Sauchinone, a Lignan from *Saururus chinensis*, Inhibits Staurosporine-Induced Apoptosis in C6 Rat Glioma Cells

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Neuronal apoptosis may contribute to pathologic neuronal loss in certain disease states such as neurodegenerative diseases. Staurosporine (ST), a nonselective protein kinase inhibitor, has been shown to induce apoptosis in a variety of cells including nerve cell lines. In this study, we investigated the neuroprotective effect of sauchinone, which is a unique lignan from *Saururus chinensis*, on ST-induced apoptosis in C6 rat glioma cells. Sauchinone attenuated ST-induced apoptosis of C6 glioma cells as evidenced by DNA fragmentation. We also provide evidence that the inhibitory effect of sauchinone on ST-induced apoptosis involves a dose-dependent upregulation of an antiapoptotic protein, Bcl-2. Mounting evidence shows that the activation of caspases, especially caspase-3, triggers the apoptotic process. The activity of caspase-3 of ST-pretreated cells was significantly decreased upon sauchinone treatment in a dose-dependent manner. Taken together, the data demonstrate that sauchinone protects C6 glioma cells from ST-induced apoptosis in a caspase-3 dependent manner. Our findings may be critical for developing a strategy to protect nerve cells from apoptosis, suggesting the potential development of sauchinone as a neuroprotective agent.

Key words *Saururus chinensis*; sauchinone; neuroprotective activity; C6 rat glioma cell; apoptosis; caspase-3

Increased attention has been focused on neuronal apoptosis which may contribute to pathologic neuronal loss in neurodegenerative diseases such as Alzheimer's disease,¹⁾ Parkinson's disease,²⁾ and Huntington's disease.^{3–5)} Staurosporine (ST), a nonselective protein kinase inhibitor, has been shown to induce apoptosis in a variety of cell lines including neuronal cells.^{6–9)}

Apoptotic cell death plays an important role in neuronal cell death and is regulated by the Bcl-2 family and caspase family. Bcl-2 prevents apoptotic death in neurons and several neuron-like cell lines.^{10,11)} The death-promoting protein Bax counteracts the antiapoptotic effects of Bcl-2 by forming a heterodimer with Bcl-2.¹²⁾ Mounting evidence shows that activation of caspases triggers the apoptotic process in various cells.¹³⁾ Caspase-3, a member of the caspase family, has been shown to play an essential role in apoptosis induced by a variety of stimuli.^{14,15)} Both *in vitro* and *in vivo* evidence implicates caspase-3 as an important factor in neuronal apoptosis, especially under pathologic conditions.¹⁶⁾

Saururus chinensis (LOUR.) BAILL. (Saururaceae) has been used in Korean folk medicine for the treatment of edema, jaundice, and gonorrhea.¹⁷⁾ Sauchinone is a unique lignan isolated from *S. chinensis*.¹⁸⁾ A significant hepatoprotective activity was reported in primary cultured rat hepatocytes exposed to CCl₄.^{19–21)} Identifying and developing pharmaceutical agents that can modulate apoptotic pathways may provide an effective strategy for the treatment of neurodegenerative diseases. In the present study, we investigated the inhibitory effect of sauchinone on apoptosis induced by ST in C6 rat glioma cells. Sauchinone efficiently protects C6 cells from ST-induced apoptosis, demonstrating a potential neuroprotective activity of sauchinone. We further suggest the molecular mechanisms for the inhibition of ST-induced apoptotic cell death by sauchinone in C6 glioma cells.

MATERIALS AND METHODS

Materials ST was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and dissolved in dimethyl sulfoxide (DMSO). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, penicillin, and streptomycin were purchased from Gibco BRL (Grand Island, NY, U.S.A.). Sauchinone was obtained as previously described.²⁰⁾

Cell Lines The C6 cell line was purchased from the American Type Culture Collection (Manassas, VA, U.S.A.). The cells were maintained at 37 °C in humidified atmosphere at 95% air and 5% CO₂ in DMEM supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl Tetrazolium Bromide (MTT) Assay Cells were treated with MTT solution (5 mg/ml) and further incubated for 4 h. After 100 μ l of supernatant was replaced with the same volume of DMSO, absorbance was read at 540 nm with a micro-ELISA reader (Molecular Devices, Sunnyvale, CA, U.S.A.). Percent of cell survival was defined as the relative absorbance of treated *versus* untreated cells.

