EVALUATION OF THE CHEMOTHERAPEUTIC AND CHEMOPREVENTIVE POTENTIAL OF TRITERPENOIDS FROM *PORIA COCOS*

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# TABLE OF CONTENTS

TITLE PAGE

ACKNOWLEDGEMENTS .................................................................i

TABLE OF CONTENTS .............................................................iii

ABBREVIATIONS ..................................................................vii

SUMMARY ........................................................................ix

LIST OF TABLES .................................................................xii

LIST OF FIGURES .................................................................xiii

PUBLICATIONS .................................................................xvii

Chapter 1: Introduction .......................................................1

1.1 Natural products as source of anticancer agents ...............4

1.2 Triterpenoids from *Poria cocos* ....................................9

1.2.1 Triterpenoids ..........................................................9

1.2.2 Triterpenoids from *Poria cocos* ..............................16

1.3 Apoptosis ..................................................................24

1.3.1 Overview of apoptosis ..............................................24

1.3.2 Characteristics of apoptosis ......................................25

1.3.2.1 Morphological features ....................................26

1.3.2.2 Biochemical features .......................................28

1.3.3 Pathways of apoptosis .............................................34

1.3.3.1 Extrinsic pathway ..........................................35
Chapter 2: Isolation and identification of triterpenoids from *P. cocos* ..........81

2.1 Introduction ..................................................................................81

2.2 Materials and methods ..........................................................83

2.3 Results .....................................................................................87

  2.3.1 Separation of alcoholic extracts into four fractions ........87

  2.3.2 Isolation of pure compounds ...........................................88

  2.3.3 Identification of purified compounds .............................90

  2.3.4 Cytotoxicity test .................................................................95

2.4 Discussion .................................................................................97

Chapter 3: Polyporenic acid C induces caspase-8-mediated apoptosis in human lung
cancer A549 cells ..........................................................................100

3.1 Introduction .............................................................................101

3.2 Materials and methods ..........................................................102

3.3 Results .....................................................................................109

3.4 Discussion .................................................................................123

Chapter 4: Pachymic acid inhibits A549 cells growth and modulates arachidonic acid
metabolism ......................................................................................129

4.1 Introduction .............................................................................130

4.2 Materials and methods ..........................................................132

4.3 Results .....................................................................................140

4.4 Discussion .................................................................................158
Chapter 5: Investigation of lanostane-type triterpenoids against breast cancer cell invasion .................................................................162

