CHAPTER III
MATERIALS AND METHODS

1. Materials

Satratoxin H, extracted from a fungus *Podostroma cornu-damae* and purified by chromatography (Saikawa *et al.*, 2001), was kindly provided by Professor Dr. Yoshiteru Oshima (Department of Natural Products Chemistry, Graduate School of Pharmaceutical Sciences, Tohoku University, Japan). Dulbecco's modified Eagle's medium (DMEM) was the product of Nissui Pharmaceutical Co., Ltd., Japan. 2′, 7′-dichlorofluorescein diacetate (DCFH-DA), fetal calf serum (FCS), chromatin dye bisbenzimide (Hoechst 33342), glutathione, *N*-acetyl-L-cysteine, poly-L-lysine, propidium iodide, and 2-[2-amino-3-methoxyphenyl]-4H-1-benzopyran-4-one (PD98059), thiobarbituric acid-reactive substances (TBARS), 1, 1, 3, 3-tetraethoxypropane, and bovine serum albumin (BSA) were obtained from Sigma-Aldrich (St. Louis, USA). Horse serum (HS), and phenol:chloroform:isoamyl alcohol (25:24:1) were obtained from Invitrogen Corporation, New Zealand. 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox) was from Fluka Chemie AG, Switzerland. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was from Research Organics, Inc. (Cleveland, Ohio, USA). Anthra(1,9-cd)pyrazol-6(2H)-one (SP600125) was from Biomol Research Labotatories (Plymouth Meeting, PA) and 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1H-imidazole (SB203580) were from Wako Pure Chemicals (Osaka, Japan). Antibodies specific for JNK, phospho-JNK, p38 MAPK, phospho-p38, ERK, phospho-ERK, and secondary anti-rabbit Ig G (H&L) horseradish peroxidase-linked were from Cell Signaling Technology, Inc. (MA, USA). The enhanced chemiluminescence Western blotting detection reagent was purchased from Amersham Biosciences, USA. All other chemicals used were commercially available reagents or analytical reagent quality.

2. Cell culture

PC12 cells were cultured in DMEM supplemented with 10% FCS, 5% HS, penicillin (50 unit/ml) and streptomycin (50 μg/ml) in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. The cells were passaged every 2-3 days. Satratoxin H and all inhibitors were dissolved in dimethyl sulfoxide (DMSO), and were subsequently

diluted with culture medium. After appropriated dilutions, the final concentration of DMSO in culture medium was kept less than 0.1% (v/v) which had no protective or toxic effect by itself (Hong and Liu, 2004). PC12 cells were growth-arrested by incubation of satratoxin H in serum-free medium (Hulleman and Boonstra, 2001). All experiments were performed with growth-arrested cells to minimize basal MAPK activity and to remove serum factors (Oberdoerster *et al.*, 1998; Panet *et al.*, 2001; Yoshizumi *et al.*, 2002).

3. Experimental design

To investigate the molecular mechanism of satratoxin H-induced apoptosis, PC12 cells were used. The methods were designed as the followings. Cell viability was measured by MTT assay. Apoptotic cell death was detected by agarose gel electrophoresis of DNA fragmentation, fluorescence microscopic assay staining with Hoechst 33342, and flow cytometry staining with PI. Mechanistic study of the MAPKs (ERK1/2, p38 MAPK and JNK) activation using MAPKs inhibitors was determined by MTT assay and Western blot analysis. Satratoxin H-generated ROS and lipid peroxide production were investigated by flow cytometry using DCFH-DA and TBARS assay, respectively. The protective effect of antioxidant(s) on satratoxin H-treated PC12 cells was also performed.

3.1 Experiment 1: cytotoxic assay of satratoxin H-treated PC12 cells

PC12 cells were seeded at a concentration of 0.5×10^5 cells/ml in 96-well plates. After 2 days, the cells were treated with 1-100 nM satratoxin H for 24 h, and the cytotoxic 50% inhibitory concentration (IC_{50}) was determined. Satratoxin H at a concentration of the IC_{50} value was added to the cells in time dependency. Cell viability was assessed by a dye staining method using MTT reduction assay (Hansen, 1989). PC12 cells were incubated with MTT at a final concentration of 1 mg/ml in a 96-well plate for 4 h at 37°C. Viable cells with active mitochondria caused cleavage of the tetrazolium ring into a visible dark blue product through formazan reaction, whereas, dead cells remained uncolored. Subsequently, the reaction was stopped by adding 100 µl of a cell lysis buffer containing 50% (v/v) *N, N*-dimethyl formamide and 20% sodium dodecyl sulfate (SDS) (pH 4.7). The terminated reaction mixture was maintained overnight at 37°C. The plate was shaken for a short time on the microplate shaker to disperse the blue MTT-formazan. The optical density (OD) was quantified at an absorbance of 595 nm using a micro-plate reader (Sunrise Classic,