DNA Fragmentation Cells in a 100-mm dish were trypsinized and collected with ice-cold PBS. After centrifugation (2000 $\times g$) for 10 min at 4 °C, cells were transferred to an Eppendorf tube and recentrifuged at 15000 rpm for 15 min at 4 °C. Cell pellets were resuspended in 0.5–1 ml of isolation buffer (10 mM EDTA, 50 mM Tris–HCl, pH 8.0, 0.5% SDS, 0.5 mg/ml proteinase K) and incubated overnight at 50 °C. The lysates were centrifuged at 15000 rpm for 15 min at 4 °C to separate the soluble fragmented DNA from the intact chromatin pellet. DNA was extracted with phenol/chloroform/isoamyl-alcohol (25:24:1) and precipitated with ethanol. Purified DNA was treated with RNase A (1 μ g/ml) for 1 h at 37 °C prior to electrophoresis on a 1.8% agarose gel containing ethidium bromide.

Immunoblot Analysis of Bcl-2 and Bax Equal amounts (100 μ g) of protein extracts in lysis buffer (1 M Tris–HCl, pH

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6.8, 10% SDS, 0.5 M EDTA, 1 M DTT) containing a protease inhibitor cocktail (Roche Mannheim, Germany) were subjected to 12% SDS-PAGE analysis and transferred to a nitrocellulose membrane. The levels of Bcl-2 and Bax were detected using anti-Bcl-2 (mouse monoclonal purchased from Cell Signaling) and anti-Bax (rabbit polyclonal from Santa Cruz Biotechnology Inc., Santa Cruz, CA, U.S.A.) antibodies.

Measurement of Caspase-3 Activity Cells were washed twice with ice-cold PBS, harvested with chilled lysis buffer, and incubated on ice for 10 min. The lysates were centrifuged for 30 min at 13000 rpm. The caspase-3 activity was measured using a fluorometric assay kit (BioVision Research Products, Mountain View, CA, U.S.A.). Briefly, equal amounts (50 μ g) of protein extracts were incubated with 50 μ M of fluorogenic peptide substrate for caspase-3 (Ac-DEVD-AFC) in 50 μ l of reaction buffer at 37 °C for 1 h. The release of AFC was measured with a fluorometer (Bio-Tek Instruments Inc., Winooski, VT, U.S.A.) at an excitation wavelength of 400 nm and an emission wavelength of 505 nm.

RESULTS AND DISCUSSION

The major aim of this investigation was to evaluate the neuroprotective potential of a lignan from *S. chinensis*, sauchinone (depicted in Fig. 1), in C6 rat glioma cells treated with ST. We first confirmed that ST effectively induced apoptosis in C6 cells as evidenced by cytotoxicity, DNA fragmentation, and nuclear condensation (data not shown). To investigate the neuroprotective activity of sauchinone, we examined the effect of sauchinone on the ST-induced apoptosis in C6 glioma cells. As shown in Fig. 2A, sauchinone alleviated cell death induced by treatment with ST (100 nM) in a dose-dependent manner. The effect of sauchinone on ST-induced apoptosis was examined by DNA fragmentation. Sauchinone inhibited DNA fragmentation induced by ST in a dose-dependent manner (Fig. 2B), showing the protective effect of sauchinone on the apoptotic cell death of ST-treated glioma cells. In addition, sauchinone reduced ST-induced nuclei condensation of C6 cells (data not shown).

