0.8 to 1.0 × 10^5 cells/ml (2 ml/dish) in basal modified Eagle’s medium containing 10% of fetal bovine serum, 25 mM KCl, 2 mM glutamine, and 100 µg/ml of gentamicin. Cytosine arabinoside (10 µM) was added to the culture medium about 20 h after plating to arrest the growth of nonneuronal cells. Culture media were not changed throughout the cultivation period to avoid neuronal death resulting from overexcitation by contaminating glutamate in the fresh medium (Schramm et al., 1990).

Assessment of Neuronal Survival. Cells were washed with Locke’s solution and double-stained with 0.0008% fluorescein diacetate (FDA) and 0.0002% propidium iodide (PI). FDA is cleaved by esterase(s) present in live cells, yielding yellowish-green fluorescein, whereas PI passes through the damaged plasma membranes of dead cells to bind to DNA, producing orange-red nuclei (Jones and Senft, 1985). Both types of fluorescent cells can be observed simultaneously in a standard fluorescence microscope (Olympus IMT-2; Olympus Corp., Hatagaya, Tokyo). In the case of cerebellar granule cells, cellular viability was measured by the ratio of the number of FDA/FDA + PI-stained positive cells in the photomicrographs of four representative squares (500 × 500 µm containing approximately 330 live and dead cells) from each dish. On the other hand, neuronal survival of CCC was assessed by counting the number of FDA-stained positive cells, because CCC readily detached from the growing surface as they were committed to death, in contrast to granule cells, which were still adhered to the culture dish after cell death (Ishitani et al., 1996b).

DNA Fragmentation Analysis. Total genomic DNA was isolated from cultured cells, and the extent of DNA fragmentation was analyzed by agarose gel electrophoresis as described by Hockenberg et al. (1990). Briefly, after treatment with RNase A (50 µg/ml) and proteinase K (0.1 mg/ml) at 37°C for 30 min, approximately 7.5 µg of soluble DNA was subjected to electrophoresis in a 1.2% agarose gel and then visualized by ethidium bromide staining.

Northern Blot Analysis. Total RNA isolation and Northern blotting were performed essentially as described previously (Sunaga et al., 1993b), except that the human GAPDH complementary DNA probe was 1.1 kb in length (Clontech, Palo Alto, CA) and high-stringency washing of the hybridized blots was performed twice in 0.1% standard saline citrate containing 0.1% SDS at 60°C for 10 min. An amount of 9 µg of total RNA from each sample was separated by electrophoresis through a 1.2% agarose-formaldehyde gel. Specific hybridization bands were quantified by charge-coupled device densitometry of the autoradiograms and then normalized to total cellular RNA in each sample, as described previously (Fukamauchi et al., 1991; Sunaga et al., 1993b).

