CHAPTER V
DISCUSSION AND CONCLUSION

The present study indicated that satratoxin H modulated neurotoxicity by induction of cell death in PC12 cells. The characteristic apoptotic changes were similar to those observed in other cell types, including nuclear condensation, DNA fragmentation, and apoptotic bodies. We also provided evidences suggesting that satratoxin H-induced apoptosis was mediated by the generation of ROS linked to the p38 MAPK and JNK activation and subsequently the production of lipid peroxidation leading to necrotic cell death. Because satratoxin H caused cell death in PC12 cells, a cell line originated from pheochromocytoma, as well as in the previously reported myeloid cell lines (Nagase *et al.*, 2002; Shifrin and Anderson, 1999; Yang *et al.*, 2000), it would injure neural cells and induced disorders of dopaminergic cells.

Incubation of satratoxin H caused PC12 cell death in concentration-dependently reduced cell viability with the IC_{50} about 50 nM at 24 h. Treatment with satratoxin H (50 nM) resulted in significant decrease cell viability of approximately 25% from control within the first 6 h and gradually decreased until 24 h. Cytotoxic measurement of mycotoxin trichothecenes including macrocyclic trichothecenes demonstrated by Yang *et al.*, 2000 suggested that the toxic effect of trichothecenes depended on cell type and type of trichothecenes. The present results correlated well with data by Yang *et al.*, 2000 that incubation of satratoxin H (MW 528.24) at a concentration of 10 ng/ml (about 19 nM) for 24 h resulted in decrease in cell viability at the IC_{50} in U937 cells whereas less toxic to RAW 264.7 cells. Although, it has been reported that macrocyclic ring containing trichothecenes including satratoxin H was the most toxic of all trichothecenes (Ehrlich and Daigle, 1987; Lee and Li, 1999), incubation of satratoxin H in myeloid cells showed less toxicity than other macrocyclic trichothecenes: verrucarin A, roridin A and satratoxin F (Yang *et al.*, 2000). Together with the present data suggested that satratoxin H may be more toxic to myeloid cell line than dopaminergic cell line. Additionally, eating toxic mushroom *Podostroma cornu-damae* containing satratoxin H showed severe toxicity to nervous system (Saikawa *et al.*, 2001), study of satratoxin H-induced apoptosis in PC12 cells was therefore elucidated.

Since incubation of satratoxin H significantly decreased cell viability in PC12 cells, the type of cell death was determined. Staining with Hoechst 33342 found the significant decrement ($P<0.01$) in vital cell numbers with accompanied by the chromatin condensation and nuclear cleavage, typical morphological features and a later event in the apoptotic cascade, after 24-h coincubation with satratoxin H in serum-free medium and in complete medium (Figures 7). Hoechst 33342 has been used to determine the early stage and the late stage of apoptosis (Piiper *et al.*, 2002), meanwhile the indicator of late apoptosis, DNA fragmentation by agarose gel electrophoresis and flow cytometry using PI, were determined. PC12 cells dramatically showed DNA fragmentation by agarose gel electrophoresis after incubation of satratoxin H in medium with serum for 72 h and incubation of satratoxin H in serum-free medium for 24 h. Additionally, DNA ladder gradually decreased after 48-h incubation and it was difficult to be observed after 72-h incubation of satratoxin H (Figure 8). The induction of late apoptosis by satratoxin H was confirmed by flow cytometric analysis of DNA content staining with PI that satratoxin H (50 nM) significantly induced an accumulation of cells in the pre G_0/G_1 phase ($P<0.05$) at 24 h when compared with control cells cultivated in serum-free medium then slightly decreased in the percentage of apoptosis. In contrast, incubation of satratoxin H (50 nM) in complete medium for 48 h showed a significant increase in the percentage of apoptotic cell death (Figure 9). The result implied that incubation of PC12 cells in serum-free medium was more sensitive to satratoxin H-induced apoptosis than incubation in medium with serum (Figure 9). Incubation PC12 cells in serum-free medium was 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). The decrement in apoptosis detected by agarose gel electrophoresis and flow cytometry using PI, as seen after 24-h incubation of satratoxin H in serum-free medium, may be from some apoptotic cells changed into secondary necrosis as mentioned by Kinloch *et al.*, 1999 and Padanilam, 2003. Eventhough it has been reported that the key morphological changes in apoptosis did not correlate with DNA fragmentation produced by endonuclease activity and apoptosis can occur without DNA degradation (Schulze-Osthoff, *et al.*, 1994), incubation of satratoxin H on PC12 cells for 72 h (medium with serum) and for 24 h (serum-free medium) clearly showed the pattern of oligonucleosomes resulting from DNA cleavage in apoptotic cells detected by agarose gel electrophoresis. In addition,

