CHAPTER II
LITERATURE REVIEW

Trichothecenes

The trichothecene mycotoxins, groups of sesquiterpenoid fungal metabolites produced by *Stachybotrys*, are commonly found as contaminants of grain-based foods and indoor air. The trichothecene mycotoxins have a 9, 10 double bond and a 12, 13 epoxide group which is responsible for their toxicological activity (Sudakin, 2003). Trichothecenes are divided into 3 groups as shown in Figure 2: (i) type A trichothecenes which have isovaleryl, hydrogen, or hydroxyl moieties at the C-8 position such as T-2 toxin, (ii) type B trichothecenes which have a carbonyl group at the C-8 position such as vomitoxin and nivalenol, and (iii) the macrocyclic trichothecenes which have a cyclic diester or triester ring linking C-4 to C-15 such as satratoxins including satratoxin H, varrucarin and roridin A (Yang *et al.*, 2000). LD_{50} studies in mouse, HeLa cell cytotoxicity test, and rabbit reticulocyte protein synthesis inhibition assay, have been shown that macrocyclic trichothecenes were 10 to 100 times more toxic than type A and type B trichothecenes (Ueno, 1989).

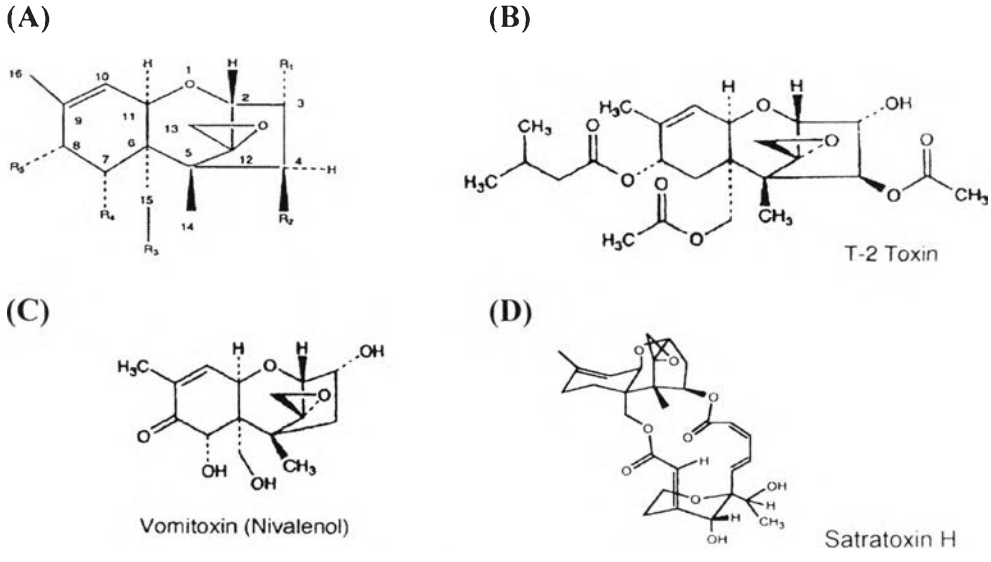

Figure 2 The general structure, numbering system, and variable side groups of the tetracyclic trichothecene nucleus (A); and structures of type A trichothecene (T-2 toxin) (B), type B trichothecene (vomitoxin) (C), and the macrocyclic trichothecene (satratoxin H) (D) (Kuhn and Ghannoum, 2003; Nagase *et al.*, 2002).