5.1 Introduction ........................................................................163

5.2 Materials and methods ......................................................165

5.3 Results .............................................................................169

5.4 Discussion .........................................................................184

Chapter 6: General discussion, conclusion and future work ...........188

6.1 General discussion .............................................................188

6.2 Conclusion ........................................................................193

6.3 Future work ......................................................................195

REFERENCES .........................................................................198

APPENDICES .........................................................................237
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AIF</td>
<td>apoptosis inducing factor</td>
</tr>
<tr>
<td>AP-1</td>
<td>activator protein-1</td>
</tr>
<tr>
<td>BCA</td>
<td>bicinchoninic acid</td>
</tr>
<tr>
<td>CAD</td>
<td>caspase-activated DNase</td>
</tr>
<tr>
<td>CCK-8</td>
<td>cell counting kit-8</td>
</tr>
<tr>
<td>CDDO</td>
<td>2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid</td>
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<tr>
<td>CDDO-Me</td>
<td>methyl 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oate</td>
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<tr>
<td>c-FLIP</td>
<td>FADD-like IL-1β-converting enzyme (FLICE)-inhibitory protein</td>
</tr>
<tr>
<td>COX</td>
<td>cyclooxygenase</td>
</tr>
<tr>
<td>DEDA</td>
<td>7,7-dimethyl-5,8-eicosadienoic acid</td>
</tr>
<tr>
<td>DISC</td>
<td>death-inducing signaling complex</td>
</tr>
<tr>
<td>DMBA</td>
<td>7, 12-dimethylbenz[a]anthracene</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>FADD</td>
<td>Fas-associated death domain</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>GAPDH</td>
<td>glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>IAP</td>
<td>inhibitors of apoptosis proteins</td>
</tr>
<tr>
<td>ICAD/DFF45</td>
<td>inhibitor of caspase activated DNase or DNA fragmentation factor 45</td>
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<tr>
<td>IKK</td>
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<tr>
<td>IkBα</td>
<td>inhibitor of kappaBα</td>
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<tr>
<td>JNK</td>
<td>c-Jun NH2-terminal kinase</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate dehydrogenase</td>
</tr>
<tr>
<td>MAC</td>
<td>mitochondrial apoptosis-induced channel</td>
</tr>
<tr>
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<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MMPs</td>
<td>Matrix metalloproteinases</td>
</tr>
<tr>
<td>MP</td>
<td>melting point</td>
</tr>
<tr>
<td>MS</td>
<td>mass spectrometry</td>
</tr>
<tr>
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<td>membrane-type MMPs</td>
</tr>
<tr>
<td>mTOR</td>
<td>mammalian target of rapamycin</td>
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<tr>
<td>MTT</td>
<td>3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>NF-κB</td>
<td>nuclear factor kappa B</td>
</tr>
<tr>
<td>NMR</td>
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</tr>
<tr>
<td>NSAIDs</td>
<td>non-steroidal anti-inflammatory drugs</td>
</tr>
<tr>
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<td>non-small cell lung cancer</td>
</tr>
<tr>
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<td>pachymic acid</td>
</tr>
<tr>
<td>PAK2</td>
<td>p21-activated kinase 2</td>
</tr>
<tr>
<td>PARP</td>
<td>poly-ADP ribose polymerase</td>
</tr>
<tr>
<td>PGE₂</td>
<td>prostaglandin E2</td>
</tr>
<tr>
<td>PI</td>
<td>propidium iodide</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
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</table>
PLA$_2$  phospholipase A2

$c$PLA$_2$  calcium-dependent cytosolic PLA$_2$

$i$PLA$_2$  calcium-independent PLA$_2$

$s$PLA$_2$  secretary PLA$_2$

PPAC  polypropionic acid C

PS  phosphatidylserine

RIP-1  receptor interacting protein 1

RT-PCR  reverse transcription - PCR

SCLC  small cell lung cancer

SP-1  stimulatory protein-1

STATs  signal transducers and activators of transcription

TIMPs  tissue inhibitors of metalloproteinases

TLC  thin layer chromatography

TNF  tumor necrosis factor

TPA  12-O-tetradecanoylphorbol-13-acetate

TRADD  TNF receptor-associated death domain

TRAF2  tumor necrosis factor receptor associated factor 2

TRAIL  TNF-related apoptosis inducing ligand

uPA  urokinase plasminogen activator
SUMMARY

*Poria cocos* (also known as Fuling) is one of the most famous herbs used in Traditional Chinese Medicine for its diuretic, sedative and tonic effects. The aim of this PhD project is to examine the efficacies of triterpenoids from *Poria cocos* against human cancers. This project was initiated with separation and isolation of triterpenoids contained in alcoholic extracts of *Poria cocos* using flash column chromatography. In total, eight compounds were obtained and identified as (1) pachymic acid, (2) dehydropachymic acid, (3) 3-acetyloxy-16α-hydroxytramenetolic acid, (4) polyporenic acid C, (5) 3-epi-dehydropachymic acid, (6) 3-epi-dehydrotumulosic acid, (7) tumulosic acid, and (8) 29-hydroxypolyporenic acid. The antiproliferative activity of these triterpenoids was examined using a cell proliferation assay.

Due to its relatively stronger antiproliferative activity, polyporenic acid C (PPAC) was subjected to further evaluation for its apoptosis-inducing effect. PPAC was found to exhibit inhibition against anchorage-dependent and –independent growth of human lung cancer cells, which was accompanied by apoptosis induction as evident from increase in sub-G1 cell population, positive annexin V staining, and increase in cleavage of procaspase-8, -3 and poly-ADP ribose polymerase. Experiments using specific caspase inhibitors confirmed the involvement of caspase-8, but not caspase-9 in PPAC-induced apoptosis. Thus it was suggested that PPAC induced apoptosis through the death receptor-mediated apoptotic pathway.
without the involvement of mitochondria. Furthermore, PPAC was shown to suppress PI3-kinase/AKT signal pathway and enhance p53 activation, implying the involvement of an additional mechanism by which apoptosis was induced by this triterpenoid.