the percentage of satratoxin H-induced apoptosis detected by Hoechst 33342 (Figure 7) and flow cytometry using PI (Figure 9) was less than the percentage of cell death detected by MTT assay (Figure 1) measured the function of mitochondrial dehydrogenase enzyme. It was possible that satratoxin H might disturb the mitochondrial function leading to partially induced apoptosis.

Since satratoxin H caused apoptotic cell death in PC12 cells, the molecular mechanism of satratoxin H-induced apoptosis was further elucidated. Trichothecene-induced apoptosis has been documented to cause GSH depletion in myeloid cells (Ji *et al.*, 1998; Pae *et al.*, 2003) and *in vivo* model (Rizzo *et al.*, 1994). Trichothecenes, such as AETD and vomitoxin, have been reported to induce apoptosis through the increment of intracellular ROS formation on human apromyelocytic HL-60 cells and RAW 264.7 murine cell line, respectively (Ji *et al.*, 1998; Pae *et al.*, 2003). Trichothecenes caused a severe loss of neurons of the brain in human and animal (Croft *et al.*, 2002; Saikawa *et al.*, 2001). Taken together, it was highly potential that ROS might take a role in satratoxin H-induced apoptosis in PC12 cells, a model of dopaminergic cells. Exposure of PC12 cells to satratoxin H resulted in the generation of ROS, the phosphorylation of p38 MAPK and JNK, and the production of lipid peroxidation. The incubation of PC12 cells with satratoxin H at a concentration of 50 nM starting increased the intracellular ROS after 3 h and significantly increased ($P<0.05$) after 6-h incubation then maintained that level until 24 h detected by flow cytometer using DCFH-DA (Figure 10). The generation of ROS from satratoxin H might be caused by GSH depletion as shown by Ji *et al.*, 1998; Pae *et al.*, 2003, and Rizzo *et al.*, 1994. Satratoxin H caused DNA fragmentation and the decrement of MTT absorbance in the present study. DNA fragmentation has been recognized that DNA was cleaved by DFF40 which was activated by caspase 3, and caspase 3 was activated by apoptosome (the complex of caspase 9, Apaf-1, ATP, and cytochrome *c* released from mitochondria). Meanwhile, satratoxin H can penetrate through mitochondrial membrane, MTT assay was measured the function of mitochondrial dehydrogenase enzyme. Since satratoxin H decreased MTT absorbance, it was possible that the increment of ROS may be generated from mitochondria as well. It has been demonstrated that ROS also caused DNA damage by both directly attacking DNA and by activation of endonucleases that degraded DNA and leading to apoptotic cell death (Cadet *et al.*, 1997; Floyd and Carney, 1992; Fernandez *et al.*, 1995). The