Tecan GmbH, Austria). Cell viability was expressed as a percentage of the control culture. The percentage of cytotoxicity was calculated by the following equation:

$$\% \text{ cytotoxicity} = \frac{OD_{595nm} \text{ (sample-blank)} \times 100}{OD_{595nm} \text{ (control-blank)}}$$

3.2 Experiment 2: measurement of apoptotic effect

Fluorescence microscopic assay using Hoechst 33342, DNA gel electrophoresis, and DNA flow cytometry method using PI were performed to investigate whether satratoxin H could induce apoptotic cells

3.2.1 Fluorescence microscopic assay using Hoechst 33342 staining

Hoechst 33342 is a non-cytotoxic DNA dye that preferentially binds to triplet adenine and thymine base pairs in the minor groove outside of the double helix. Nuclear morphological changes of apoptotic cells using the fluorescent dye Hoechst 33342 were determined as described previously (McKeague *et al.*, 2003). PC12 cells were seeded onto 24-well plates and treated with satratoxin H at a concentration of the IC_{50} value for 12, 24, 36, and 48 h. Treated cells were stained with Hoechst 33342 (2.5 μg/ml) and incubated in dark for 15 min. The cells were visualized and photographed under a fluorescent microscope. A minimum of 100 cells was counted, and the number of apoptotic cells was calculated as a percentage of the total population. Three replicate wells were analyzed for each treatment of three random fields in each well. The healthy cell showed an oval-shaped cell body, and its chromatin stains dimly and occupied the majority of the cell body volume. The chromatin of an apoptotic cell was condensed, intensely stained by Hoechst 33342 (blue color), and shifted to the periphery of the cell body.

3.2.2 DNA fragmentation analysis using agarose gel electrophoresis

To measure oligonucleosome-sized fragments resulting from cleavage of nuclear DNA, the DNA fragmentation was performed as described previously (Chen *et al.*, 1997). PC12 cells were seeded in 100-mm dish and treated with satratoxin H at a concentration of the IC_{50} value for 24, 48, and 72 hours. Floating and attached cells were harvested, washed and resuspended in lysis buffer (10 mM Tris-HCl, pH 7.5, 10 mM NaCl, 10 mM EDTA, and 0.5% SDS) supplemented with 100 μg/ml proteinase K. The cells were incubated for 1 h at 50 °C. The lysates were extracted with equal volumes of phenol/chloroform/isoamyl alcohol

(25:24:1), followed by extraction with 2 volumes of chloroform/isoamyl alcohol (24:1). The supernatants containing fragmented DNA were precipitated with 2.5 volumes of ice-cold ethanol plus 10% volume of 3 M sodium acetate, pH 5.2 at -20 °C overnight. The DNA precipitates were obtained by centrifugation at 12,000 rpm for 10 min. After washing the pellets with 70% ethanol, the DNA was resuspended in 50 µl of TE buffer (Tris 10 mM, EDTA 1 mM, pH 7.5) supplemented with 0.1 µg/ml RNase A. After 1-h incubation at 37 °C, the samples were electrophoresed in a 1.5% agarose gel in TBE buffer (Tris-borate 45 mM, and EDTA 1 mM), at 100 V for 45 min. After electrophoresis, the gels were stained with ethidium bromide and photographed under UV light.

3.2.3 Flow cytometry analysis for measurement of subG$_1$ phase

PC12 cells were seeded in 6-well plates at a concentration of 5×10^5 cells/ml. The cells were further cultivated for 24 h and treated with satratoxin H at a concentration of the cytotoxic IC_{50} value for 12, 24, and 48 h. After satratoxin H exposure, flow cytometric analysis was performed as described previously (Li *et al.*, 2003). Treated cells were harvested and washed with PBS twice, then fixed in 1 ml cold 70% ethanol and stored at 4 °C for 24 h. After washing with PBS, the cells were resuspended in PBS containing RNase A 200 µg/ml and incubated at 37 °C for 30 min, then centrifuged at 1,000×g for 10 min. The cells were stained with 300 µl PBS containing 100 µg/ml PI at 4 °C for 30 min (avoiding light). The cells were washed and subjected to flow cytometric analysis of DNA content. DNA content was determined using a FACSort flow cytometer (Becton Dickinson, San Jose, CA.). Excitation was done at 488 nm, and emission filter at 600 nm. CELLQuest™ software (Becton Dickinson), for subG$_1$ peak detection of the apoptotic effect was used. Ten thousand cells in each sample were analyzed and expressed as percentage of total cells.