Pachymic acid (PA), the main triterpenoid isolated from *Poria cocos*, was examined for its anticancer activity with a focus on its modulation of arachidonic acid metabolism. A multi-factorial anticancer property of PA toward human lung cancer was demonstrated. At high concentrations, PA induced apoptosis in lung cancer cells, accompanied by perturbation of mitochondrial membrane potential. At non-lethal levels, PA decreased IL-1β-induced activation of cPLA₂ and COX-2 by suppressing MAPKs activation and inhibiting NF-κB signaling. Consequently, arachidonic acid and its downstream product prostaglandin E2 were downregulated.

In view that arachidonic acid metabolism plays an important role in promoting cancer progression, these findings had indicated the chemopreventive potential of PA against lung carcinogenesis.

The anticancer potential of *Poria cocos*-originated triterpenoids was further explored by examining their efficacy against phorbol ester-stimulated matrix metalloproteinase secretion and breast cancer cell invasion. PPAC, PA and dehydropachymic acid were found to reduce the gelatinolytic activity of matrix metalloproteinase-9 with different efficacies. PA, with the strongest anti-invasive potential, was demonstrated to significantly inhibit phorbol ester-stimulated migration of MDA-MB-231 cells in an *in vitro* Matrigel invasion assay. The inhibition of
MMP-9 by PA was found to occur at the transcriptional level through the inhibition of NF-κB signaling. It was thus concluded that by targeting NF-κB signaling, PA inhibited MDA-MB-231 cell invasion through decreasing MMP-9 expression.

Together, the findings presented in this PhD study had expanded on the current understanding of the anticancer potential of triterpenoids from *Poria cocos.*
LIST OF TABLES

Table 1.1 Differences between apoptosis and necrosis .................................. 25

Table 2.1 $^{13}$C-NMR spectral data for compound 1-8 (75 MHz in C$_2$D$_2$N; $\delta$ in ppm).95

Table 2.2 IC$_{50}$ values of compounds 1-8 against A549 cell growth ...................97
LIST OF FIGURES

Figure 1.1 Antineoplastic triterpenoids manuscripts abstracted in PubMed for the period 1987-2001 .................................................................10

Figure 1.2 Morphological features of cell death by apoptosis ....................27

Figure 1.3 Mechanisms of caspase activation ........................................31

Figure 1.4 Extrinsic and intrinsic apoptotic pathways ..............................34

Figure 1.5 Model of the two CD95 signaling pathways ..........................38

Figure 1.6 Prostaglandin biosynthesis cascade .................................51

Figure 2.1 Effects of four pooled fractions obtained from crude extract of *Poria cocos* against A549 cell proliferation .........................................................88

Figure 2.2 Schematic diagram illustrating the extraction schemes leading to isolation of eight triterpenoids from *Poria cocos* ..........................89

Figure 2.3 Chemical structures of compounds 1-8 .................................91

Figure 2.4 Effects of compounds 1-8 on A549 cell proliferation ...............96

Figure 3.1 PPAC decreased the cell viability of human NSCLC A549 cells in a dose-dependent manner. .................................................................109

Figure 3.2 Effect of PPAC on the proliferation of SCLC H82 and H187 cells....110
Figure 3.3 PPAC reduced colony formation of A549 cells…………………………112

Figure 3.4 PPAC induced apoptosis in A549 cells as evaluated by sub-G1 analysis113

Figure 3.5 PPAC induced apoptosis in A549 cells as evaluated by Annexin V labeling assay …………………………………………………………………………………114