generation of ROS also preceded the production of lipid peroxidation demonstrated by TBARS assay, which appeared after 36-h exposure of the cells to satratoxin H (50 nM) as shown in Figure 11. Consequently, the significant increase ($P<0.01$) in membrane lipid peroxidation following satratoxin H exposure is thought to result from the excess intracellular production of ROS as found in previous study (Arai *et al.*, 2000; Yang *et al.*, 2003). The prolong accumulation of ROS by satratoxin H treatment is likely to be responsible for the increase in lipid peroxidation product such as 4-HNE, and MDA. Many cellular compounds were easily oxidized by ROS, lipid peroxides have been wildly used as a marker for oxidative stress in many systems including PC12 cells (Hong and Liu, 2004). Apoptotic cell death induced by satratoxin H seemed to be not involved in the production of lipid peroxidation. These results were similar to previous studies in PC12 cells that exposure of 200 μM H_2O_2 for 24 h or exposure of toxic chemical dieldrin increased ROS production subsequently increased the production of lipid peroxides (Hong and Liu, 2004; Kitazawa *et al.*, 2001). It has been mentioned that lipid peroxidation caused membrane deterioration leading to necrotic cell death (Dalal and Khanna-Chopra, 1999; Higuchi, 2004). Therefore, the data were probably concluded that lipid peroxidation induced by satratoxin H may cause necrotic cell death in PC12 cells.

It has been reported that the classification of trichothecene mycotoxins based on their ability to inhibit protein synthesis, activate p38 MAPK/JNK, and induce apoptosis (Shifrin and Anderson, 1999). The macrocyclic trichothecenes including verrucarin A and satratoxin G have been shown to induce apoptosis through activation of p38 MAPK/JNK phosphorylation in the RAW 264.7 cell, U937 cell line, the Jurkat human T-lymphoid cell line (Yang *et al.*, 2000; Shifrin and Anderson, 1999) and caspase 3, DFF40/CAD, caspase 8, as well as cytosolic accumulation of cytochrome *c* in a human promyelotic leukemia cell line (Nagase *et al.*, 2002). The results suggested that ROS generated by satratoxin H following by the activation of p38 MAPK and JNK is one of the critical pathways of satratoxin H-induced apoptosis in PC12 cells, and the accumulation of ROS then damaged cell membrane by activation of lipid peroxidation (Esterbauer *et al.*, 1991). Although satratoxin H has been etiologically associated with neurological disorder, no information exists regarding the specific molecular mechanism on apoptosis in neuronal cells involving the activity of MAPKs. The activity of JNK, but not p38 MAPK and ERK1/2, has been

previously reported in satratoxin H-induced apoptosis in RAW 264.7 murine macrophage cells, which might be through ROS (Yang *et al.*, 2000). As indicated by Western blotting analysis in the present study, p38 MAPK, JNK, and ERK1/2 were phosphorylated by satratoxin H in PC12 cells. The activation of p38 MAPK and JNK appear after 3 h, and clearly seen after 6-h exposure of the cells to satratoxin H detected by Western blot analysis (Figure 12). Satratoxin H-induced the generation of ROS accompanied by the activation of p38 MAPK and JNK tightly correlated with the subsequent apoptosis in PC12 cells, since apoptotic cell death significantly increased after 24 h of treatment with satratoxin H in serum-free medium detected by fluorescence microscope using Hoechst 33342, agarose gel electrophoresis, and flow cytometer using propidium iodide. The functional significance of these responses was revealed by the finding that SB203580, a p38 MAPK-specific inhibitor, or SP600125, a specific inhibitor of JNK increased cell survival after 48-h exposure of satratoxin H, detected by MTT assay (Figure 13). Therefore, satratoxin H can be thought to induce apoptosis through the generation of ROS starting after 3 h exposure of satratoxin H following by the activation of p38 MAPK and/or JNK. In contrast to these two MAPKs, ERK1/2 showed no apparent role in satratoxin H-induced apoptosis, because a specific MAPK/ERK kinase inhibitor, PD98059, did not affect cell survival. Although the phosphorylation of ERK 1/2 was increased by satratoxin H, the less and slower phosphorylation of ERK 1/2 might not overcome satratoxin H-induced apoptosis on PC12 cells. The present result was similar to Yang *et al.*, 2000 that phosphorylated ERK1/2 was observed as early as 1 h after incubation of satratoxin G (10 ng/ml) for 4 h, but not satratoxin H (10 ng/ml) in murine macrophage cells. Previous study reported that induction of apoptosis by trichothecenes in Jurkat T cells was strongly activated both p38 MAPK and JNK via inhibition of the peptidyltransferase reaction by binding to the 60S ribosomal subunit in eukaryotic cells (Middlebrook and Leatherman, 1989; Shifrin and Anderson, 1999). Both p38 MAPK and JNK were recently shown to be essential components of a signal transduction pathway involving oxidative stress-induced apoptosis in PC12 cells (Sarker *et al.*, 2003; Yoshizumi *et al.*, 2002; Zhang and Jope, 1999). Recent studies have demonstrated that ROS served as a signal for apoptosis by activating an array of cell-signaling molecules including p38 MAPK and JNK, caspase, cytochrome c, and endonuclease (Junn and Mouradian, 2001; Kling *et al.*, 2005; Luo *et al.*, 1998). A known neurotoxic agent, 1-methyl-4-phenylpyridinium MPP^+, has been shown to

