Danthron Inhibits the Neurotoxicity Induced by Various Compounds Causing Oxidative Damages Including β-Amyloid (25—35) in Primary Cortical Cultures

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Oxidative stress caused by an elevation in reactive oxygen species (ROS) plays an important role in Alzheimer’s disease and other neurodegenerative diseases. In this study, we examined the neuroprotective effect of danthron (1,8-dihydroxyanthraquinone) against neurotoxicities induced by β-amyloid (25—35), excitotoxins, apoptosis, and oxidative stress in primary cortical cultures. Danthron dose-dependently reduced neuronal injury induced by 30 μM β-amyloid (25—35). Danthron significantly inhibited oxidative injury induced by 100 μM Fe³⁺ and decreased membrane lipid peroxidation induced by 100 μM Fe³⁺ as measured by thiobarbituric-acid-reactive substance (TBARS). Danthron (0.5—50 μM) ameliorated the effects of buthionine sulfoximine (BSO, 1 mM), which depletes endogenous glutathione by 10—73%. Danthron also dose-dependently inhibited neuronal injury mediated by nitric oxide (NO) radicals, but failed to inhibit injury due to superoxide radicals (O₂⁻). These results suggest danthron treatment may, in part, reduce neurotoxicity related to β-amyloid protein by both dominant inhibitory effects on membrane lipid peroxidation and glutathione deprivation.

Key words danthron; β-amyloid protein; glutathione deprivation; lipid peroxidation; cortical culture

Alzheimer’s disease (AD) is known to induce deficits in learning and memory in older patients. The neuropathological hallmarks of AD are senile plaques and neurofibrillary tangles containing β-amyloid protein.

Although the neurodegenerative mechanism of AD remains unclear, oxidative insults are important factors in the acceleration of neuronal death.1) Some investigators have reported that β-amyloid itself does not directly contribute to the pathway of oxidative stress.2,3)

However, other investigators have demonstrated that β-amyloid (25—35) induces a depletion of neuronal glutathione (GSH) in neuronal culture.4,5) In addition, both βA25—35 and βA1—42 Ca²⁺-dependently depleted endogenous glutathione in hippocampal astrocytes and secondarily depleted glutathione in neuronal cells.5) Akama and his colleagues have demonstrated that the β-amyloid protein stimulates nitric oxide (NO) production in astrocytes by a cytokine-dependent pathway.6) Recently, the abnormal accumulation of iron and redox-active iron was found to contribute to β-amyloid-mediated neurotoxicity in AD.7,8) Iron ions promote the aggregation of β-amyloid in some situations.9) Brains from patients with AD exhibit a disruption of iron metabolism.10)

We recently observed that danthron, a component of Rumex japonicus, senna, and aloe, attenuates β-amyloid-induced neurotoxicity in a murine cortical culture system. Danthron (1,8-dihydroxyanthraquinone) is known to be a potential mutagen or carcinogen and is present in laxatives and Chinese herbal medicines, such as senna (Cassia senna) and aloe.11,12) Before we evaluated the protective effect of danthron in β-amyloid neurotoxicity, we screened the plants used in 128 kinds of Chinese herbal medicines for the ability to rescue neuronal cells from β-amyloid (25—35) neurotoxicity and found that Rumex japonicus extract ameliorated the neuronal injury induced by β-amyloid in a mixed cortical culture system. Therefore, we examined the inhibitory action of danthron on β-amyloid neurotoxicity in primary cortical cultures. In addition, we looked for an inhibitory action of danthron on apoptosis- or excitotoxin (N-methyl-D-aspartate (NMDA) or kainite)-induced neurotoxicity using the same culture paradigms.

MATERIALS AND METHODS

Neuronal and Glial Cultures Mixed cortical cell cultures containing both glia and neurons were prepared from ICR mice at gestation day 15—16, as previously described.13) Briefly, dissociated neocortical cells (2.5—3.0×10⁵ cells/well) were plated onto primaria-coated 24-well plates (Falcon) containing a glial bed in a plating medium consisting of Eagle’s minimal essential medium (MEM; Earle’s salts, supplied glutamine-free) supplemented with 20 mM glucose, 2 mM l-glutamine, 5% fetal bovine serum, and 5% horse serum. Cytosine arabinoside (10 μM) was added 5 d after plating to halt the growth of non-neuronal cells. Cultures were maintained at 37°C in a humidified CO² incubator and used for experiments between days 14 and 16 in vitro. Glial cultures were prepared from postnatal (1—3 d) mice and plated at 0.5—0.75 hemispheres/24-well plate in plating medium supplemented with 10% horse serum/10% fetal bovine serum and 10 mg/ml epidermal growth factor. After two weeks in vitro, cytosine arabinoside was added to the cultures, which were fed weekly with the same medium with 10% horse serum used for mixed cultures. β-Amyloid (25—