SDS-Polyacrylamide Gel Electrophoresis (PAGE) and Western Blot Analyses. Cerebellar granule cells harvested at the end of the experiment were ruptured by sonication in 50 mM Tris-HCl, pH 7.4, and the homogenates were centrifuged at 200,000g for 30 min. The cytosolic and particulate fractions (i.e., supernatants and pellets) were redissolved in a small volume of SDS-containing (2%) sample buffer. An aliquot of these preparations was loaded onto each lane of the gel (8–16% linear gradient) for SDS-PAGE analysis, as described by Laemmli (1970). The separated protein bands on the gel were visualized by staining with 0.1% of Coomassie brilliant blue. Western blotting was performed after transferring proteins on the gel to a polyvinylidene difluoride membrane (Polyscreen; DuPont-New England Nuclear, Boston, MA) as described previously (Ishitani et al., 1997). For specific immunostaining of GAPDH protein, a
Protection by ONO-1603 and THA Against Age-Induced Apoptosis of Cultured Neurons. Primary cultures of CCC and cerebellar granule cells were prepared from 1- and 8-day-old rats, respectively, and cultured in the presence of 25 mM KCl without medium change and periodic glucose supplements. As shown in Fig. 2, cell death of both cultures was almost undetectable within the first 15 or 16 days in vitro (DIV) and was increased abruptly thereafter, confirming our previous reports (Ishitani et al., 1996a, b). A single addition of ONO-1603 (0.03 \( \mu M \)) or THA (10 \( \mu M \)) to both cultures at 7 DIV effectively delayed the neuronal death. Thus, at 17 DIV, the percentage of survival of CCC was reduced to 50.8 \( \pm \) 3.1\% in untreated cultures, and pretreatment of ONO-1603 and THA increased cell survival to 91.4 \( \pm \) 5.1 and 90.8 \( \pm \) 4.9\%, respectively (Fig. 2A). Similarly, in the culture of cerebellar granule cells, the percent survival at 17 DIV was increased from 28.5 \( \pm \) 2.2\% to 92.0 \( \pm \) 4.4\% and 91.0 \( \pm \) 4.6\% in the presence of ONO-1603 and THA, respectively (Fig. 2B). After 18 and/or 19 DIV, the neuroprotective potencies of these drugs in both types of neurons declined rapidly. The neuroprotective effects of ONO-1603 and THA were dose-dependent (Fig. 3). The assessment of cell survival at 17 days in cultures of CCC and cerebellar granule neurons undergoing age-induced apoptosis indicated that the protection by ONO-1603 was evident at low nanomolar concentrations, reached a maximum of near-complete effect at doses between 0.03 and 1.0 \( \mu M \), and then gradually declined at doses between 10 and 100 \( \mu M \). On the other hand, THA protection showed a very narrow range of dose dependence in both cultures; that is, apoptotic neuronal death in these cultures was almost completely prevented at 10 \( \mu M \) of THA, but at 100 \( \mu M \), this drug produced severe neuronal cytotoxicity.

Visualization of cell morphology after toluidine blue staining of CCC (Fig. 4A) and FDA-PI double staining of cerebellar granule cells (Fig. 4B) revealed that pretreatments of ONO-1603 and THA robustly blocked the loss of live cells and the extensive degeneration of nerve fibers resulting from age-induced neuronal death.

Inhibition by ONO-1603 and THA of Internucleosomal DNA Cleavage Associated with Age-Induced Neuronal Apoptosis. We reported previously that internucleosomal DNA cleavage detected as DNA laddering on agarose gels occurs during the age-induced apoptosis of CCC (Ishitani et al., 1996b) and cerebellar granule cells (Ishitani et al., 1996a). As shown in Fig. 5, ONO-1603 and THA added at 7 DIV robustly attenuated the enhancement of DNA laddering and degradation of high-molecular-weight DNA detected in cortical and granule cells at 16 and 17 DIV, respectively. These inhibitory effects on both types of cultures were concurrent with the rescue of neuronal death.

Suppression by ONO-1603 and THA of GAPDH mRNA and Protein Overexpression in Cultured Neurons Undergoing Apoptosis. We reported previously that an increase in GAPDH mRNA level and accumulation of particulate GAPDH protein are involved in age-induced apoptosis of CCC and cerebellar granule cells (Ishitani et al., 1996a, b). Therefore, as a first step for the exploration of the mechanisms of neuroprotective effects of these drugs, we examined the effects of ONO-1603 or THA pretreatment on GAPDH mRNA and protein overexpression during age-induced apoptosis. As shown in Fig. 6, ONO-1603 and THA added at 7 DIV completely blocked the increment (2- to 3-fold) of GAPDH mRNA levels detected at 13 DIV in CCC and at 15 DIV in cerebellar granule cells.

