CHAPTER 4
DISCUSSION AND CONCLUSION

Chemoprevention by edible phytochemicals is now considered to be an inexpensive, readily applicable, acceptable and accessible approach to cancer control and management. With increasing molecular mechanistic evidences coupled with considerations of quality, safety, and efficacy, phytochemicals from dietary and medicinal plants have emerged as very promising sources of potential anticancer agents and new chemotherapy adjuvants (318, 319).

Several studies indicate that dietary factors play an important role in the prevention of several types of cancer, including lung cancer, inflammatory bowel disease and colorectal cancer (22, 24, 49, 320-323). Rice is the most important staple food for a large part of the world's human population, especially in Asia and the West Indies. Although, there are many species and varieties of rice, but most varieties come from the most common rice species *Oryza sativa*, which cultivates in Asia. *Oryza sativa* contains two major subspecies, including *japonica* and *indica*. *Japonica* is a sticky and short grained variety, which usually cultivated in dry fields, in temperate East Asia, upland areas of Southeast Asia and high elevations in South Asia, while *Indica* is a nonsticky and long-grained variety, which cultivated mainly in Lowland, grown mostly submerged, throughout tropical Asia. Several studies have shown that rice contains various constituents that exhibited the chemopreventive and anti-cancer effects (289, 324-326).

Phase 1: Fermented brown rice and rice bran (FBRA), a rice product, prevented the nicotine-derived nitrosamine ketone (NNK)-induced lung tumorigenesis in *in vivo* model

The data presented here demonstrate that the administration of 10% FBRA in the diet during both initiation and post-initiation phase significantly suppressed the total number of lung proliferative lesions of NNK-treated A/J mice. The results also showed that the administration of 10% FBRA in the post-initiation phase significantly decreases the mean size of tumor. This inhibitory action is consistent with the previous reports about the chemopreventive effect of FBRA in the carcinogenesis of the colon, stomach, bladder and esophagus (50-52, 270).

NNK initially presents itself in the body as a procarcinogen, an inert form that requires activation to exert its full effects. There are three primary pathways responsible for NNK activation: i) carbonyl reduction, ii) pyridine N-oxidation and iii) α-hydroxylation (14, 327, 328). Cytochrome pigment 450 (CYP450) enzymes belonging to the CYP multigene family catalyze the α-hydroxylation of NNK in the oxidative metabolism pathway (14, 16, 18). It is interesting to note that mRNA expression levels of *Cyp2a5* in lung tissues were significantly decreased by the administration of FBRA. It is possible that the inhibitory effect on the *Cyp2a5* expression leads to a decreased production of the active form of NNKs such as α-hydroxyNNKs and α-hydroxyNNALs, and is associated with the suppressive effects of FBRA on the lung tumorigenesis at the initiation phase.

In general, hyperproliferation is suggested to be relevant to carcinogenesis of many organs (329-331). Indeed, carcinomas of the lung had the higher indices for Ki67 (332, 333). Therefore, the control of cell proliferation in the target organs is regarded as an important strategy for chemoprevention. The results showed that 10% FBRA administration at the post-initiation phase decreased the size of tumors. It is important that the number of Ki67 positive cells in lung lesions was significantly decreased by the post-initiation treatment of FBRA. Similar results have been obtained in other studies with various chemopreventive agents (334, 335) and previous study of FBRA (50). Decreasing the cell proliferative index of lung tumor cells might be one of the important chemopreventive effect of FBRA.

FBRA is a processed food prepared by fermenting brown rice and rice bran with *Aspergillus Oryzae*. The nutritional and sanitary advantage of fermentation has been recognized, although the details are not well known. Fermented soy bean paste or soy sauce, which prepared by fermenting with *Aspergillus Oryzae*, has been found to be more stable against lipid peroxidation than unfermented soybeans, because *Aspergillus Oryzae*-fermented soybean products contain several antioxidants like 6-hydroxydaidzein, 8-hydroxydaidzein and 8-hydroxygenistein more abundantly than unfermented soybeans (336). These 8-hydroxyisoflavones are reported to possess greater 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging activity and antiproliferative activity than the corresponding isoflavone analogs (336, 337). Furthermore, these 8-hydroxyisoflavones and 6-hydroxydaidzein showed high antimutagenic activity (337, 338). Several epidemiological and preclinical studies suggested the chemopreventive effects of such fermented soybean products (339, 340). Dihydroferulic acid and dihydrosinapic acid, which were isolated from unpolished rice vinegar (Kurosu), were suggested as the major constituents responsible for radical scavenging activity of Kurosu (341). These acids are produced in Kurosu through the process of the fermentation from ferulic acid and sinapic acid. Rice bran contains approximately 20% oil which contains several bioactive polyphenols including ferulic acid, protocatechuic acid, sinapic acid, and vanillic acid (342). Thus, it is possible that FBRA has similar mechanistic aspects to the fermented soybeans or Kurosu.