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Measurement of Neurotoxicity and Immunocytochemistry  Neuronal injury was quantitatively estimated by measuring lactate dehydrogenase (LDH) released from damaged cells into the bathing medium 24 h after the β-amyloid (25—35), ferric chloride, buthionine sulfoximine (irreversible inhibitor of γ-glutamylcysteine synthase), xanthine/xanthine oxidase, sodium nitroprusside (NO donor), NMDA, and kainate treatments, or 48 h after treatment with apoptosis-inducing agents (staurosporine, dextromethorphan, and cytosine arabinoside), as previously described.14) For morphological confirmation, neuronal cells were stained with neuron-specific enolase antibody (NSE, BioGenex). Briefly, mixed cortical cultures were fixed overnight at 4 °C in a freshly prepared solution of 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.4), washed three times with PBS, and permeabilized with 0.25% Triton X-100 in PBS for 10 min at room temperature. Cultures were washed with PBS and incubated in 10% normal goat serum (NGS) in PBS for 30 min at room temperature; cultures were then labeled with mouse anti-neuron-specific enolase (1:400 dilution) overnight at 4 °C. After three washes, the appropriate biotinylated secondary antibody (goat anti-mouse IgG) was diluted 1:200 and added to the cultures for 1 h at room temperature. All antibodies were diluted in PBS containing 2% NGS. The reaction product was visualized using 3,3-diaminobenzidine tetrahydrochloride (Vector, Burlingame, CA, U.S.A.). Images were photographed with an Olympus IX-70 microscope.

Measurement of Lipid Peroxidation Lipoperoxides were quantitated according to the method of Ohkawa et al.15) In brief, cortical cells were lysed with 900 μl of 2% sodium dodecyl sulphate solution, and the protein concentrations of cell lysates were measured by the Bradford method. Lysates were added serially to 25 μl of 4% butylated hydroxytoluene in ethanol, 500 μl of 10% phosphotungstic acid in 0.5 M sulfuric acid, and 250 μl of 0.7% thiobarbituric acid. The mixtures were boiled for 50 min, added to 1 ml of n-butanol, and centrifuged. The supernatant was collected, and the amount of thiobarbituric-acid-reactive substance (TBARS) was measured at 535 nm. TBARS were scaled to a standard curve using a mixture of 1 mM tetrahydroxypropane in 1% sulfuric acid.

Statistical Analysis Data were expressed as the mean ± standard error of the mean (S.E.M.) and analyzed for statistical significance by one-way analysis of variance (ANOVA) using a post-hoc Student–Neuman–Keuls test for multiple comparisons.

RESULTS AND DISCUSSION

The exposure of mixed cortical cell cultures to 30 μM β-amyloid (25—35) for 24 h caused moderate neuronal injury (40—55%). When 0.5—50 μM danthron and 30 μM β-amyloid (25—35) were added simultaneously to the cortical cultures, neuronal damage was significantly inhibited (36—75%) in a dose-dependent manner (Fig. 1). In the same β-amyloid neurotoxic paradigm, cycloheximide (0.5 μM), a protein synthesis inhibitor, decreased β-amyloid-induced neurotoxicity by 80%.

Morphologically, β-amyloid-induced neurotoxicity manifested as early shrinkage and late loss of cell bodies (Fig. 2A); cycloheximide and danthron rescued many neurons from β-amyloid (25—35) neurotoxicity (Figs. 2B, C).

The exposure of neuronal cultures to 40 μM or 100 μM Fe3+ induced 70% or greater than 80% neuronal injury, respectively. The co-treatment of cultured neurons with 40 μM Fe3+ and either 100 μM deferoxamine, an iron chelator, or 0.5—5 μM danthron almost completely blocked neuronal injury (Fig. 3A). Morphologically, we confirmed a massive loss of neurites and cell bodies at 24 h after treatment with iron (Fig. 3C); deferoxamine and danthron rescued most of the neurons from iron-induced neurotoxicity (Fig. 3D, E). At a higher concentration of Fe3+ (100 μM), danthron (1—30 μM) attenuated neuronal damage in a concentration-dependent fashion (Fig. 3B). Danthron (30 μM) also significantly de-

![Fig. 1. Danthron Attenuated β-Amyloid (25—35)-Induced Neurotoxicity in Mixed Cortical Cultures in a Concentration-Dependent Manner](image1)

Bars represent LDH release into the bathing medium (mean±S.E.M.; n=4) in sister cultures 24 h after exposure to 30 μM β-amyloid (25—35) alone (Control) or in the presence of 0.5 μM cycloheximide (CHX) or danthron at the indicated concentrations. *p<0.05, **p<0.01, ***p<0.001 vs. controls.