Satratoxin H, a very potent toxin, is generally found in *Stachybotrys atra* and a poisonous mushroom, *Podostroma cornu-damae*, found in Japan and Java, (Harrach *et al.*, 1981; Saikawa *et al.*, 2001). *Stachybotrys atra* (also called *S. chartarum and S. alternans*) is a dark-coloured fungus that grows well on cellulose materials with high humidity over long periods of time such as straw, wood, and the paper covering of gypsum wallboard in bathroom. Fungal bio-aerosols in indoor environment caused symptoms common to "sick building syndromes" including allergic rhinitis, difficult in breathing, headaches, flu-like symptoms, and watering of eyes (Mishra *et al.*, 1992). The common symptoms included vomiting, diarrhea, skin irritation and itching, rash or blisters, bleeding, and upper respiratory symptoms such as sore-throat, cough, dyspnea, nausea, anorexia. Continuous exposure of farm animals and humans to low level of trichothecene mycotoxins from *S. atra* caused severe illness stachybotryotoxicosis, characterized by leukopenia, severe thrombocytopenia, hemorrhage, arrhythmic heartbeat and dead (Bata *et al.*, 1985; Harrach *et al.*, 1981, 1983). Acute toxicological effects of oral, parenteral, dermal, or aerosol exposure to trichothecenes produced gastric and intestinal lesions; immunosuppressive effects; suppression of reproductive function; vascular effects leading to hypotension, and CNS toxicity. It has been reported that some Japaneses drank cups of sake containing some pieces of *Podostroma cornu-damae* or ate the fried mushroom causing gastrointestinal disorder, decrease in the number of leukocytes and thrombocytes, erroneous perception, atrophy of the cerebellum, and some leading to die the next day (Saikawa *et al.*, 2001). There was an evident that trichothecenes, DON or T-2 toxin, produced lipid peroxidation in rat brain (Rizzo *et al.*, 1994). Even if, it has some documents of trichothecene-induced neurotoxicity in mammals, until now the molecular mechanism of trichothecene particularly satratoxin H-induced cytotoxicity on neuron cells has not been clarified.

Previous study revealed that the trichothecene mycotoxins, entered the cells easily by moving across the plasma membrane and interacted with a number of targets including ribosomes and mitochondria. They inhibited protein synthesis at either the initiation or the elongation process of translation by interfering with peptidyl transferase activity as the principal mechanism of action (Middlebrook and Leatherman, 1989); inhibited mitochondria protein synthesis (Pace *et al.*, 1988),

altered mitochondria membrane, and inhibited mitochondrial electron transport activity.

Reactive oxygen species

Reactive oxygen species (ROS), regarded as by-products of metabolism, are species of oxygen which have a more reactive state than molecule oxygen. ROS are usually referred to free radicals, any molecule or atom which contains one or more unpaired electrons in their outer orbit, such as superoxide anion radical ($O_2^{-\bullet}$), hydroxyl radicals ($OH^{\bullet}$), peroxynitrite ($ONOO^-$), and alkoxyl radicals ($RO^{\bullet}$); and other oxygen-related reactive compounds, such as singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), and hydroperoxides (ROOH). ROS including the first oxidant superoxide anion characterized by the one electron reduction of molecule oxygen is generated in several metabolic pathways, especially mitochondria. H_2O_2 and $O_2^{-\bullet}$, through generating $OH^{\bullet}$ (the most reactive species within the ROS family), are known to cause lipid peroxidation, which at least in part mediated cellular signaling, and cell injury. Hydroxyl radical is produced from at least two chemical processes known as the iron-catalyzed Fenton reaction, catalyzed by the transition metals ion or copper and H_2O_2 (I); and the Haber-Weiss reaction, the interaction of $O_2^{-\bullet}$ and H_2O_2 (II) as the followings.

(I) $Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^{\bullet} + OH^-$

(II) $O_2^{-\bullet} + H_2O_2 \longrightarrow O_2 + OH^{\bullet} + OH^-$

H_2O_2, easily passed across biological membrane, has been reported to cause apoptosis at low levels as well as necrosis or caspase-independent apoptosis at high levels (Creagh *et al.*, 2000; Hampton and Orrenius, 1997). Massive amounts of ROS are generated in various conditions such as inflammation, exposure to UV light, hypoxia, transforming growth factor-β (Herrera *et al.*, 2004) and toxins including 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (Shimoke *et al.*, 2003) and AETD (Pae *et al.*, 2003), which generate cell death through caspase 3.