Figure 3.6 PA induced cleavage of caspase-8, caspase-3 and PARP ……………115

Figure 3.7 Effect of caspase inhibitors on PPAC-induced apoptosis ………117

Figure 3.8 Effect of caspase inhibitors or JNK inhibitor on PPAC-induced PARP cleavage…………………………………………………………………………….118

Figure 3.9 PPAC failed to affect $\Delta \Psi_m$ while pachymic acid caused disruption of $\Delta \Psi_m$ in a dose- and time-dependent manner……………………………………….120

Figure 3.10 Effect of PPAC on JNK activation……………………………………121

Figure 3.11 PPAC treatment suppressed Akt activation and increased the activation of p53………………………………………………………………………………123

Figure 4.1 Discrepancy between cell viability results obtained from the MTT assay and Trypan blue exclusion assay………………………………………………...141

Figure 4.2 Differences in formazan formation in a MTT assay between cells treated or untreated with PA…………………………………………………………...142

Figure 4.3 Effect of PA on A549 cell viability as evaluated by CCK-8…………143

Figure 4.4 Effect of PA treatment on LDH release of A549 cells into culture medium………………………………………………………………………………….144
Figure 4.5 PA inhibited anchorage-independent growth of A549 cells.............145

Figure 4.6 PA induced apoptosis in A549 cells...........................................146

Figure 4.7 PA induced cleavage of PARP in A549 cells.........................147

Figure 4.8 Dose and time-dependent disruption of $\Delta \Psi_m$ by PA treatment.......148

Figure 4.9 PA inhibited IL-1$\beta$-induced cPLA$_2$ protein activation in A549 cells.....149

Figure 4.10 PA inhibited IL-1$\beta$-induced cPLA$_2$ gene activation ...............150

Figure 4.11 PA suppressed IL-1$\beta$-enhanced cPLA$_2$ enzyme activity...............151

Figure 4.12 PA reduced IL-1$\beta$-stimulated PGE$_2$ production......................152

Figure 4.13 PA inhibited IL-1$\beta$-induced COX-2 mRNA and protein expression.....153

Figure 4.14 PA inhibited IL-1$\beta$-induced MAPKs activation in A549 cells.........154

Figure 4.15 Effect of specific MAPK inhibitors on IL-1$\beta$-induced phosphorylation of cPLA$_2$ and COX-2 protein expression..................................................155

Figure 4.16 PA inhibited IL-1$\beta$-induced NF-$\kappa$B activation in A549 cells.........157

Figure 5.1 Dose-dependent decrease in MMP-9 gelatinolytic activity mediated by PA, PPAC and dehydropachymic acid.........................................................170

Figure 5.2 PA reduced extracellular expression of MMP-9 .........................171
PUBLICATIONS

Journal publications and preprints:


Conference publications:

1. **Hui Ling**, Liang Zhou, Leslie Gapter, Rajesh Agarwal, Ka-yun Ng.
   “Polyporenic acid C induces caspase-8-mediated apoptosis in human lung cancer A549 cells.”
   *2008 American Association of Pharmaceutical Sciences (AAPS) Annual Meeting and Exposition, Atlanta, USA, Nov 2008.*

2. **Hui Ling**, Yin-shan Lim, Leslie A. Gapter, Liang Zhou, Rajesh Agarwal, Ka-Yun Ng.
   “Inhibition of PLA2 activity and non-small cell lung cancer A549 cell growth by pachymic acid.”
   *American Association for Cancer Research (AACR) Special Conference, Chemical and Biological Aspects of Inflammation and Cancer, Hawaii, USA, Oct 2008.*

   “Pachymic acid from *Poria cocos* induces apoptosis in lung cancer cells.”