Phase 2: Gamma-tocotrienol (γ-T_3) but not phytic acid (IP_6) suppressed the NNK-induced lung tumorigenesis in *in vitro* model, and induced apoptosis in lung cancer cell line A549

The results in this study showed that treatment with 10μmol/L of NNK significantly induced the cell viability at both 48 and 72 hours and further significantly induced the cell viability at 24 hours in the human alveolar epithelial adenocarcinoma A549 cells. Moreover, this study suggested that NNK may induce the cell proliferation through increasing transduction of MAPK signal. The results showed that NNK treatment increased level of MAPK signal-mediated proteins, such as K-ras and phosphorylated-MEK1/2 (p-MEK1/2). Furthermore, overexpression of the cell cycle regulatory proteins, such as cyclinD1 and cyclinE, and the S phase

marker protein, such as proliferating cell nuclear antigen (PCNA), were appeared due to NNK treatment. Pre-treatment with γ-tocotrienol but not phytic acid for 12 hours before the treatment with 10μmol/L of NNK significantly inhibited these NNK-induced the cell viability and proliferation in A549 cells. The IC50 values for the cell viability were 43.8±0.2 and 37.0±0.1 μmol/L at 24 and 48 hours, respectively. Furthermore, the IC50 for the cell proliferation was 43.2±0.2 μmol/L at 24 hours. The results in this study suggested that pre-treatment with γ-tocotrienol inhibited the NNK-induced cell cycle progression through blockage of p-MEK1/2 but not K-Ras, which may led to decreasing transduction of MAPK signal, and inhibited the NNK-induced overexpression of cyclinD1, cyclinE and PCNA proteins. In addition, NNK treatment also induced the overexpression of NNK-activating proteins, such as cytochrome P450 isotype 2A6 (CYP2A6) and cytochrome P450 isotype 2A13 (CYP2A13), and also induced the nuclear accumulation of DNA methyl transferase1 (DNMT1), which involved in the hypermethylation at the promoter sequence of tumor suppressor genes and result in their silencing. Pre-treatment with γ-tocotrienol prevented the NNK-induced overexpression of CYP2A6 and CYP2A13 proteins, and the NNK-induced nuclear accumulation of DNMT1 protein. Interestingly, this study found that the relative mRNA expression of *CYP2A6* and *CYP2A13* genes were not significantly changed after NNK treatment, which suggested that NNK had no effect on the transcription of *CYP2A6* and *CYP2A13* genes. Recently, a study in 2010 has been shown that NNK increased expression and activity of DNMT1 protein by reducing ubiquitination of DNMT1 protein, which prolonged the stabilization of DNMT1 protein, but did not affect the mRNA expression level of this protein (98). Therefore, NNK induced the overexpression of CYP2A6 and CYP2A13 proteins may be the effect of NNK on the stabilization of these proteins. Finally, this study showed that NNK treatment induced DNA damage in A549 cells and pre-treatment of γ-tocotrienol had no effect on the NNK-induced DNA damage. These results indicated that the chemopreventive effect of γ-tocotrienol did not involve in DNA adduction or DNA repairing.

Together, this study suggested that γ-tocotrienol but not phytic acid has the anti-cancer activity against lung cancer progression. Treatment with γ-tocotrienol also significantly inhibited both the cell viability and proliferation of A549 cells

(NNK-untreated cells). Treatment of γ-tocotrienol in A549 cells induced G0/G1 cell cycle arrest due to decreasing expression of cyclinD1 protein, and further induced apoptosis. It is well known that many chemopreventive agents induce apoptosis in cancer cells (75, 343-346). This study showed that γ-tocotrienol treatment induced the cleavage of poly (ADP-ribose) polymerase (PARP), the activation of caspase-3 (decreasing expression of pro-caspase-3). This study further showed that the morphology of γ-tocotrienol-treated A549 cells were changed and cell blebing, a sign of apoptosis, was appeared. Finally, this study found that γ-tocotrienol treatment induced apoptosis in A549 cells via both intrinsic and extrinsic pathways. Treatment with γ-tocotrienol decreased the expression of pro-caspase-8 and pro-caspase-9, which involved in the activation of these caspases. Treatment with γ-tocotrienol increased expression of pro-apoptotic protein, bax, but decreased expression of anti-apoptotic protein, bcl-xL. These results suggested that γ-tocotrienol also induced apoptosis in A549 cells by increasing bax/bcl-xL ratio (347, 348). It is well known that the activation of caspase-8 can cleave the BH3-interacting domain death agonist (Bid), a pro-apoptotic member of the B-cell CLL/lymphoma 2 (Bcl-2) family. This cleaved or truncated Bid (tBid) engages the intrinsic apoptotic pathway by binding itself to the Bcl-2-associated X protein (BAX) and Bcl-2 homologous antagonist killer (BAK), resulting in their oligomerization and translocation to the mitochondrial outer membrane. BAX and BAK oligomers then promote a decrease in the mitochondrial membrane potential and subsequent/concomitant formation of pores leading to the outer membrane permeabilization (348). Therefore, an increase of the bax and caspase-9 activation may result from the activation of caspase-8. To prove that the γ-tocotrienol directly induced the intrinsic pathway of apoptosis, further studies with the caspase-8 inhibitor, for example Z-IETD-FMK, should be conducted. Furthermore, γ-tocotrienol has been reported for its ability to induce apoptosis in other cancers, such as gastric cancer (279), T cell lymphoma (349), and in leukemic cells (350). The sensitivity for γ-tocotrienol- induced apoptosis is different among these types of cancer. From our results, lung cancer is more sensitive than gastric cancer but less sensitive than T cell lymphoma, and leukemic cells.