Under normal physiological conditions, a number of cellular defense systems have evolved to combat the accumulation of ROS. There are two groups of defense systems including: (i) non-enzymatic molecules such as GSH and vitamin E and (ii) enzymatic scavenger of ROS such as superoxide dismutase (SOD), catalase, and

glutathione peroxidase (GPx) (Martindale and Holbrook, 2002). Superoxide dismutase detoxifies $O_2^{-\bullet}$ by converting it to H_2O_2, which is subsequently reduced to water and molecule oxygen by catalase, presented in peroxisome, or decomposed in the cytosol and mitochondria by GPx in the present of GSH, the electron donor, giving oxidized glutathione (GSSG) and preventing the formation of $OH^{-\bullet}$. Intracellular GSH is maintained by glutathione reductase (GR) required NADPH, generated through glucose-6-phosphate dehydrogenase (G6PD) in the pentose phosphate pathway in cytosol and through transhydrogenase in mitochondria (Curtin *et al.*, 2002).

$$2O_2^{-\bullet} + 2H^+ \xrightarrow[\text{cytosol, mitochondria}]{\text{SOD}} H_2O_2 + O_2$$

$$2H_2O_2 \xrightarrow[\text{peroxisome}]{\text{catalase}} 2H_2O + O_2$$

$$H_2O_2 + 2GSH \xrightarrow[\text{mitochondria}]{\text{GPx}} 2H_2O + GSSG$$

$$GSSG + NADPH + H^+ \xrightarrow[\text{cytosol, mitochondria}]{\text{GR}} 2GSH + NADP^+$$

$$NADP^+ + \text{G-6-P} \xrightarrow[\text{cytosol}]{\text{G6PD}} \text{6-P-gluconolactone} + NADPH + H^+$$

$$NADH + NADP^+ \xrightarrow[\text{mitochondria}]{\text{transhydrogenase}} NAD^+ + NADPH$$

γ-L-Glutamyl-L-cystinylglycine (GSH), a major cellular reductant, is presented at high concentration in most living cells including eukaryotic cells. GSH is synthesized by consecutive action of two enzymes: (i) γ-glutamylcysteine (γGluCys) synthetase, the rate limiting enzyme of GSH biosynthesis, used glutamate and cysteine as substrates to form the dipeptide γGluCys, can be feedback inhibited by the endproduct GSH; and (ii) glutathione synthase combines γGluCys with glycine to generate GSH (Dringen, 2000; Curtin *et al.*, 2002). GSH, the most abundant intracellular thiol, is found in various mammalian tissues in a physiological range of 0.5-10 mM: the brain in a range of 1-3 mM, and astroglia culture about 8 mM (Dringen, 2000). Almost 90% of cellular GSH are in the cytosol, 10% are found in the mitochondria, and small percentages are in the endoplasmic reticulum (Lu, 1999). GSH performs both as a nucleophilic scavenger of numerous compounds and as a substrate in the GPx-mediated destruction of hydroperoxides (Dringen, 2000). It has

been reported the involvement of GSH in apoptotic cell death causing from GSH efflux through GSH-specific membrane channel or carrier-mediated GSH extrusion (Ghibelli *et al.*, 1998; van den Dobbelsteen *et al.*, 1996), and from oxidative stress (Herrera *et al.*, 2004; Lizard *et al.*, 1998; Pae *et al.*, 2003). Extracellular GSH hardly penetrated the cell membrane through the amino acid transporting pathways, whereas NAC, acetylated variant of L-cysteine, does (De Flora *et al.*, 1995; Mazor *et al.*, 1996). GSH, like NAC and methionine, was a cysteine delivery agent. In contrast, it has been reported that GSH transported in an immortalized mouse brain endothelial cell line (MBEC-4) and Cos-7 cells was Na^+ dependency and Na^+ independency, respectively (Kannan et al., 1999).