promote dopaminergic cell death through the activation of similar cellular signaling mechanism (Chun *et al.*, 2001). The findings of these studies suggested that activation of p38 MAPK or JNK, but not ERK1/2, contributed to cell death machinery induced by satratoxin H-induced apoptosis via the generation of ROS.

Oxidative stress has been implicated as a possible signaling in apoptotic cell death through p38 MAPK and JNK (Osone *et al.*, 2004; Shin *et al.*, 2004). The activation of ROS-dependent p38 MAPK and JNK pathway which further leading to cell death has been shown to activate through an alternative pathway, modulated by manipulating the lipid peroxidation level (Yang *et al.*, 2003). The administration of trichothecene mycotoxins, DON or T-2 toxin has been reported to increase lipid peroxides such as MDA and 4-HNE, in rats which received a feed deficient in vitamins C and E and selenium (Rizzo *et al.*, 1994). MDA, less toxicity than 4-HNE, has been shown to induce cell death in cultured rat brain endothelial cells detected by intracellular lactate dehydrogenase (LDH) and MTT assay (Hipkiss *et al.*, 1997). A line of evidence demonstrated that the 4-HNE-induced apoptosis in PC12 cells was likely to be mediated though the activation of ASK1-SEK1-JNK pathway without activation of p38 MAPK and ERK1/2 (Soh *et al.*, 2000). 4-HNE-induced JNK activation promoted its translocation into the nucleus where JNK-dependent phophorylation of c-Jun and the transcription factor activator protein (AP-1) binding taken place leading to transcriptional regulation of a number of genes having consensus sequences in their promoter region (Yang *et al.*, 2003). Under these conditions, JNK did not appear to be the direct target of 4-HNE since peroxidation end products such as 4-HNE have been shown to cause apoptosis with coincident of intracellular GSH depletion leading to activation of JNK pathway (Uchida *et al.*, 1999; Yang *et al.*, 2003). GSH depletion was also closely associated with HNE-induced apoptotic cell death through Fas-independent activation of caspase 8, caspase 9, and caspase 3, possibly through a mitochondria-dependent pathway (Liu *et al.*, 2000) which was similar to satratoxin G-induced apoptosis in human leukemic HL-60 cells (Nagase *et al.*, 2002). In present study, the generation of ROS and the activation of p38 MAPK/JNK appeared at 3-6 h after satratoxin H treatment, and the apoptosis increased significantly after 24-h incubation of satratoxin H. The prolong production of ROS was likely to be responsible for the significant increase in lipid peroxidation observed at 36 h after satratoxin H treatment. It is possible that p38 MAPK/JNK-

induced apoptotic cell death in satratoxin H-treated PC12 cells through the generation of ROS was lipid peroxidation-independent, since the apoptotic cell death preceded the production of lipid peroxidation. In our knowledge, however, there is no evidence showing the participation of ROS in the satratoxin H-induced apoptotic cell death in PC12 cells. The present results suggested that satratoxin H stimulated intracellular stress signaling pathway including p38 MAPK and JNK mainly through the ROS generation.