This study founded that all Thai rice samples contained γ-tocotrienol, a subgroup of vitamin E that exhibits chemopreventive activities (351-354). However,

its content varied widely between lines and cultivated areas. Many factors such as temperature, soil-quality and rice varieties, may influence the γ-tocotrienol level in rice (295). Moreover, the contents of γ-tocotrienol in Thai rice are relatively high compared with other rice (295, 355). Therefore, this study highlighted the new economic properties of Thai rice for the chemoprevention.

Phase 3: Fermented brown rice and rice bran (FBRA) prevented the inflammation-related colorectal tumorigenesis in *in vivo* model

A number of reports have shown the chemopreventive effect of FBRA on several types of cancer, including colon cancer (49-52). In contrast to the findings that FBRA significantly suppressed azoxymethane-induced colorectal carcinogenesis in rats, in the present study, FBRA has no effect on the tumor development of the colon in *Apc*$^{Min/+}$ mice, a heredetary carcinogenesis model of the colon. These results indicated that the suppressive effects of FBRA on colorectal carcinogenesis were not involved in the Wnt signaling-induced colorectal tumorigenesis. It was noteworthy that the administration of FBRA significantly suppressed the total number of colonic tumors in DSS-treated *Apc*$^{Min/+}$ mice. The results clearly indicated that FBRA specifically inhibits inflammatory-related colorectal carcinogenesis in *Apc*$^{Min/+}$ mice. The study results supported the study in 2008 in which FBRA suppresses DSS-induced inflammation in the colon (356). Therefore, the mode of chemopreventive action of FBRA may be attributable to be the anti-inflammatory activity

Generally, hyperproliferation is suggested to be relevant to carcinogenesis of many organs (329-331). Therefore, the control of cell proliferation in the target organs is regarded as an important strategy for chemoprevention. Indeed, a number of compounds consisting FBRA revealed chemopreventive actions that are accompanied by the decreased proliferative activities in the target organs. It was important to note that the administration of FBRA significantly decreased the Ki67 positive cell index in colonic crypts of DSS-treated mice, whereas such effects did not observed in colonic crypts of non-treated mice. Thus, the suppressive effect on cellular proliferation in tumor cells might be one of the important mechanisms of the chemopreventive effects of FBRA.

Nitric oxide (NO) is involved in many of the pathophysiological processes that lead to colon cancer development and progression. The role of NO in carcinogenesis

is not well defined and appears to be complex due to divergent functional activities under normal and pathophysiological conditions (357-359). It is probable that the high sustained levels of NO generated by iNos, the inducible and Ca^{2+}-independent isoform of Nos, can produce multiple types of damage and lead to an accumulation of gene mutations that contribute to malignant transformation (359, 360). Evidence from both *in vitro* and *in vivo* experiments support that NO and its reactive metabolite peroxynitrite stimulate *Cox2* activity leading generation of tumor growth enhancing prostaglandins and influence colon tumorigenesis (361-363). This study founded that the relative mRNA expression of *iNos*, *Cox2* and *Tnfα* were strongly increased in the colonic tissues of mice treated with DSS in comparison with the untreated mice, suggesting that these genes are involved in the DSS-induced inflammation. It was important that the administration of 10%FBRA significantly decreased the relative mRNA expression of both *iNos* and *Cox2* but not *Tnfα* genes in the colon tissues of DSS-treated mice.