4. **Hui Ling**, Liang Zhou, Leslie A. Gapter and Ka-yun Ng.
   “Lanostane-type triterpenoids from *Poria cocos* induces mitochondria-mediated apoptosis.”
   *The First Japanese Cancer Association (JCA) - American Association for Cancer Research (AACR) Special Joint Conference, Nagoya, Japan, Mar 2007.*
Chapter 1: Introduction

Cancer is a class of diseases caused by abnormal and uncontrolled cell division that eventually invade nearby tissues and spread to other parts of the body through the blood and lymphatic circulation systems (Clark 1991). Six essential characteristics have been proposed to differentiate cancer from normal tissue: “self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of programmed cell death, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis” (Hanahan and Weinberg 2000). Cancer affects all parts of the body and is a major public health concern worldwide. According to a recent statistical report, the most frequently occurring cancers among men are prostate cancer (25%) and lung cancer (15%), while the most common cancers in women are breast cancer (27%) and lung cancer (14%) (Jemal et al. 2009). The transformation of normal human cells into highly malignant tumor cells is a multi-step process and can be represented by three stages that often overlap: initiation, promotion, and progression phases (Farber 1984; Clark 1991). Cancer initiation begins when normal cells are exposed to a carcinogen and their genomic DNA undergoes damage that remains unrepaired or misrepaired. In the cancer promotion stage, the cell damages are expanded, and eventually lead to the appearance of benign tumors. Finally, during the progression phase, new clones of tumor cells with increased proliferative capacity, invasiveness, and metastatic potential are produced.

To combat cancer, a variety of strategies have been proposed and developed. The conventional treatments of cancer include surgery, chemotherapy, radiotherapy
and immunotherapy. More recently, gene therapy emerged as a promising alternative method for cancer treatment, owing to increased understanding of tumor biology and the process of tumorigenesis. In addition, the concept of cancer prevention has received much attention since increasing evidence has shown that more than 30% of cancer can be prevented by modifying or avoiding key risk factors such as smoking (Danaei et al. 2005). Therefore, chemoprevention, defined as the use of synthetic or natural agents to inhibit, retard, or reverse the process of carcinogenesis, has been employed as a strategy against cancer along with therapeutic treatments.

In the search of effective chemotherapeutic and/or chemopreventive agents against cancer, natural products have proved to be valuable sources for discovery of lead drug compounds. More than half of the currently used anticancer agents are developed in one way or another from natural products (Gordaliza 2007). These anticancer drug candidates include vinblastine, vincristine, the camptothecin derivatives, topotecan and irinotecan, etoposide, and paclitaxel (Cragg and Newman 2003). One class of natural products that have attracted interest as potential anticancer candidate in recent years is the triterpenoids.

Triterpenoids refer to a large and structurally diverse group of compounds derived from squalene or related acyclic 30-carbon precursors (Xu et al. 2004). Due to their diverse biological effects, naturally occurring cyclic triterpenoids such as ursolic acid, tubeimoside and oleanolic acid have attracted much attention in cancer research (Connolly and Hill 2008). Recently, two semisynthetic triterpenoids
developed from oleanolic acid were believed to be among the most effective anti-inflammatory and anti-carcinogenic agents (Liby et al. 2007).

Triterpenoids are widely distributed in many traditionally used medicinal herbs, such as *Poria cocos*. Alcoholic extracts of *Poria cocos* contain various lanostane-type triterpenoids which have been shown to possess anti-inflammatory and anticancer properties (Kaminaga et al. 1996; Cuellar et al. 1997; Akihisa et al. 2007). However, due to limited research on *Poria cocos*-originated triterpenoids, the cancer-preventive and -therapeutic potential of these lanostane-type triterpenoids remains largely unknown.

The subsequent sections provide (1) an overview of natural products-derived anticancer agents, (2) a review on anticancer triterpenoids, and (3) a literature review on triterpenoids from *Poria cocos*. In addition, three cancer-related topics, namely apoptosis, prostaglandins in cancer development, and matrix metalloproteinases and tumor invasion are also discussed in this chapter.
1.1 Natural products as source of anticancer agents

Natural products have been used for treatment of various kinds of diseases for thousands of years. Since the beginning of modern scientific research, natural products have been the source of most of the active components in medicines (Harvey 2008). Before 1996, more than 80% of medicinal drugs were derived from natural products or inspired by a natural product (Harvey 2008). The continuing importance of natural products as sources in drug discovery can be seen from the statistical analysis showing that half of the drugs approved from 1994 to 2007 were derived from natural products (Newman and Cragg 2007).