![Fig. 2. Morphological Evidence of Neuroprotection against β-Amyloid (25—35)-Induced Neurotoxicity by Cycloheximide (CHX) or Danthron](image2)

Phase-contrast photomicrograph of sister cultures 24 h after exposure to 30 μM β-amyloid (25—35) alone (A) or in the presence of 0.5 μM cycloheximide (B) or 50 μM danthron (C). Neurons were stained with neuron-specific enolase antibody (BioGenex, U.S.A.). Scale bar, 50 μm.
creased membrane lipid peroxidation measured as TBARS by 24% at 24 h after treatment with 100 μM ferric chloride (Table 1).

A concentration of 1 mM buthionine sulfoximine (BSO), an inhibitor of endogenous glutathione synthesis, induced 65—70% neuronal injury as evaluated by an increase in LDH released into the culture medium after 20—24 h. Co-treatment cultured neurons with BSO and 100 μM α-tocopherol, a free radical scavenger, reduced neuronal injury by 98%; co-treatment with 5—50 μM danthron reduced neuronal injury by 10—73% (Fig. 4A). We also confirmed that the neuroprotective actions of both compounds, α-tocopherol and danthron, were consistent with morphological findings (Figs. 4B—D). Furthermore, danthron concentration-dependently attenuated the neuronal damage induced by 300 μM sodium nitroprusside, a nitric oxide (NO) donor, and 100 μM hydrogen peroxide (Figs. 5A, B). However, danthron did not inhibit several other neurotoxic injuries, including zinc neurotoxicity, xanthine/xanthine oxidase-induced superoxide radical injury, NMDA- or kainate-induced excitotoxic injury, or staurosporine- or dextromethorphan-induced apoptotic injury (data not shown).

In this study, danthron, a naturally occurring anthraquinone derivative from Rumex species plants or senna laxatives, provided significant neuroprotective action against the oxidative insults of iron, BSO, nitric oxide, and hydrogen peroxide. Although danthron is considered potentially genotoxic or carcinogenic in some respects,11,12) it nevertheless provides a distinct alleviatory action against neuronal damage caused by free radicals related to the Fenton reaction, such as hydrogen peroxide- or iron-mediated neurotoxicity, and by membrane lipid peroxidation.1,7) The well-known redox active metals, e.g., iron and zinc, may play a pivotal role in the pathogenesis of AD through the acceleration of β-amyloid aggregation, although their exact neurotoxic mechanism remains controversial.8,9) However, some antioxidants that contribute to the rescue of neuronal cells from oxidative injury are known to be useless in blocking β-amyloid toxicity.3) For this reason, danthron may act as a potential selective antioxidant against β-amyloid-induced neuronal damage, but not against excitotoxicity- or apoptosis-mediated injuries, through the inhibition of membrane lipid peroxidation. Furthermore, the β-amyloid peptide is known to calcium-dependently deplete endogenous glutathione levels of neurons and astrocytes,23) and danthron alleviated neuronal injury induced by GSH depletion.

![Fig. 3. Danthron Inhibited Fe^{3+}-Induced Neuronal Injury](image)

Bars represent LDH released into the bathing medium (mean±S.E.M.; n=4) in sister cultures 24 h after exposure to 40 μM Fe^{3+} alone or with 100 μM deferoxamine or danthron at the indicated concentrations (A). Dose-dependent inhibition by danthron of 100 μM Fe^{3+}-induced neuronal injury (B). *p<0.05, **p<0.01, ***p<0.001 vs. controls. Morphological evidence of neuroprotection with danthron against Fe^{3+}-induced neurotoxicity. Phase-contrast photomicrograph of sister cultures 24 h after exposure to 40 μM Fe^{3+} alone (C) or in the presence of 100 μM deferoxamine (D) or 0.5 μM danthron (E). Neurons were stained with neuron-specific enolase antibody (BioGenex, U.S.A.). Scale bar, 50 μm.

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<th>Table 1. Danthron Attenuated Membrane Lipid Peroxidation Induced by Ferric Chloride (100 μM for 24 h)</th>
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<td><strong>Treatment</strong></td>
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Lipid peroxidation is reported as TBARS (nmol/mg protein). Data represent mean±S.E.M. *p<0.05, two tailed t-test.
In conclusion, danthron may be a potential free radical scavenger protecting against β-amyloid-mediated neuronal injury.

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REFERENCES