Although an investigation suggested that trichothecene-generated ROS, GSH depletion and lipid peroxidation was protected by NAC in the human promyelocytic cell line (Pae *et al.*, 2003), and by selenium, vitamin C, and α-tocopherol in rat liver (Rizzo *et al.*, 1994), there was lack of evidence to show the protective effect of vitamin E, GSH, and NAC on satratoxin H-induced apoptosis in PC12 cells. To evaluate the protective effect of GSH, NAC, and vitamin E analog (trolox) in satratoxin H-induced apoptosis, the generation of ROS, and lipid peroxidation, various concentrations of antioxidant was cotreated with satratoxin H (50 nM) in serum-free medium in PC12 cells. It has been recognized that pretreatment was important to investigate the protective effect of antioxidants *in vivo*. For example, to maintain a high concentration of selenium in the tissues of Wistar rats, selenium, vitamin C, and vitamin E were pretreated for 2 weeks before administration of T-2 toxin and DON (Rizzo *et al.*, 1994). Pretreatment of GSH or NAC has been used for protection against oxidative stress in a variety of cell systems (Li *et al.*, 2003; Pae *et al.*, 2003; Tan *et al.*, 2003). A line of evidence demonstrated that increasing of the preincubation time of trolox decreased the rate of cell death following ultraviolet B exposure to human keratinocytes (Peus *et al.*, 2001). On the other hand, cotreatment of GSH with toxins has been shown effectively to protect cell death in numerous cell systems. For examples, immediate addition of 2 mM GSH before the incubation with vanadate protected the epithelial cell lines Ma104 from vanadate toxicity (Capella *et al.*, 2002). Exogenous GSH (3-10 mM) addition for 24 h prevented 6-hydroxydopamine-induced neuronal apoptosis in the human dopaminergic neuroblastoma cell line SK-N-SH detected by DNA fragmentation enzyme-immunoassay and Hoechst 33258 staining (Shimizu *et al.*, 2002). In addition, it has been shown that hepatocytes were coincubated with carpet dust containing trichothecenes and vitamin E (10-15 μM) showed a significant decrease in TBARS

production (Ameen *et al.*, 2004). Under our conditions, cotreatment with GSH (1, 5, and 10 mM), NAC (1, 5, and 10 mM) or trolox (0.01, 0.1, and 1 mM) and satratoxin H (1-100 nM) for 6-24 h did not significantly increase the percentage of cell viability detected by MTT assay (Figure 15). Since, the time of the production of lipid peroxidation (36-48 h) appeared after later than the time of apoptotic cell death (24 h) induced by satratoxin H, lipid peroxidation may not involve apoptotic cell death. And trolox has been well known in protection of the propagation of lipid peroxidation (Cao and Cutler, 1993; Horáková et al., 2003), which involved in necrotic cell death (Higuchi, 2004). Therefore only GSH and NAC were used to investigate the protective effect of satratoxin H-induced apoptosis detected by flow cytometer using propidium iodide. Since satratoxin H significantly increased ($P<0.05$) the generation of ROS at 6 h and maintained at the same level until 24 h (Figure 10), the effect of GSH and NAC in the presence of satratoxin H was demonstrated at 6 h. Coincubation of GSH (1 mM), but not NAC, and satratoxin H (50 nM) in PC12 cells for 6 h significantly decreased ($P<0.05$) ROS level (Figure 17). NAC, acetylated variant of L-cysteine, was well-known as a precursor of GSH synthesis using two enzymes, γ-glutamylcysteine synthetase and GSH synthetase. The result suggested that satratoxin H might affect enzymes involving GSH synthesis, γ-glutamylcysteine synthetase and/or GSH synthetase. Cotreatment of GSH or trolox with 50 nM satratoxin H for 48 h did not significantly restore the MDA level when compared to control group (Figure 18).