Extracellular administration of NAC has been shown to increase intracellular GSH in PC12 cells by approximately 10-folds via reduction of extracellular cystine to increase available levels of cysteine, but not by direct metabolic conversion (Yan *et al.*, 1995), subsequently enhancing GSH biosynthesis (Dringen, 2000), and also by replenishing other soluble and protein thiol-groups. Thiols scavenge free radicals, hydroxyl radicals, hydrogen peroxide, and singlet oxygen by direct interaction with cysteine residues in proteins or by raising the intracellular concentration of GSH (Lu, 1999). NAC, an aminothiol reducing agent, has a broad range of actions potentially relevant in the prevention of cell death through several mechanisms such as induction of immediate early response genes, such as *c-fos* and *c-jun* (Yan and Green, 1998), reduction of the level of DNA damage (Morley *et al.*, 2003), enhancing mitochondrial protection (Hart *et al.*, 2004), increasing the number of tyrosine hydroxylase-positive neurons as a producer of dopaminergic cells (Rodriguez-Pallares *et al.*, 2001), donating electron for the reduction of peroxides in the GPx reaction (Chance *et al.*, 1979; Winterbourn and Metodiewa, 1994), inhibiting the activation of c-Jun N-terminal kinase, p38 MAP kinase, and redox-sensitive activating protein-1, nuclear factor kappa B transcription factor activities regulating expression of numerous genes as well as activating the Ras-ERK pathway (Natoli *et al.*, 1997; Yan and Green, 1998; Zafarullah *et al.*, 2003). NAC-promoted survival has been recognized to be due to its reducing property rather than an antioxidant or a glutathione-enhancing activity (Yan *et al.*, 1995). Meanwhile, the survival-promoting actions of NAC from apoptosis in PC12 cells caused by withdrawal of trophic support have been shown to use high concentration of NAC (40-60 mM) for 2 days (Yan and Greene, 1998).

α-Tocopherol (vitamin E), a lipid-soluble radical-scavenging antioxidant located mainly in cellular membrane, effectively protected protein and lipid peroxidation against free radicals on cellular membrane (Heller *et al.*, 2004; Kamat and Devasagayam, 1995). In normal human plasma, α-tocopherol concentration was ranging from 10 to 40 μM (Cao and Cutler, 1993). α-Tocopherol and trolox possessed the same functional structure, which was responsible for their oxygen-radical absorbing quality and trapped two peroxyl radicals per molecule (Burton *et al.*, 1983; Cao and Cutler, 1993). Trolox, a hydrophilic analog and a water-soluble vitamin E, was used as the representator of a chain-breaking antioxidant that inhibited lipid peroxidation by donating a hydrogen atom in its chromanol nucleus to the lipid radical thereby terminating the propagating process (Tsuchiya *et al.*, 1992) which has been used in rat treated with T-2 toxin (Rizzo *et al.*, 1994). Vitamin E has been demonstrated to prevent apoptotic cell death by many manners such as by acting as scavenger of radicals via the H-donating group in its chromanol nucleus (Tsuchiya *et al.*, 1992); by blocking the release of cytochrome c; and by modification of the expression of genes or proteins, or promoting (Bcl-2) or preventing (Bax) apoptosis (Haendeler *et al.*, 1996; Wu *et al.*, 2004).

Lipid peroxidation

Oxidative stress, an imbalance between ROS and antioxidants, induced excessive production of the ROS, which may cause severe damage to DNA, protein, and lipid leading to cellular dysfunction and cell death in a variety of disorders including neuronal diseases such as Alzheimer's disease and Parkinson's disease (Gibson *et al.*, 2000; Giasson *et al.*, 2002). Hydroxyl radical, one of the strong oxidants, particularly reacts with nucleic acid at sugars, purines and pyrimidines (Breen and Murphy, 1995) and also produces lipid peroxidation through the abstraction of hydrogen atoms from the methyl groups of polyunsaturated fatty acid in membrane lipid bilayers to obtain the carbon radical group which further react with molecule oxygen to generate a peroxyl radicals and other oxidants such as conjugated dienes, lipid peroxyl radicals, and hydroperoxides. The peroxyl radical can directly abstract a hydrogen atom from another fatty acid molecules cause a chain reaction (Figure 3). The products of lipid peroxidation are complex molecules such as lipid aldehydes: 4-hydroxynonenal (4-HNE), and malondialdehyde (MDA) (Young and