The high-speed particulate and cytosolic fractions of cere-
bellar granule cell homogenates were electrophoresis on SDS-PAGE for Western blotting. Both SDS-PAGE and Western blotting analyses (Fig. 7) confirmed that aging of cerebellar granule cell cultures increased the level of a particular 38-kDa protein identified as GAPDH. Importantly, ONO-1603 or THA pretreatment completely suppressed this protein increase. It is also noteworthy that the level of GAPDH protein in the supernatant fraction was unaffected by aging and/or treatment with these drugs. Western blotting for GAPDH protein in the CCC was not performed because these cells readily detached from the growing surface as they committed age-induced apoptosis.

Discussion
Prolyl endopeptidase hydrolyzes several proline-containing peptide hormones including vasopressin, oxytocin, Substance P, neuropeptide Y, bradykinin, angiotensin II, and thyrotropin-releasing hormone (for review, see Wilk, 1983). Given that some of these neuropeptides have been suggested to play a role in memory and learning (for review, see Zager and Black, 1985), it is conceivable that an inhibitor of this enzyme may be useful for the therapeutic treatment of cognitive dysfunction disorders. Based on this working hypothesis, ONO-1603 was discovered as a potential antidementia drug (Katsube et al., 1994). Recently, we found that ONO-1603 at a low concentration (30 nM) promotes neuronal survival and neurite outgrowth against cell death of immature cerebellar neurons (i.e., 2–8 DIV) induced by lowering KCl to 15 mM in culture medium. This effect appears to be mediated through the induction of m₂-muscarinic acetylcholine receptor and potentiation of the receptor-mediated phosphoinositide turnover (Katsube et al., 1996). Interestingly, these actions of ONO-1603 are also induced by THA, but the latter is about 1000 times less potent (Sunaga et al., 1993a, b). Additionally, ONO-1603 restores a decrease of choline and serotonin levels in the aged rats and improves cognitive performance in several neuronal injury models (i.e., aged and medial septum-lesioned animals) (N. Katsube, Michitaka Yamamoto, and H. Aishita, unpublished data).

In the present study, we systematically compared the neuroprotective effects of ONO-1603 versus THA against age-induced apoptosis of central nervous system neurons in cultures. This paradigm seems to be more appropriate than the low K⁺ (5 mM-serum-free paradigm (Ishitani et al., 1997) for studying the actions of antidementia drugs, because both age-induced apoptosis of cultured cerebellar neurons and apoptotic cell death in the brains of AD patients involve abnormal activation of excitotoxic glutamate receptors (Su et al., 1994; Ulas et al., 1994; Lin et al., 1997). On the other hand, exposure of mature cerebellar neurons to 5 mM KCl/serum-free medium induces both apoptosis and necrosis, and only the apoptotic component involves overexpression of GAPDH (Ishitani et al., 1997). In this study, we found that both ONO-1603 and THA markedly delay age-induced apoptosis of CCC and cerebellar granule cells (Fig. 2). Thus, both drugs robustly inhibit neuronal death detected morphologically by toluidine blue staining and FDA/PI double staining (Fig. 4) and biochemically by DNA laddering analysis on agarose gels (Fig. 5). However, ONO-1603 is superior to THA as a neuroprotective agent for the following reasons. 1) ONO-1603 is about 300 times more potent than THA in inhibiting age-induced apoptosis, with a maximal effect at 0.03 and 10 μM for ONO-1603 and THA, respectively. 2) ONO-1603 shows a wide effective concentration range of 0.03 to 1 μM in contrast to a narrow effective range (3–10 μM) for THA (Fig. 3). 3) The effective concentration of ONO-1603 in our study is comparable with the drug concentration in the cerebral cortex of rats orally administrated a dose of 10 mg/kg (N. Katsube, Hitoshi Maegawa, and H. Aishita, unpublished data). 4) ONO-1603 is not significantly cytotoxic to these two types of neurons even at a dose of 100 μM, whereas THA elicits a severe neurotoxicity at a dose of ≥30 μM (Fig. 3). In this context, we found that E-2020, another drug used clinically for AD, also shows a cytoprotective effect against age-induced apoptosis of cerebellar neurons at 0.3 μM but undesirable cytotoxicity at 10 μM (data not shown). Taken together, these results suggest that the protection against this apoptotic paradigm could be a common feature for potentially active antidementia drugs. Moreover, among these three drugs tested, ONO-1603 appears to be most potent, robust, nontoxic, and reliable. Therefore, future clinical trials of ONO-1603 as a drug for AD treatment seem to be warranted.