In the cancer field, natural products have been widely screened by researchers in search for new and effective anticancer agents. According to a recent statistical report, of the 155 anticancer drug substances from 1940s to 2006, only 27% are synthetic compounds, with 47% essentially being natural products or derived forms of natural products (Newman and Cragg 2007). These naturally occurring compounds are used as anticancer drugs themselves, precursors for semisynthesis or templates for synthesis of more potent and safer analogs. In addition, more compounds derived from natural products are currently under preclinical or clinical evaluation as promising anticancer candidates.

The clinically used anticancer agents with a natural product source include several groups of molecules with diverse chemical structures. The first anticancer agents in clinical use are a group of vinca alkaloids, which were isolated from the Madagascar periwinkle, *Catharanthus roseus* and discovered as potential anticancer
agents in the 1950s. Two natural occurring compounds of this group, vincristine and vinblastine were found to exert their anticancer activity through inhibiting tubulin polymerization and approved by Food and Drug Administration (FDA) as anticancer agents in 1963 and 1965 respectively (Cragg et al. 2009). They have been largely used in combination with other cancer chemotherapeutic drugs for the treatment of a variety of cancers including leukemia, lymphoma, Kaposi’s sarcoma, advanced testicular cancer, as well as breast and lung cancers (Cragg et al. 2009).

The second group of anticancer agents was developed based on the identification of podophyllotoxin, a bioactive lignan contained in *Podophyllum* species, as a potent cytotoxic agent (Damayanthi and Lown 1998). Podophyllotoxin shows strong cytotoxic activity against various cancer cell lines, but its complicated and severe side effects make it unsuitable for clinical use as anticancer agent (Cragg and Newman 2005). Further chemical modification successfully yielded two clinically effective anticancer agents, etoposide and teniposide. Their anticancer mechanism has been revealed to be through inhibition of topoisomerase II and they are clinically used in the treatment of neoplasia such as lymphomas and bronchial and testicular cancers (Hande 1998).

Derivatives of camptothecin constitute the third class of anticancer agents in clinical use. Camptothecin was first extracted from the Chinese ornamental tree, *Camptotheca acuminata*, also known as the “tree of joy”. Despite its remarkable anticancer activity, camptothecin failed clinical trial due to its adverse drug reactions including vomiting, diarrhea and severe bladder toxicity (Kehrer et al. 2001).
However, extensive research on analogues of camptothecin has produced two clinically used anticancer drugs, topotecan and irinotecan (Kehrer et al. 2001). Acting through their inhibitory effects on topoisomerase I, these agents are currently used for treatment of ovarian and colon cancers (Kehrer et al. 2001).

The discovery of paclitaxel in the late 1960s has bred a new class of anticancer agents with unique molecular mechanism (Kingston 2009). Paclitaxel was initially isolated from the bark of *Taxus brevifolia*, a pacific yew, and its cytotoxicity against cancer was discovered under a systematic screening program organized by the National Cancer Institute of the United States (NCI) (Wani et al. 1971). The following preclinical evaluation and clinical trials not only confirmed paclitaxel as an effective anticancer agent, but also revealed its novel anticancer mechanism as a stabilizer of microtubules (Kingston 2009). Though hindered by its shortage of supply from natural source, paclitaxel was finally approved by FDA (as Taxol®) for the treatment of metastatic ovarian cancer in 1992. In the following year, the successful semi-synthesis of paclitaxel using a readily available precursor solved the long outstanding supply problem (Kingston 2007). Today, Taxol® is one of the most important anticancer drugs; it is used either alone or in combination with other anticancer agents in chemotherapy for the treatment of various cancers including ovarian, breast, and non-small cell lung cancer.