The role of GSH in preventing apoptosis is ambiguous. Meanwhile GSH possessed protective effect against oxidative stress-induced apoptosis (Morley *et al.*, 2003; Pae *et al.*, 2003; Yamamasu *et al.*, 1997), GSH monoester-mediated enhancement of hypoxic apoptosis through inhibition of nuclear factor-κB in murine embryonic fibroblasts (Qanungo *et al.*, 2004). The present results showed that antioxidants GSH had no protective effect on satratoxin H-induced apoptosis detected by MTT assay (Figure 15) and flow cytometric analysis using PI (Figure 16); and GSH did not significantly change the MDA level detected by TBARS assay (Figure 18). GSH is a known antioxidant to neutralize H_2O_2 in cytosol and mitochondria (Curtin *et al.*, 2002), and to detoxify the lipid peroxidation product, 4-HNE (Yang *et al.*, 2003). GSH at concentrations ranging from 1-10 mM was used which was in the physiological concentration, and 0.1 up to 12 mM in mammalian cells (Schroder *et al.*;

1996). It has been reported the interaction between sesquiterpene lactone and GSH that sesquiterpene lactone of kava-kava, extracted from the roots of the kava plant *Piper methysticum*, reacted with the sulfide group of GSH (Whitton et al., 2003). After cotreatment, sesquiterpene lactone ring of satratoxin H may interact with GSH intracellularly and extracellularly resulting in contradictory effect. Interaction between satratoxin H and exogenous GSH may decrease free satratoxin H and lead to decrease the toxicity of satratoxin H. Extracellular GSH does not penetrate the cell membrane (De Flora *et al.*, 1995; Mazor *et al.*, 1996), it is degraded to its constituent amino acids by the membrane bound γ-glutamyl transpeptidase (γGT) and dipeptidylpeptidase (DPP), whose active site is located on the outer plasma membrane, give rise γ-glutamyl amino acid; and cysteine and glycine, respectively which are subsequent taken up by cells and serve as substrates for intracellular GSH synthesis. GSH is synthesized from glutamic acid, L-cysteine, and glycine in two steps which catalysed by γ-glutamylcysteine synthase and glutathione synthase. However, recently it has been reported for the existence of a sodium-dependent GSH transporter in brain endothelial cells, and a sodium-independent GSH transporter in Cos-7 cells (Kannan *et al.*, 1999). GSH plays an important role in controlling the redox state of cells by maintenance the enzyme activity of GSH peroxidase in a reduced state and protection against oxidative stress by scavenging of hydrogen peroxide even if GSH depletion has been shown to induce apoptosis by itself (Domenicotti *et al.*, 2000; Dringen, 2000; Higuchi, 2004). The role of GSH replacement in preventing lipid peroxidation is also unclear, as GSH has been shown to reduce lipid peroxidation in isolated liver microsomes (Palamanda and Kehrer, 1992). It has been demonstrated that trichothecene mycotoxin T-2 freely passed through mammalian cells (Middlebrook and Leatherman, 1989). The structure of satratoxin H ($C_{29}H_{36}O_9$) containing a macrocyclic dilactone derivative (a macrocyclic ring between C_4 and C_{15} with two ester linkages) of the sesquiterpene 12,13-epoxy-$\triangle^9$-trichothecene and an epoxide functional group (Eppley and Mazzola, 1975; Josephs *et al.*, 2004; Saikawa *et al.*, 2001) may conjugate with the sulfhydryl groups of intracellular thiols including GSH leading to apoptotic cell death from depletion of intracellular GSH (Anazetti *et al.*, 2004; Lu, 1999). GSH depletion has been demonstrated to induce apoptosis in human leukemic HL-60 cells by rapid activation of caspase-3 at 0.5 h after treatment of AETD from *Isaria japonica* (Pae *et al.*, 2003), and from nitrofen (Kling *et al.*,

