CHAPTER IV

DISCUSSION AND CONCLUSIONS

Ginger, the rhizome of *Zingiber officinale*, one of the most widely used species of the ginger family. Various compounds in ginger such as gingerol, paradol and shogoal have been reported to have anti-cancer properties in a wide range of experimental models. For example, 6-gingerol suppresses colon cancer growth by targeting leukotriene A4 hydrolase [74]; stimulates apoptosis through up-regulation of NSAID-activated gene-1 (NAG-1) and cell cycle arrest by down-regulation of cyclin D1 in human colorectal cancer cells [78]; inhibits cell adhesion, invasion, and motility as well as the activities of MMP-2 and MMP-9 in MDA-MB-231 human breast cancer cell lines [79]. 6-Shogaol induces autophagy by inhibiting the AKT/mTOR pathway in A549 human lung cancer cells [80]; inhibits proliferation of the transgenic mouse ovarian cancer cell lines [81]; and induces apoptosis in human colorectal carcinoma cells via ROS production, caspase activation, and GADD 153 expression [82]. 6-Paradol decreases the incidence and the multiplicity of skin tumors initiated by 7,12-dimethylbenz anthracene (DMBA) and promoted by 12-O-tetradeca-noylphorbol-13-acetate (TPA) [83]; exerts inhibitory effects on the viability and DNA synthesis of human promyelocytic leukemia (HL-60) cells [84]; and induces apoptosis in an oral squamous carcinoma cell line [85].

Our previous report showed that the ethyl acetate fraction of ginger inhibited hTERT and c-Myc mRNAs expression in A549 lung carcinoma cells [86]. In the present study, we attempted to identify the active compound from this fraction.

We further purified this fraction by column chromatography and obtained another 4 fractions: E1-E4. The E2 fraction was found to be the most active and we further fractionated by column chromatography into 3 more fractions: E2.1-E2.3, as shown in Figure 2.1. We also fractionated the E2.2 into 3 more subfractions: E2.2.1, E2.2.2, and E2.2.3. We then tested these 4 subfractions (E2.1, E2.2.1, E2.2.2 and E2.2.3; except E2.3 fraction due to this fraction is highly toxic to cells) for their activities in suppressing hTERT and c-Myc expression. Each extract was tested for its cytoxicity by Sulforhodamine B colorimetric assay, using A549 human lung cancer cells. The IC_{50} values of each fraction were shown in Table 3.1. The effect of these *Z. officinale* extracts to modulate the *hTERT* and *c-Myc* expression using semi-quantitative and real-time quantitative RT-PCR showed that most of these fractions down-regulated *hTERT* and *c-Myc* expression. The fold change in down-regulation of the *hTERT* mRNAs expression by E2.1, E2.2.1, E2.2.2, and E2.2.3 at 32 µg/ml was 0.7 ± 0.17, 0.68 ± 0.13, 0.5 ± 0.25, and 0.5 ± 0.14, respectively; while the fold change in down-regulation of c-Myc mRNAs expression was 0.5 ± 0.08, 0.45 ± 0.16, 0.3 ± 0.07 and 0.4 ± 0.3, respectively. We confirmed that the down-regulation of *c-Myc* mRNAs expression by each subfraction extracts led to the reduction of c-Myc at the protein level expression, using western blotting analysis. The results show that the c-Myc protein was significantly reduced after treatment with E2.1 and E2.2.1 in a dose dependent manner, while E2.2.2 and E2.2.3 had a little effect on the

c-Myc protein expression in A549 treated cells. In addition, telomerase activity in the cells treated with the each subfraction was reduced in a concentration-dependent manner. These telomerase inhibition is likely arisen from the down-regulation of hTERT, not from the direct telomerase inhibition because these extracts had no effect on telomerase activity in a cell-free system.

In an attempt to identify the active compound that is responsible for the down-regulation of *hTERT* and *c-Myc* expression that led to a reduction in telomerase activity, we employed TLC, HPLC, and GC/MS to examine the composition of the *Z. officinale* extract in each fraction. The TLC fingerprints and HPLC chromatograms are useful as a proof in the purification step and as a reference for the quality control. The HPLC analysis found that E1 and E2 fractions contain less than 1% of 6-gingerol, while E3 and E4 fractions contain about 30% and 40% of 6-gingerol, respectively. Since the E2 fraction was found to be active, while E3 and E4 were not. We ruled out that 6-gingerol is not the active compound in the down-regulation of *hTERT* in A549 cells. The result from the commercial available 6-gingerol (Sigma) also confirmed that it is not the active compound. In order to identify compounds in E2 subfractions, we identified the major compounds in each subfraction using GC-MS. We found that paradols, in various forms, were found as the major chemicals in these subfractions. We wonder whether these paradols could be responsible for the down-regulation of *hTERT* and *c-Myc* gene in A549 cells. Therefore, we tested the effect of 6-paradol (Because of the material we have, and detected in the E2 subfractions) on *hTERT* and *c-Myc* mRNAs expression in A549 cells. The results showed that 6-paradol could significantly reduce *hTERT* and *c-Myc* mRNA expression in a

dose-dependent manner. Therefore, it can be concluded that the compounds that are responsible for the down-regulation of *hTERT* and *c-Myc* expression is paradols.

The observation that telomerase is active in cancer cells but not in normal somatic cells has generated a great deal of interest in the development of telomerase inhibitors for use as anticancer agents. Telomerase inhibition is probably most useful in chemoprevention, the early stages of carcinogenesis, or maintenance therapy after treatment by cytotoxic chemotherapy due to its specificity and delayed response in cancer cells. Recent studies suggest that cancer stem cells already possess some telomerase activity; therefore, rather than telomerase reactivation, enzyme activity might increase in the terminal stages of carcinogenesis due to an increased expression or efficient assembly of telomerase components [87]. Since the ginger extract used in this study down-regulates *hTERT* and reduces telomerase activity, it should prove useful in cancer prevention and maintenance therapy.

There are at least 430 genes that have been found to possess a Myc-bound promoter; 13% of these genes are related to protein synthesis, and 12% are related to cell cycle proteins [88]. c-Myc increases cell size by means of an increase in global protein synthesis. It is speculated that the increase in cell growth might promote cell proliferation downstream of Myc activation, in addition to Myc's ability to regulate key cell cycle proteins [88]. Although Myc, in principle, should be an attractive target for cancer therapy, the lack of direct evidence and the concern about the safety issues have hindered the development of Myc inhibitors as anticancer agents. Recently, there is renewed interest in Myc inhibition for cancer treatment. The experiments in a conditioned-mouse model showed that Myc inhibition triggered

rapid regression of a Ras-dependent tumor, with tolerated and reversible effects on normal regenerating tissues [89]. These experiments demonstrate the feasibility of Myc inhibition as an effective and efficient cancer therapy. Since our ginger extract can clearly down-regulate c-Myc in a dose- and time-dependent manner, our results indicate that ginger extract might prove to be an effective chemopreventive anticancer agent.

Taken as a whole, the results from this study reveal that the paradols contained in ginger (*Z. officinale Roscoe*) rhizome extract are likely the active substances that are responsible for the down-regulation of *hTERT* and *c-Myc* expression in A549 human non-small cell lung cancer cells. The down-regulation of *hTERT* leads to a reduction in telomerase activity. The down-regulation of *hTERT* might follow the down-regulation of *c-Myc* since *c-Myc* regulates *hTERT* transcription. As both telomerase inhibition and Myc-inhibition are cancer-specific targets for cancer therapy, paradols or ginger extract might prove to be beneficial as a complementary agent in cancer prevention and maintenance therapy.