It has been shown that the ST-induced apoptosis involves mitochondrial caspase activation that is inhibited by Bcl-2.^{6,22,23} In an attempt to reveal the molecular mechanisms underlying the neuroprotective effect of sauchinone in ST-treated C6 cells, we measured the protein levels of two key apoptosis-linked gene products, Bcl-2 and Bax, which are known to regulate the cell death/survival in opposite manners.²⁴ As shown in Fig. 3A, expression of the antiapoptotic oncoprotein Bcl-2 was prominently decreased by treatment with ST (250 nM), while the death-promoting Bax expression remained unchanged. Sauchinone treatment increased the level of Bcl-2 in a dose-dependent manner, while it had little

Fig. 1. Chemical Structure of Sauchinone

Fig. 2. Attenuation of ST-Induced Apoptosis by Sauchinone

Cells were treated with ST (100 nM) with or without sauchinone for 48 h. (A) Cytotoxicity was determined by MTT assay. The results presented are mean $\pm$ S.E. of triplicates. *, ** Statistically different at $p < 0.05$ and $p < 0.01$, respectively, from the cells treated with ST (100 nM) alone by the independent two-sample Student's *t*-test. (B) DNA was extracted from the treated cells and analyzed by agarose gel electrophoresis. Internucleosomal DNA fragmentation was observed.

Fig. 3. Increase in the Ratio of Bcl-2 to Bax with Sauchinone

Cells were treated with ST (250 nM) with or without sauchinone for 48 h. (A) Immunoblot analysis was performed with lysates from the treated cells for Bcl-2 and Bax. Relative band intensities were determined by quantitation of each band. (B) Cytotoxicity was determined in the MTT assay. The results presented are mean $\pm$ S.E. of triplicates. * Statistically different at $p < 0.05$ from cells treated with ST (250 nM) alone by the independent two-sample Student's *t*-test.

effect on the expression of Bax. Pro- and antiapoptotic Bcl-2-related proteins are often coexpressed within the same cells and the ratio between pro- and antiapoptotic proteins is a major determinant of the fate of the cell.²⁵⁾ Since the ratio of Bcl-2 to Bax, rather than the levels of the individual proteins, is considered to be critical in determining the cell survival and death,^{24,26)} we quantitated the expression levels of Bcl-2 and Bax using the Image Analysis System to determine the ratio of the two proteins. The ratio of Bcl-2 to Bax was decreased in the cells treated with ST. A dose-dependent increase in the ratio was obtained with sauchinone treatment, suggesting that the inhibition of ST-induced apoptotic cell death by sauchinone involves upregulation of the antiapoptotic protein, Bcl-2. We also examined the effect of sauchinone on cell death induced by the same concentration (250 nM) of ST. Treatment with sauchinone (5, 10 μ M) significantly attenuated the death of C6 cells treated with ST (250 nM) (Fig. 3B).

It has been demonstrated that caspase-3 catalyzes the formation of beta-amyloid peptide, a hallmark of Alzheimer's disease.¹⁶⁾ To test the possible involvement of caspase-3 in ST-induced apoptosis and the inhibition by sauchinone, we measured the caspase-3 activity of C6 cells treated with ST in the presence or absence of sauchinone by measuring fluorescence generated from proteolytic cleavage of Ac-DEVD-AFC (50 μ M). As shown in Fig. 4, ST treatment (250 nM) markedly increased the activity of caspase-3 in C6 glioma cells. The activity was significantly decreased upon sauchinone treatment in a dose-dependent manner.

Taken together, we demonstrated that sauchinone exerts a significant neuroprotective effect on C6 rat glioma cells. Our results suggest that sauchinone protects C6 glioma cells from ST-induced apoptosis in a caspase-3-dependent manner. It would be worthwhile to investigate further the potency of sauchinone using a known antiapoptotic and neuroprotective agent as a positive control. Understanding the basic mechanisms mediating cell death in neurodegenerative disease may

Fig. 4. Inhibition of Caspase-3 Activity by Sauchinone

Cells were treated with ST (250 nM) with or without sauchinone for 48 h. Caspase-3 activity was measured using a fluorometric assay kit. *,** Statistically different at $p < 0.05$ and $p < 0.01$, respectively, from cells treated with ST alone by the independent two-sample Student's *t*-test.

lead to the development of novel approaches for the treatment of diseases featuring apoptosis. Further studies need to be performed to elucidate the structure-activity relationship of sauchinone-induced inhibition of apoptosis.

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