2005). Caspases activation was often referred to as the apoptotic commitment point, the point in the signaling cascade where the cells commits to die (Ashe and Berry, 2003), and occur in many types of cells such as HL-60 cells by satratoxin G (Nagase *et al.*, 2002) including PC12 cells (Kitazawa *et al.*, 2001; Rong *et al.*, 1999). Therefore, it is possible that caspases may be one of important pathway in satratoxin H-induced apoptosis in PC12 cells. Trichothecene mycotoxins including satratoxin H has been demonstrated to inhibit protein synthesis by binding to the ribosomal peptidyl transferase site leading to activation of p38 MAPK/JNK and induce apoptosis in Jurkat T cells (Shifrin and Anderson, 1999). It is possible that satratoxin H-induced apoptosis through the activation of p38 MAPK/JNK is stimulated from at least two stimulators: ROS and/or a ribotoxic stress and/or mitochondrial pathway. Together with the present results implied that ROS pathway is one of the important pathways in satratoxin H-induced apoptosis in PC12 cells.

In addition, it has been suggested that trolox may play an important role in scavenging free radicals prominent in cytosol as compared with stabilizing cellular membranes (Peus *et al.*, 2001). Although it has been reported that trolox (a vitamin E analogs) inhibited induction of apoptosis (Peus *et al.*, 2001) and lipid peroxidation in PC12 cells treated with H_2O_2 (Horáková *et al.*, 2003); the present results showed that cotreatment of trolox (0.01, 0.1, and 1 mM) and satratoxin H (50 nM) incubated in serum-free medium for 24-48 h had no inhibitory effect on apoptosis detected by MTT assay and the generated lipid peroxidation for 48 h detected by the TBARS assay. Trolox (5, 10 mM) has also been reported to inhibit the generation of lipid peroxidation, but did not inhibited the induction of apoptosis in isolated Bursal cells of Fabricius (Arai, *et al.*, 2000). The effect of vitamin E was controversial that α-tocopherol did not decrease the toxicity of lipid peroxide, HNE (Kruman *et al.*, 1997) meanwhile cotreatment of carpet dust contaminated endotoxins and vitamin E (10-15 μM) in hepatocytes protected lipid peroxidation (Ameen *et al.*, 2004). Together with the incubation time which satratoxin H-induced apoptosis (24-48 h) was prior to the production of lipid peroxides (36-48 h), it implied that lipid peroxidation may not play a crucial role in satratoxin H-induced apoptosis. Lipid peroxidation may be generated from the accumulation of intracellular ROS (6 h). Coincubation with antioxidant GSH or vitamin E was not sufficient to protect the propagation of chain reaction in cell membrane. Antioxidants did not show protective effect on satratoxin H-induced

apoptosis in the present study was probably due to the generation of other radical species such as $O_2^{\bullet-}$ and/or deficiency of enzyme in GSH redox cycle and/or intracellular localization of the ROS responsible for induction of apoptosis. Although we do not know how ROS are generated by satratoxin H so far, our results suggest that apoptosis by satratoxin H requires the generation of ROS which coincides of the activation of p38 MAPK and JNK, and consequent causes the production of lipid peroxidation (Figure 19).