3.3 Experiment 3: measurement of intracellular generation of ROS and lipid peroxidation

3.3.1 Measurement of intracellular ROS level

2′, 7′-dichlorofluorescein diacetate (DCFH-DA), an oxidation-sensitive fluorescent probe and a stable nonpolar compound, readily diffused through the cell membrane and yields 2′, 7′-dichlorodihydrofluorescein ($DCFH_2$) after rapid

de-acetylation by esterases. In the presence of ROS, $DCFH_2$ was oxidized to the highly fluorescent compound 2′, 7′-dichlorofluorescein (DCF) (Arai *et al.*, 2001; Curtin *et al.*, 2002; Myhre, *et al.*, 2003), as the following:

$$\text{DCFH-DA} \xrightarrow{\text{esterase}} \text{DCFH}_2 \xrightarrow{\text{ROS}} \text{DCF}$$

The fluorescence intensity of DCF was estimated by flow cytometry according to the procedure described previously (Pae *et al.*, 2003). After treated with satratoxin H at the concentration of the IC_{50} value for various time points (0.5, 1, 3, 6, 12, and 24 h), the cells were washed three times with ice-cold phosphate-buffered saline (pH 7.4). The cells were incubated with 10 μM DCFH-DA for 30 min at 37 °C. After the cells were washed twice with PBS to remove the extracellular DCFH-DA, the fluorescence intensity was analyzed by a FACSort flow cytometry (excitation 488 nm and emission 525 nm for DCF). The relative amount of intracellular ROS production by satratoxin H was expressed as the fluorescence ratio of the treatment to control.

3.3.2 Lipid peroxidation assay using TBARS method

PC12 cells (10^7 cells) were exposed to satratoxin H at the concentration of the cytotoxic IC_{50} value for 12, 24, 36, and 48 h. Lipid peroxides were determined by the spectrophotometric method measuring the amount of TBARS formation during the decomposition of lipid hydroperoxides (Lee *et al.*, 2000). Treated PC12 cells were harvested by scrapping, washed, and re-suspended in deionized water then mixed with 8.1% SDS, 20% acetic acid solution, and 0.8% thiobarbituric acid. The mixtures were incubated in a boiling water bath for 1 h. After cooling the tubes, n-butanol:pyridine mixture (15:1, v/v) was added and the reaction mixture was centrifuged at 3,000 rpm for 15 min. The absorbance of the upper organic layer containing MDA, one of the end products of lipid peroxidation, was determined at 532 nm. The concentration of TBARS was expressed as nanomoles/mg protein using 1, 1, 3, 3-tetraethoxypropane as a reference standard. The protein concentration was measured by the method of Bradford (Bradford, 1976). All determinations were done in duplicates in each sample.

3.4 Experiment 4: mechanistic study of satratoxin H-induced apoptosis via activation of MAPKs

3.4.1 Determination of MAPKs protein expression by Western blot analysis

To demonstrate the expression of MAPK(s) in PC12 cells, MAPKs and phosphorylated MAPKs were determined by Western blotting using antibodies specific for MAPKs and phosphorylated MAPKs.