Apart from the abovementioned clinically used anticancer agents, some anticancer candidates currently under clinical trials are also derived one way or another from natural products. These include, but not limited to, flavopiridol,
Combretastatin A-4 and roscovitine (Cragg and Newman 2005). Based on the novel chemical structure of rohitukine from Dysoxylum binectariferum, flavopiridol was synthesized as a potential anticancer agent acting through inhibition of cyclin-dependent kinase. It is the first cyclin-dependent kinase inhibitor under clinical development (Senderowicz 1999). Combretastatin A-4 is a natural stilbenoid phenol isolated from the bark of the South African tree Combretum caffrum. Acting as an inhibitor of tubulin polymerization, combretastatin A-4 is one of the most cytotoxic phytomolecules toward cancer cells with remarkable inhibitory effect on tumor’s blood supply (Griggs et al. 2001). Roscovitine is a derivative of olomucine, a natural compound originally isolated from the cotyledons of the radish, Raphanus sativus. Roscovitine is a cyclin-dependent kinase inhibitor and currently under clinical evaluations for its effect against lung cancer and lymphoma (Meijer and Raymond 2003). Although these agents are still under clinical assessment, a larger inventory of anticancer agents derived from a natural source is expected in the near future.

On the other hand, epidemiologic studies have indicated the potential anticancer activity of compounds contained in natural products such as fruits and vegetables (Block et al. 1992). Consumption of such products was found to significantly reduce cancer occurrence. In addition, many natural occurring compounds such as resveratrol from grapes, curcumin from turmeric, and isothiocyanates from cruciferous vegetables have been shown to possess preventive and/or therapeutic effects against various cancer types in vitro and
in vivo (Beliveau and Gingras 2007). These findings further reinforce the importance of natural products as sources of agents with anticancer potential.
1.2 Triterpenoids from *Poria cocos*

1.2.1 Triterpenoids

The triterpenoids are a large and structurally diverse group of natural compounds derived from squalene or related acyclic 30-carbon precursors (Xu et al. 2004). To date, more than 20,000 naturally occurring triterpenoids have been isolated and identified (Liby et al. 2007). For centuries, natural products containing high content of triterpenoids have been a main source of folk medicines. Scientific research also showed that triterpenoids have a wide range of biological activities, such as anti-inflammatory, anticancer, antimicrobial and antiviral effects (Dzubak et al. 2006). The intense research interest on triterpenoids can be evidenced from the large amount of literature published. Notably, the enthusiasm on antineoplastic triterpenoids has been increasingly prevailing over the past 20 years. As shown in Figure 1.1, from 1987 to 2001, the number of publications on antineoplastic triterpenoids has dramatically increased from 3 to 74 (Setzer and Setzer 2003), and this number is still increasing.
Among the triterpenoids identified so far, a number of triterpenoid structures including lupane, lanostane, dammarane, ursane and oleanane triterpenoids, have attracted much attention due to their remarkable anticancer activities.

Lupeol and betulinic acid (structures are shown after this paragraph) are two natural pentacyclic triterpenes belonging to the lupane family. The anticancer activity of lupeol has been demonstrated both in vitro and in vivo. To prostate carcinoma cell lines, lupeol has been reported to inhibit the proliferation of LNCaP cells (IC\textsubscript{50} = 75 µM) and cause arrest of PC-3 cells at G1-S phase (Prasad et al. 2008). In animal models, lupeol significantly reduces testosterone-induced prostate changes in mice and effectively attenuates tumor formation in nude mice bearing 451Lu melanoma xenograft (Prasad et al. 2008). In addition, lupeol is found to induce
mitochondria-mediated apoptosis through elevation of Bcl-2/Bax ratio and cleavage of caspase-3 in prostate cancer and melanoma cells (Prasad et al. 2008; Prasad et al. 2008; Saleem et al. 2008). Apart from its cytotoxic effect, lupeol has also been demonstrated to be effective against carcinogenesis, evident from its preventive effect on 7, 12-dimethylbenz[a]anthracene (DMBA)-induced DNA breakage in a mouse model (Nigam et al. 2007). Betulinic acid, also a lupane-type triterpenoid, is known to inhibit DNA polymerase β, topoisomerases I and II and to possess broad cytotoxicity against lung, colorectal, breast, prostate and cervical cancer cell lines (Kuo et al. 2009). Betulinic acid has been shown to be an effective anticancer agent both in vitro and in vivo by inducing apoptosis through increasing permeability of the mitochondrial membrane (Kuo et al. 2009). Intriguingly, as compared to clinically used anticancer agents such as cisplatin and doxorubicin, betulinic acid has demonstrated better anticancer activity while exerting less toxic effect on non-cancer cells (Sarek et al. 2003; Jeremias et al. 2004). Owing to its remarkable anticancer activities, betulinic acid is now under development under the Rapid Access to Intervention Development program at the NCI (Tan et al. 2003).
Lanostane-type triterpenoids include those contained in *Ganoderma lucidum* and *Poria cocos*. Ganoderic acid D, a lanostane triterpenoid isolated from *Ganoderma lucidum*, is reported to inhibit proliferation of human cervical carcinoma HeLa cells with an IC$_{50}$ value of 17.3 µM (Yue et al. 2008). Flow cytometric analysis revealed that ganoderic acid D caused cell cycle arrest at G2/M phase and resulted in apoptotic cell death of HeLa cells (Yue et al. 2008). Lanostane-type triterpenoids isolated from *Poria cocos* have also been demonstrated to have anticancer activities and their pharmacological activities will be reviewed in details in the following section 1.2.2.