McEneny, 2001). MDA is wildly used as an indicator of lipid peroxidation by measuring with the TBARS assay. Aldehydes are biologically active, for examples; they directly damage cells by interaction with cellular macromolecules, inhibit various enzyme reactions, and rapidly interact with thiols such as cysteine and GSH. Previous study showed that HNE mediated apoptosis by activation of SAPK/JNK in several cell lines through the redox state-sensitive MAPK kinase cascade (Uchida *et al.*, 1999), and in PC12 cells through apoptosis signal-regulating kinase 1-stress-activated protein kinase kinase-JNK (Ask1-SEK1-JNK) pathway followed by activation of caspase 3 (Soh *et al.*, 2000). This phenomenon could be prevented by NAC (Ramachandran *et al.*, 2003). 4-HNE induced JNK activation and promoted its translocation in the nucleus where JNK-dependent phosphorylation of c-Jun and the transcription factor activator protein (AP-1) binding take piace, leading to the transcription of a number of genes having AP-1 consensus sequences in their promoter regions (Yang *et al.*, 2003).

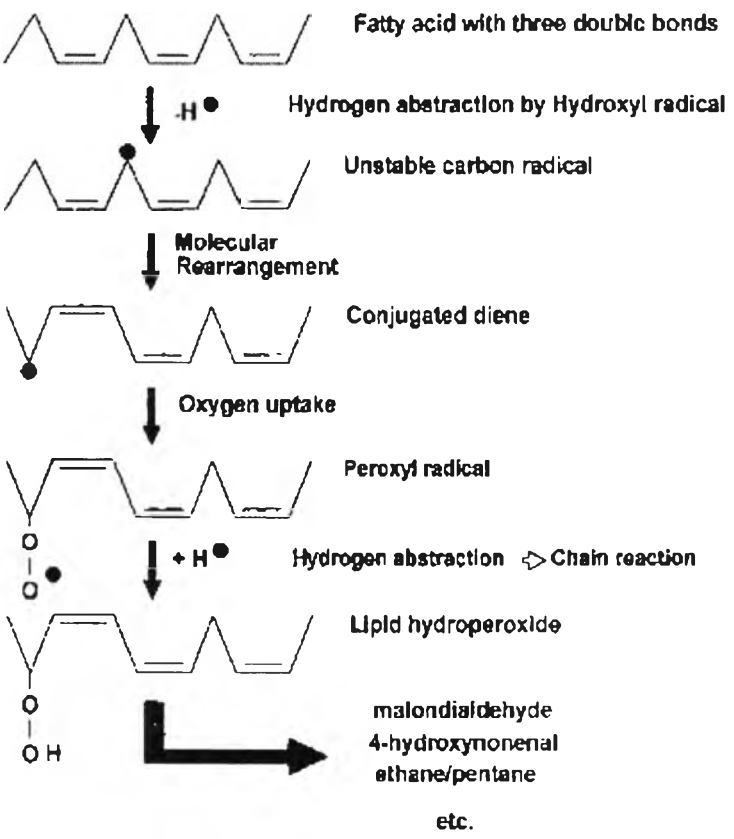

Figure 3 Basic reaction sequence of lipid peroxidation (Young and McEneny, 2001).

Mitogen-activated protein kinases (MAPKs)

Mitogen-activated protein kinases have been shown to be important intermediate signal transducer of extracellular signals to intracellular responses. Three MAPKs subfamilies have been clearly identified and widely studies: (i) ERK1/2, also known as p44/p42 MAPK; (ii) p38 kinase, and (iii) p54 and p46 stress-activated protein kinase (SAPK), also referred to JNK1 and JNK2. ERK1/2 is generally associated with proliferation and growth. In contrast, JNK and p38 MAPKs induced by stress responses and cytokines mediate differentiation and cell death. MAPK pathways relay, amplify and integrate signals from diverse stimuli and elicit an appropriate physiological response depending on the cell types, activating signals and cellular responses as shown in Figure 4 (Ichijo, 1999; Yang *et al.*, 2000).