PC12 cells were seeded at a concentration of 5×10^5 cells/ml in 6-well plates. After 24-h incubation, PC12 cells were treated or untreated with satratoxin H in serum-free medium at a concentration of IC_{50} value for various times (0.5-6 h). Treated cells were washed with ice-cold PBS and solubilized in lysis buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 20 mM EDTA, 50 mM NaF, 1% NP-40, 0.02% NaN_3 with protease inhibitors (1 mM phenylmethanesulfonyl fluoride, 2 μg/ml leupeptin and 2 μg/ml aprotinin). Lysates were briefly sonicated. Protein concentrations in the samples were determined by the Bradford method. Whole cell extracts were mixed with Laemmli loading buffer (225 mM Tris-HCl, pH 6.8, 6% SDS, 30% glycerol, 9% 2-mercaptoethanol and 0.009% bromphenol blue), boiled for 5 min, and stored at -20 °C until use. Equal amounts of protein were loaded each lane and resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto PVDF membrane using a semi-dry blotting system as described previously (Datta and Long, 2002). The membranes were blocked with 5% skimmed milk in TBST (10 mM Tris, 0.1 M NaCl and 0.01% Tween 20) for 2 h at room temperature. Blots were probed with specific antibodies, ERK, phospho-ERK, p38 MAPK, phospho-p38 MAPK, JNK or phospho-JNK at 4 °C overnight. After washing with TBST, the membranes were incubated with the corresponding secondary antibodies conjugated with horseradish peroxidase for 2 h at room temperature. Blots were treated with an enhanced chemiluminescene detection reagent and exposed to CL-X films.

To verify the effect of MAPKs inhibitors (SB203580, p38 inhibitor; PD98059, ERK inhibitor; SP600125, JNK inhibitor) on satratoxin H-induced activation of MAPKs, MAPKs inhibitors were added to PC12 cells 1 h before incubation with satratoxin H at the concentration of the IC_{50} value. Treated cells were collected,

washed and lysed. The lysates were subjected to immunoblotting analysis with an anti ERK/JNK/p38 and antiphospho-ERK/JNK/p38 antibody as described above.

3.4.2 Determination of MAPKs inhibitors on satratoxin H-induced apoptosis by MTT assay

To support the effect of satratoxin H on the activity of MAPKs, the cells were treated with each inhibitor (p38 inhibitor; SB203580, JNK inhibitor; SP600125, and ERK inhibitor, PD98059) for 1 h, and further incubated with indicated concentration of satratoxin H for 48 h. The cells were washed out and replaced with new medium before adding MTT solution because these antioxidants at high concentration induced the formazan reaction as described above.

3.5 Experiment 5: Effect of antioxidants on satratoxin H-induced apoptosis in PC12 cells

The antioxidants, GSH, NAC, or trolox, was added immediately before the application of satratoxin H at the concentration of IC_{50} value in serum-free medium for indicated times.

3.5.1 Effect of antioxidants on satratoxin H-induced cytotoxicity

To determine whether antioxidant(s) could protect satratoxin H-induced cytotoxicity, antioxidants, GSH, NAC, and trolox, were used. PC12 cells were treated with antioxidants immediately before adding satratoxin H at the concentration of IC_{50} value in serum-free medium for 6-24 h. The cells were washed out, replaced with new medium, and measured by MTT assay.

3.5.2 Effect of antioxidants on satratoxin H-induced apoptosis

To determine whether antioxidant(s) could protect satratoxin H-induced apoptosis, antioxidants, GSH, and NAC were used. PC12 cells were treated with antioxidants immediately before adding satratoxin H at the concentration of IC_{50} value for 24 h. Treated cells were stained with Hoechst 33342, visualized, and photographed under a fluorescent microscope. The number of apoptotic cells was calculated as a percentage of the total population.

3.5.3 Effect of antioxidants on the generation of ROS in satratoxin H-treated PC12 cells

To determine whether antioxidant(s) could protect satratoxin H-induced the generation of ROS, antioxidants, GSH, and NAC were used. PC12 cells were treated with antioxidants immediately before adding satratoxin H at the concentration of IC_{50} value for 6 h. The cells were stained with DCFH-DA, and the relative fluorescence intensity was analyzed by flow cytometry.

3.5.4 Effect of antioxidants on satratoxin H-induced the production of lipid peroxidation in PC12 cells

To determine whether antioxidant(s) could protect satratoxin H-induced the production of lipid peroxidation, antioxidants, GSH, and trolox were used. PC12 cells were treated with antioxidants immediately before adding satratoxin H at the concentration of IC_{50} value for 48 h. The production of lipid peroxides was determined by TBARS assay.

4. Statistical analysis

Values were expressed as means ± SEM. The reproducibility of the results was confirmed in at least three independent sets of experiments. Data shown in figures were from a representative set of experiments. Time- and concentration-dependency were analyzed by using one-way analysis of variance followed by Dunnett's multiple comparisons post hoc test. All the other data were evaluated by one-way analysis of variance followed by Scheffé's test. Values of $P < 0.05$ were regarded to be statistically significant.