Phase 4: Phytic acid but not γ-tocotrienol inhibited the inflammation-related colon tumorigenesis in *in vitro* model

This study founded that lipopolysaccharide (LPS) treatment significantly increased the number of migrated leukemic cells from upper compartment of Boyden chamber to culture medium of LPS-treated colon cancer cell line, SW480, in lower compartment in dose-dependent manner, which suggested that LPS induced secretion of chemoattractants from LPS-treated SW480. Pre-treatment with phytic acid but not γ-tocotrienol for 12 hours before LPS treatment significantly decreased the number of migrated cells, which suggested that pre-treatment with phytic acid but not γ-tocotrienol reduced the secretion of chemoattractants in LPS-treated SW480. Furthermore, pre-treatment with phytic acid clearly inhibited the overexpression of iNOS protein and the nuclear accumulation of NF-κB protein, which are the signature of inflammation, in LPS-treated SW480. These results indicated that phytic acid had anti-inflammatory effects against LPS-induced inflammation response in colonic cells. Additionally, pre-treatment with phytic acid also inhibited the overexpression of cyclinD1 protein, the gatekeeper protein for cell cycle transition from G1 to S phase, in LPS-treated SW480, which suggested that phytic acid may prevented the inflammation-induced colon tumorigenesis.

Phytic acid (also called phytate when in salt form) or inositol hexaphosphate (IP_6) is the principal storage form of phosphorus, polyphosphorylated carbohydrate, in many plant tissues. The IP_6 is found in substantial amounts in whole grains, cereals, legumes, nuts, and seeds, and is the primary energy source for the germinating plant (282, 283). The IP_6 and its lower phosphorylated forms are also found in most mammalian cells, where they assist in regulating a variety of important cellular functions (283, 284). Phytate is indigestible for humans or non-ruminant animals due to deficient in expression of phytase, the enzyme that neutralizes phytic acid and liberates the phosphorus. Brown rice is high in phytates, which represents about 1-2.5 mg/g in brown rice grains, but their contents in rice are upon the genetic and environmental variation (47). The IP_6 exhibits anti-cancer activities, anti-oxidant activities, and anti-inflammation activities (43, 45, 284-290). Phytic acid's chelating effect may serve to prevent, inhibit, or even cure some cancers by depriving those cells of the minerals, especially iron, which they need to reproduce (45). Phytic acid is also used as one of few chelating therapies for uranium removal (364). However, the strong binding affinity to important minerals of phytic acid can therefore contribute to mineral deficiencies in people (365).

In conclusion, this study demonstrated that the fermented brown rice and rice bran (FBRA), a rice product, had the chemopreventive effect against both NNK-induced lung tumorigenesis and inflammation-related colorectal tumorigenesis (due to DSS treatment) but not *Apc* mutation induced colorectal tumorigenesis in *in vivo* model. The chemopreventive effect of FBRA against NNK-induced lung tumorigenesis was involved with reducing mRNA expression of *Cyp2a5* gene and the cell proliferative index in lung tissues of female A/J mice. In case of inflammation-related colorectal tumorigenesis, the chemopreventive effect of FBRA was involved with reducing the cell proliferative index and mRNA expression of the inflammatory-related genes, including *Cox2* and *iNos* but not *Tnfα*, in colonic tissues of DSS-treated $Apc^{Min/+}$ mice. Furthermore, this study demonstrated that rice constituents, such as γ-tocotrienol and phytic acid, had the chemopreventive effects against NNK-induced lung cancer and inflammation-induced colon cancer (due to LPS treatment) in *in vitro* model. In case of NNK-induced lung cancer, pre-treatment with γ-tocotrienol but not phytic acid for 12 hours before NNK treatment significantly inhibited both NNK-

induced cell viability and proliferation in lung cancer cell line A549. These inhibitory effects were involved with inhibiting overexpression of p-MEK1/2 but not K-ras protein, the MAPK signal mediators, which resulted in preventing overexpression of the cell cycle regulatory proteins, such as cyclinD1, cyclinE and PCNA, in NNK-treated A549 cells. The NNK-induced overexpression of NNK-activating proteins, CYP2A6 and CYP2A13, and the NNK-induced nuclear accumulation of the tumor suppressor genes-silencing protein, DNMT1 were also suppressed by γ-tocotrienol pre-treatment. However, γ-tocotrienol pre-treatment had no effect on NNK-induced DNA damage. Moreover, γ-tocotrienol induced G0/G1 cell cycle arrest via decreasing expression of cyclinD1, and further induced apoptosis via both intrinsic and extrinsic pathways in A549 cells. In case of inflammation-induced colon cancer, pre-treatment with phytic acid but not γ-tocotrienol for 12 hours before LPS treatment significantly inhibited the secretion of chemoattractants from LPS-treated colon cancer cell line SW480. The LPS-induced nuclear accumulation of NF-κB protein and the LPS-induced overexpression of iNOS and cyclinD1 proteins were clearly suppressed by phytic acid pre-treatment.