CHAPTER V
DISCUSSION

The *in vitro* results showed that methomyl could reduce mitochondrial transmembrane potential ($\Delta\Psi$) and induce apoptosis in MM6, THP-1, and Jurkat cell lines at different concentrations. The decrease in $\Delta\Psi$ indicated the PT pore generation, which allowed the mitochondria to release caspase activating proteins to activate caspases and induce apoptosis. The results showed that apoptosis induced by methomyl is a caspase dependent process as indicated by using benzyloxycarbonyl-Val-Ala-Asp(Ome)-fluoromethylketone (zVAD-fmk) and immunoblotting assay. The zVAD-fmk, a pan-caspase inhibitor, could block apoptosis induced by methomyl, and the immunoblot also confirmed the caspase activation by showing the enzyme cleavage in the cells exposed to the apoptotic dose of methomyl. Therefore, the possible mechanism of methomyl-induced apoptosis proposed in this study is that methomyl reduces the $\Delta\Psi$, leading to the opening of PT pore which in turn may lead to cytochrome *c* release, caspase activation, and apoptosis. However, time-related study may need to be conducted to clarify the proposed mechanism.

The mechanisms as to how methomyl reduces $\Delta\Psi$ leading to apoptosis are not known. There are many possibilities:

(i) ROSs generation: Methomyl can deplete an important free radical scavenger, glutathione (IPCS, 1996), therefore ROSs are generated. It is well-established that ROSs have two roles in the apoptotic process, the inducers of PT pore and the consequences of PT pore (Jabs, 1999).

(ii) Genotoxicity: The capability of methomyl to cause damages in DNA via ROSs was shown in a previous study (Bonatti et al., 1994). The p53 protein is a key sensor of DNA damage. Once the p53 is activated, it induces cell-cycle inhibition and apoptosis (Bratton and Cohen, 2001)

(iii) Role of methomyl as a sulfhydryl group-binding agent may explain the observed effects on mitochondria and apoptosis. The modification of a critical cysteine residue at adenine nucleotide translocator (ANT), an important protein that controls the opening of PT pore, by sulfhydryl reacting agents can lead to the $\Delta\Psi$ collapse and apoptosis (Belzacq et al., 2001; Moreno et al., 2001). Therefore, ANT modification can be affected by methomyl and may be the cause of apoptosis seen.

(iv) From the studies in male sterile maize, methomyl may interfere with the activity of mitochondrial aldehyde dehydrogenase (mtALDH) enzyme, whose activity is required to inhibit mitochondrial PT pore generation (Irwin et al., 2002). In addition, the enzyme is important in the control of NADH, which is the key to cell death protection, within the cells. The interference with the enzyme will cause the NADH depletion and cell death. Therefore, it is possible that methomyl might induce PT pore generation and cell death by the mechanism concerned with energy metabolism.

The result also demonstrated that apoptosis in the cell lines induced by methomyl was related to interleukin-6 (IL-6) since IL-6 could partly block the apoptosis observed.

There are various pathways involved in the IL-6-regulation of cell growth, survival, and differentiation: JAK/STAT pathway; Ras/MAPK; Src family tyrosine kinases; and phosphatidylinositol-3-kinase (PI-3 kinase) pathway. However, how IL-6 is related to the apoptosis seen is still unclear, and there must be other pathways related to methomyl-induced apoptosis.

It was found that methomyl could not induce apoptosis in Raji cells and the DNA fragmentation was not detected. Instead, the Raji cells showed a cell cycle arrest in the G_0/G_1 phase. Various mechanisms have been proposed to cause arrest in the G_0/G_1 phase as follows:

(i) The inhibition of cyclin E-CDK2 complex, whose activity is required at the G_1-S transition. The inhibitors of the cyclin E-CDK2 complex include p21 and p27 (Karp, 1999; Normal and Lodwick, 1999; Stuart et al., 2000)

(ii) Inhibition of JAK/STAT 3 signaling pathway. This pathway, regulated by IL-6, is important in the step of driving cells from the G_1 to S phase (Taga et al., 1989; Fukada et al., 1996; Urashima et al., 1997; Xu et al., 1998; and Chauhan et al., 2000).

From the results, the sensitivity of each cell type to methomyl was different. Raji did not show apoptosis, whereas others did. It has been long known that resistance to apoptosis among cancer cells has been established as an important mechanism of the continuous cell growth. In the mitochondria, IF_1 inhibitor protein of the F_1F_0-ATPase plays an important role to conserve ATP in the cancer cells in the anoxic necrotic center (Green and Grover, 2000). In addition, the different ability of

each cell type to initiate apoptosis after DNA damage depends on a number of factors: the ability of that cell to 'sense' DNA damage, the way in which the DNA damage sensor(s) are coupled to the pathways initiating apoptosis (or alternative responses such as cell cycle checkpoints and repair) and the difference in survival signals acting upon that cell (Yarnold, 1997). For Raji cells, although they can be induced apoptosis by some chemicals (Voorzanger-Rousselot et al., 1998; Kurita-Ochiai, Ochiai, and Fukushima, 1998), it is reported that growth inhibition in Raji cells can be uncoupled from apoptosis, or signal transduction correlated with growth inhibition in Raji cells is an insufficient link to apoptosis (Kaptein et al., 1996).