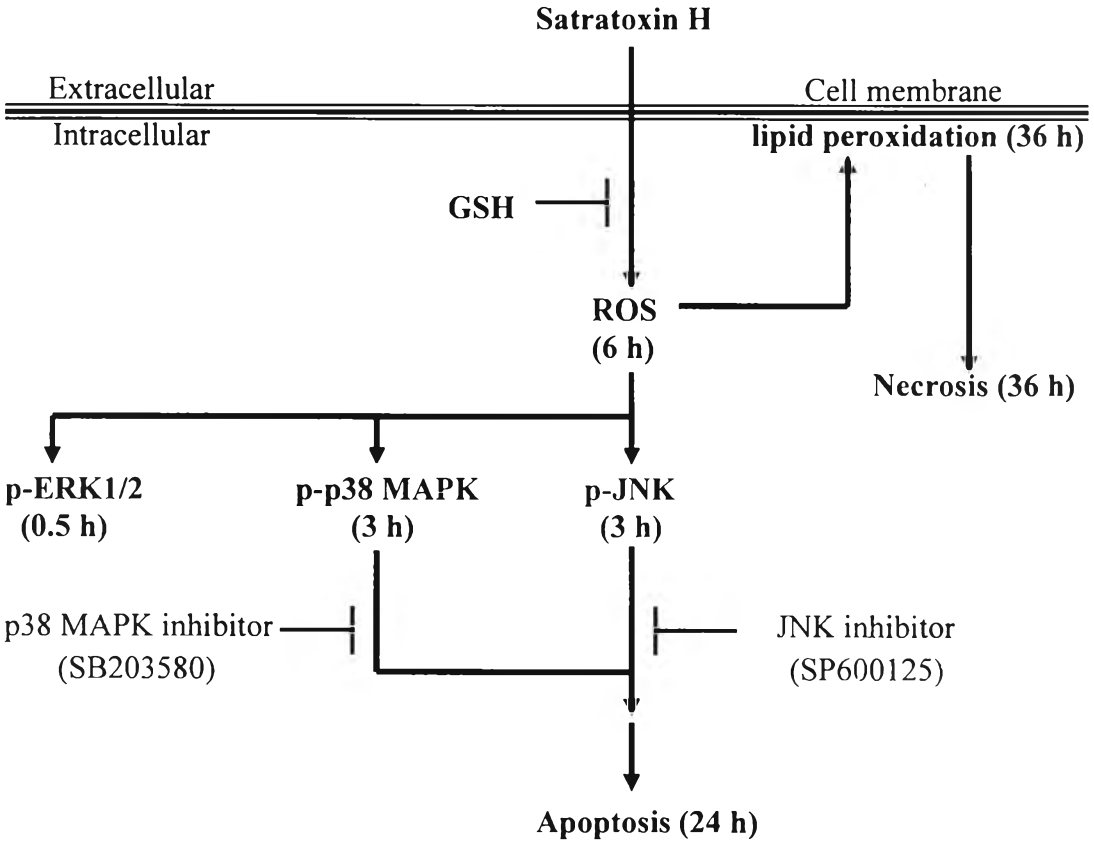

Figure 19 Hypothetical signaling pathways of satratoxin H-induced cell death in PC12 cells.

In conclusion, this study clearly demonstrated that satratoxin H, a trichothecene mycotoxin, can cause cell death in time- and concentration-dependent manner. Incubation of satratoxin H at the concentration of the IC_{50} (50 nM) for 24 h, showed the induction of apoptosis following by secondary necrosis detected by fluorescence microscopic assay using Hoechst 33342, DNA fragmentation analysis using agarose gel electrophoresis, and flow cytometry using propidium iodide. Determination by flow cytometry using DCFH-DA found that apoptotic cell death induced by satratoxin H increased intracellular ROS starting from 3 h and significant augmented at 6 h then maintained that level until 24 h. The generation of ROS coincided with the activation of p38 kinase and JNK. Since the time that the lipid peroxidation product stimulated by satratoxin H (at 36 h) appeared after the time that apoptotic cell death occurred (24 h), satratoxin H-induced apoptosis in PC12 cells was lipid peroxidation-independent. So satratoxin H-induced apoptosis in PC12 cells was through the MAPKs pathway (p38 and JNK pathway), may be involving the ROS production, but not the generation of lipid peroxidation. The lipid peroxidation induced by satratoxin H may cause necrotic cell death in PC12 cells. The addition of antioxidants did not confirm ROS-induced cell death from satratoxin H-treated PC12 cells. The results implied that another important pathway, the inhibition of protein synthesis at the peptidyl transferase site, was the upstream to p38 MAPK/JNK pathway in satratoxin H-induced apoptosis. Additionally, satratoxin H may induce cell death through the activation of mitochondrial pathway and/or through receptors. The present data supported the hypothesis that cell death induced by satratoxin H partially required the generation of ROS which coincides of the activation of p38 MAPK and JNK, and subsequent caused the production of lipid peroxidation. These findings may be important in explaining the molecular mechanism of satratoxin H-induced cell death in PC12 cells, a model of dopaminergic cells. To clarify the radical species including the source of ROS generated from satratoxin H-treated PC12 cells may be useful for further study.