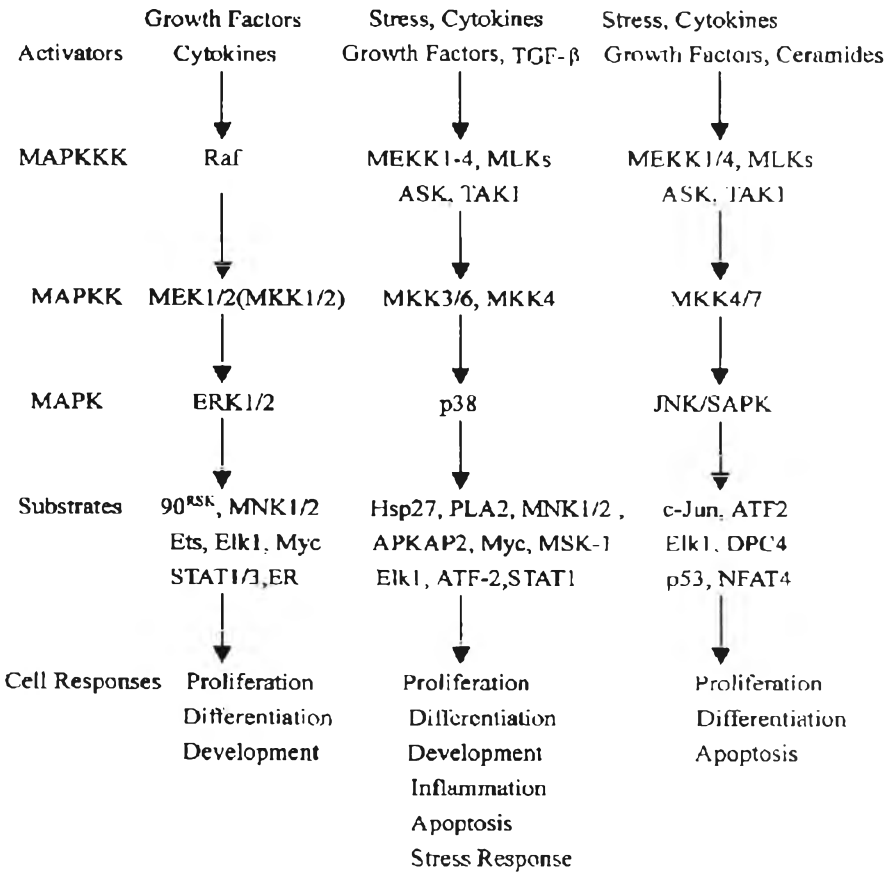

Figure 4 Major MAP kinase cascades in mammalian cells. The ERK, JNK, and p38 signaling pathways regulated by MEKK family members are illustrated (Zhang and Liu, 2002).