The mechanisms underlying the neuroprotective effects of ONO-1603 are unclear. However, it seems unlikely that the protection is due to inhibition of prolyl endopeptidase activity, because this enzymatic activity is unaltered during age-induced apoptosis (data not shown). Additionally, ONO-1603...
does not interfere with the activity of more than 30 different surface receptor types including the NMDA receptor (N. Katsume, H. Maegawa, and H. Aishita, unpublished data), which appears to be overstimulated during age-induced apoptosis of cerebellar neurons (Lin et al., 1997). It is important to point out that the neuroprotective effects of ONO-1603 and THA are associated with robust inhibition of GAPDH mRNA and protein overexpression (Figs. 6 and 7), which has been shown to have a prominent role in neuronal apoptosis induced by multiple insults, including aging of the cultures (Ishitani and Chuang, 1996; Ishitani et al., 1996a, b; 1997). GAPDH is a glycolytic enzyme that exists as multiple forms in various subcellular compartments and has been shown to have many nonglycolytic functions (for review, see Sirover, 1997). In the case of apoptosis of cerebellar granule cells induced by low K+/serum-free culture, culture aging, and cytosine arabinoside exposure, the overexpressed GAPDH is translocated to the nucleus (Saunders et al., 1997; Ishitani et al., 1998). This translocation process appears to be intimately linked to apoptosis of neurons, as evidenced by our antisense studies. It has also been reported that GAPDH overexpression and subsequent nuclear translocation participate in the apoptosis of nonneuronal cells (Sawa et al., 1997). Thus, it seems possible that the neuroprotective effects of ONO-1603 and THA are mediated through inhibition of GAPDH overexpression and its downstream processes. GAPDH has been shown to bind specifically the carboxy-terminal of the β-amyloid precursor protein (β-APP) (Schulze et al., 1993). Accumulating evidence suggests that, in addition to amyloid β-protein, the carboxy-terminal fragment(s) of β-APP is also involved in inducing neuronal loss in AD (for review, see Suh, 1997). In this context, we found that GAPDH cross-interacts with a monoclonal antibody raised against amyloid plaques from Alzheimer’s patients brain (Sunaga et al., 1995). It is con-
remarkable that GAPDH overexpression results in enhanced binding of GAPDH to the β-APP carboxyl fragment and subsequent neurodegeneration.

It should be noted that CGP 3466, an analog of R(-)-deprenyl (one of the drugs used for the treatment of Parkinson’s disease), protects apoptotic death of PAJU cells, and the putative molecular target responsible for its antiapoptotic, neuroprotective effects was identified as GAPDH (Kragten et al., 1998). GAPDH also selectively binds to gene products of other neurodegenerative diseases (Burke et al., 1996; Koshy et al., 1996). These include huntingtin of Huntington disease, atrophin of dentatorubropallidoluysian atrophy, ataxin of spinocerebellar ataxia type-1, ataxin-3 of Machado-Joseph disease, and androgen receptor of spinobulbar muscular atrophy. It is interesting to note that NHL-terminal fragments of huntingtin and ataxin-3 are located to intranuclear inclusions in neurons of affected brain regions (Davies et al., 1997; DiFiglia et al., 1997; Paulson et al., 1997). This raises the possibility that GAPDH serves as a “carrier” to mediate the translocation of these disease gene products to the nucleus. It remains to be studied whether the truncated fragment(s) of β-APP is translocated to the nucleus of neurons by a GAPDH-dependent mechanism and, if so, whether this process is suppressed by treatment with potential antidementia drugs such as ONO-1603.

**References**


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