The G_0/G_1 phase arrest seen in Raji cells could imply that serine/threonine protein kinases (CDKs) and CDK inhibitors possibly have roles in methomyl-induced the inhibition of cell proliferation and apoptosis.

The downregulation of IL-6 or the inhibition of IL-6 related pathways in Raji cells could be suggested as a pathway affected by methomyl since IL-6 is an important cytokine used in the step of driving cells from the G_1 to the S phase through signaling via JAK/STAT 3 pathway. Role of methomyl on the inhibition of IL-6-related pathways is shown in other cell lines tested (MM6, THP-1, and Jurkat).

Due to presenting the cell cycle arrest, Raji might be an important cell line useful for further studies related to mechanism of growth inhibition and apoptosis induced by methomyl exposure.

For the acetonitrile exposure, MM6, THP-1, and Jurkat cells showed a reduction of $\Delta\Psi$ detected by TMRE, and mostly necrotic cell death with some apoptosis were detected by Annexin V-FITC. However, the DNA fragmentation shown in the sub-G_1 area was detected increased with a low percentage. Whereas in Raji cells, a reduction of $\Delta\Psi$ detected by TMRE and necrotic cell death detected by annexin V-FITC were shown without DNA fragmentation.

The result could be explained by the action of acetonitrile, which is similar to cyanide, as an inhibitor of the cytochrome c oxidase, resulting in a block of oxidative phosphorylation and a subsequent insufficient utilization of oxygen (Villani and Attardi, 2000; Jensen et al., 2002). Although the type of cell death induced by cyanide is still controversially discussed, this study demonstrated that it could induce both necrosis and apoptosis, however with higher percentage of necrosis. The necrosis observed was

probably induced by the ATP depletion caused by the disruption at the electron transport chain. However, apoptosis could still be induced from the hypoxic state, which could lead to the cytochrome *c* release (Shen and White, 2001; Lee et al., 2002; Liu et al., 2002). A loss of cytochrome *c*, combined with a low cytochrome *c* oxidase capacity in cells are implications for the execution of apoptotic cell death (Villani and Attardi, 2000).

The *in vivo* results showed that rats orally exposed to single dose of methomyl at 8 mg/kg body weight for 6 hours had higher percentage of lymphocyte apoptosis compared to non-exposed littermates. The findings revealed that doses of methomyl used in the leukocytic cell lines were very high compared to those used *in vivo* to cause lymphocyte apoptosis. The more prominent *in vivo* effects of methomyl–even at lower doses–compared to the results from *in vitro* experiments can easily be explained by the pleiotropic effect that direct interactions of methomyl might have on a broad spectrum of cell types, including neurons and immune regulatory cells, that may add up to a much higher biological response *in vivo* than the rather isolated events occuring in a single immortalised cell line. These lines are known to have lost some of their repertoire to respond to death signaling with apoptosis induction.

The observation of sections of spleens collected from rats after 24 hour-oral exposure to methomyl at 8 mg/kg body weight under the light microscope revealed cell death. At the same dose of methomyl, the transmission electron microscope illustrated the the mitochondrial swelling and degenerative changes with cristae loss. These effects seen could be caused by the collapse of $\Delta\Psi$ and PT pore opening, a volume dysregulation of mitochondria due to the hyperosmolality of the matrix, the matrix space expansion, swelling, and rupture of the outer mitochondrial membrane (Green and Reed, 1998; Bratton and Cohen, 2001). These mitochondrial effects supported the hypothesis that methomyl could interfere with mitochondrial function leading to an interference with energy metabolism and consequently apoptotic cell death.

The mitochondrial effect was also substantiated by the increasing levels of 2,3-DPG in blood, which are an indicator of the amount of oxidized NADH; and the increase in red blood cell NADH-DCIP reductase activity, which indicated

methemoglobinemia and oxidative stress. These indicators refer to changes in energy metabolism and oxidative stress related to mitochondrial function.

The interesting aspect of the effect of methomyl on either IL-6 or its related signaling pathways may lead to further investigation of its role in spleen. Since *stat3* gene activation induced by IL-6 is an important antiapoptotic signal in T cells of the spleen, especially in the red pulp region (Narimatsu et al., 2001), the effect of methomyl on IL-6 related pathways in spleen should be studied. In addition, action of methomyl on the neuropeptide somatostatin, whose receptors are largely found in the red pulp region (Reubi et al., 1998) should be also studied. Since somatostatin has an inhibitory effect on IL-6 release (Reubi et al., 1998) and may share signaling pathways with angiotensin II–a compound inducing apoptosis in ventricular myocytes (Tone et al., 1998; Hajdu et al., 2000), studies on signaling pathways of these two compounds related to IL-6 may possibly lead to further explanation of the reported cardiotoxicity induced by methomyl which may not be related to acetylcholinesterase inhibition.

At present, it remains to be clarified how secondary effects mediated by the inhibitory activity of methomyl on cytokines such as IL- may contribute to apoptotic effects seen also in vivo. It might well involve a down regulation of the complement system by cytokine dependent effects on local complement biosynthesis. It is known- for example- that complement activation products such as C3b and C3dg exert a potent signal on the cell cycle of B lymphocytes and lack of these activation products can induce a lack of antibody responses due to the necessity of CR-2 signaling to complete the S1 phase of deviding B lymphoblasts. These aspects should be focused in the future work.