The role of MAPKs in the induction of apoptosis is controversial. While SAPK/JNK and/or p38 MAPK activations were required for the induction of apoptosis in some experimental systems (Assefa *et al.*, 2000; De Zutter and Davis, 2001; Sarker *et al.*, 2003), ultraviolet B radiation in HeLa cells showed that JNK1 and p38 MAPKs played an important role in protection of apoptosis in hypericin–induced apoptosis (Assefa *et al.*, 1999). Many lines of evidences have been reported that trichothecenes induced apoptosis via transduction of MAPKs pathway. Type A trichothecenes, T-2 toxin, has been shown to induced apoptosis in thymocyte and human leukemia HL-60 cells via independence of the Fas/Fas ligand pathway (Murshedul *et al.*, 2000), and via activation of caspase 9, caspase 3, but not caspase 8, including poly-ADP-ribose-polymerase (PARP), and DNA fragmentation factor-40/caspase-activated DNase (DFF-40/CAD) (Nagase *et al.*, 2001; 2002). Oral administration of T-2 toxin and deoxynivalenol (DON) in rats increased lipid peroxidation in liver (Suneja *et al.*, 1989) with consequent decrease of GSH content which was protected by selenium, vitamin E, and ascorbic acid (Rizzo *et al.*, 1994). Type B trichothecenes, vomitoxin, mediated immunotoxic effect by the induction of cyclooxygenase-2 gene expression leading to enhance production of PGE_2 and phosphorylation of ERK1/2 and p38 MAPK (Moon and Pestka, 2002). Vomitoxin plus TNFα, and AETD potently induced apoptosis in human promyelocytic HL-60 cells and thymus leukocyte, respectively, which were protected by NAC (Pae *et al.*, 2003; Uzarski *et al.*, 2003). Macrocyclic trichothecenes including satratoxins and other trichothecenes have been shown to induce apoptosis by activation of JNK/p38 kinases in the Jurkat human T-lymphoid cell line (Shifrin and Anderson, 1999), ERK in RAW 264.7 murine macrophage and U937 human leukemic cells (Yang *et al.*, 2000) and caspase 3 in HL-60 cells (Pae *et al.*, 2003). Satratoxin G-treated human leukemia HL-60 cells showed apoptosis through activation of caspase 8, caspase 3 and DFF-40/CAD and subsequent accumulation of cytochrome *c* (Nagase *et al.*, 2002). Previous studies demonstrated that low concentration of macrocyclic trichothecenes including satratoxins stimulated the production of TNF-α in RAW 264.7 murine macrophage (Chung *et al.*, 2003), and superinduced IL-2 production in EL-4 thymoma cells (Lee and Li, 1999) whereas higher concentrations of these toxins were markedly cytotoxic and concurrently reduced cytokine production. It has also been found that vomitoxin has been more potent in immune system than satratoxins

because it upregulated a complementary 3a receptor, a proinflammatory mediator, and macrophage inflammatory protein-2 (MIP-2), a potent neutrophil chemoattractant tissue injury (Lee and Li, 1999; Chung *et al.*, 2003), meanwhile satratoxins stimulated only MIP-2.

The stimulation of JNK and p38 kinase has been shown to be prerequisited for stress-induced apoptosis (Verheij *et al.*, 1996; Xia *et al.*, 1995). It has been shown that ROS generation caused apoptotic cell death through activation of p38 MAPK and/or JNK pathway in many cell lines, such as neuroblastoma (Osone *et al.*, 2004), murine macrophages (Kim and Sharma, 2004), UISO-Mel-1 human melanoma cells (Tan *et al.*, 2003) as well as PC12 cells (Seo *et al.*, 2001; Shin *et al.*, 2004). The change in the cellular redox state may play an important role for oxidant-induced activation of these stress pathways. Although oxidative stress causes activation of the apoptosis signal-regulating kinase1 complex (ASK1) by dissociation of the redox regulatory protein thioredoxin (Trx)-ASK1 complex (Saitoh *et al.*, 1998), ROS and/or p38 MAPK and/or JNK play a pivotal role in immune diseases as well as in trichothecene-induced apoptosis (Pae *et al.*, 2003; Yang *et al.*, 2000). The lines of evidences showed that free radicals could activate JNK and p38 kinase (Zang *et al.*, 2003) following by cytochrome *c* release from mitochondria and caspase activation (Junn and Mouradian, 2001). While caspase, an indicator of apoptosis, is well-known to be the point of no return, MAPKs are causing cell death. Therefore, acting on upstream signaling pathway involving ROS and MAPKs would be more effective in protection on satratoxin H-treated cells.

Apoptosis

The generation of reactive oxygen species can induce cell death either by apoptosis or necrosis depend on exposure time and cell type. Necrotic cell death, an unprogrammed or accidental cell death, is the consequence of acute disruption of cellular metabolism, leading to ATP depletion, ion dysregulation characterized by the loss of plasma membrane integrity, mitochondrial and cellular swelling, plasma membrane failure and cell lysis, and inflammation due to activation of macrophages and other white blood cells of the immune system. In contrast, apoptosis (a form of programmed cell death) requires the expression of specific genes taking several hours or more than a day to develop fully. Apoptosis is physiologically important process

in growth and development as well as in the removal of cells after being exposure to certain stress factors such as reactive oxygen species, ultraviolet, or toxins; and is tightly controlled by the cellular genetic apparatus. Now, it is well established that apoptosis is biochemically characterized by maintenance of membrane integrity until very late in the death process, plasma and nuclear membrane blebbing, cell shrinkage, intact lysosomal content, chromatin margination and condensation, internucleosomal DNA fragmentation, and cell disintegration forming of membrane-enclosed structure termed "apoptotic bodies" (Ashe and Berry, 2003; Kinloch *et al.*, 1999; Lemasters *et al.*, 1998) as shown in Figure 5. These fragments are eventually engulfed by macrophages or by phagocytosing adjacent cells which can be observed *in vivo*, in contrast *in vitro* systems where phagocytosis did not occur are consequently swelling and lysis, known as secondary necrosis (Padanilam, 2003). The key difference of the two types of cell death is the energy. While the apoptotic process is energy-dependent for synthesis of new proteins, the necrosis has no energy supplied and protein synthesis is halted (Kinloch *et al.*, 1999).

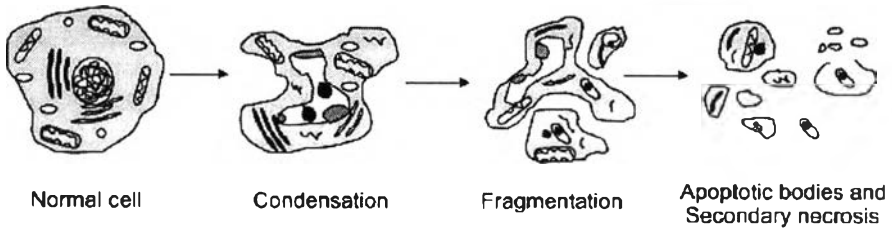

Figure 5 Hallmarks of the apoptotic cell death process. The apoptosis are characterized by cellular shrinking, condensation, forming bleb, chromatin condensation, and DNA fragmentation following by formation of membrane-bound apoptotic bodies containing organelles; and engulfed by phagocytosis without triggering inflammatory process. *In vitro* system, apoptotic bodies are consequently swelling and lysis, known as secondary necrosis (Padanilam, 2003).

Apoptotic cell death has been reported to involve with several stimulators such as receptor activation (Jung and Kim, 2004); growth factor deprivation in sympathetic neuron, PC12 cells and rat cortical neurons (Deckwerth and Johnson Jr, 1993; Satoh *et al.*, 1996); DNA damage (Huang *et al.*, 2001); and oxidative stress in many cell systems, such as human leukemia HL-60 cells (Pae *et al.*, 2003), primary human chondrocytes (Lo and Kim, 2004), neuronally differentiated mouse embryonal

carcinoma P19 cells (Ishihara *et al.*, 2000), and PC12 cells (Jiang *et al.*, 2004). Oxidative stress has been implicated as possible signaling molecules in apoptotic cell death through JNK, p38 kinase, and caspase cascade (Kim *et al.*, 2003, Lin *et al.*, 2003). Mitochondria have a critical role in mediating the apoptotic signal transduction pathway involving a family of specific cysteine proteases that cleave protein substrates after aspartic acids, known as the caspases. In the present of ATP, the release of cytochrome c triggers the interaction between the initiating apoptosis protease activating factor 1 (Apaf-1), a mammalian CED-4 homologue; and pro-caspase-9, which in turn results in the conversion of pro-caspase-9 to activated caspase-9. Caspase-9 cleaves and activates other caspases such as caspase-3. The target proteins of caspases include poly(ADP-ribose) polymerase, Bcl-2 family member, and proteases. In addition, caspase-3 has been shown to activate endonuclease activity which caused chromatin fragmentation and then formed apoptotic bodies. Also, caspase 8 up-regulated pro-apoptotic protein Bax, and down-regulated anti-apoptotic protein Bcl-2 (Lemasters *et al.*, 1998; Luo *et al.*, 1998).