![ganoderic acid D](image)

The dammarane-type triterpenoids include ginsenosides isolated from *Panax ginseng*, a famous herb widely used in Traditional Chinese Medicine. The chemopreventive effects of *Panax ginseng* have been successfully tested on a wide range of animal models which include cancers derived from the mammary glands, colon, liver, cervix and central nervous system (Dzubak et al. 2006). In addition, *Panax ginseng* has shown preventive effect in patients with esophageal and endometrial precancers (Dzubak et al. 2006). Lending support to this observation are three epidemiological studies showing the correlation of consumption of *Panax ginseng* with lower cancer risk (Yun et al. 2001). Ginsenosides isolated from *Panax*
ginseng have been shown to possess various anticancer activities; for instance, ginsenoside Rh-1 induces melanoma cells differentiation; Rh-2 inhibits proliferation of human and murine cancer lines; Rg-3 not only inhibits carcinogenesis by suppressing key enzymes involved in the origination of tumors (as demonstrated by its inhibition on phorbol ester-induced expression of pro-inflammatory enzyme cyclooxygenase-2 and ornithinedecarboxylase in mouse skin), but also inhibits cancer invasion (Dzubak et al. 2006).

The anticancer triterpenoids belonging to the ursane group include ursolic acid and boswellic acids. Ursolic acid has been shown to exert anticancer activity through its suppressive effect on the nuclear factor kappa B (NF-κB) signaling.
pathway, thereby leading to down-regulation of the downstream oncogenes (Ikeda et al. 2008). Furthermore, ursolic acid has been shown to inhibit skin tumor formation by interfering with the initiation of carcinogenesis (Ikeda et al. 2008). Boswellic acids, as inhibitors of mammalian DNA polymerases $\alpha$ and $\beta$, and human DNA topoisomerases I and II, exert antiproliferative effect on cancer cells (Dzubak et al. 2006). In one study, they have been reported to induce apoptosis in colon cancer cells through the apoptotic pathway dependent on caspase-8 activation (Liu et al. 2002). In a more recent report, acetyl-keto-$\beta$-boswellic acid is shown to induce apoptosis through a death receptor 5-mediated pathway in prostate cancer cells (Lu et al. 2008). Collectively, these two studies suggest that activation of extrinsic apoptotic pathway may be the main mechanism underlying the apoptotic-inducing effects of boswellic acids. In addition to their cytotoxicity on cancer cells, boswellic acids are found to inhibit bFGF-induced angiogenesis in an in vivo Matrigel plug assay and this effect is comparable with indomethacin and cyclophosphamide (Singh et al. 2007).
Oleanolic acid is a natural oleanane triterpenoid with weak anticancer activities including antimutagenic, antiangiogenic and differentiation-inducing effects (Dzubak et al. 2006). Chemical modifications made on the structure of oleanolic acid have yielded two more potent synthetic oleanane triterpenoids, 2-cyano-3,12-dioxoolena-1,9(11)-dien-28-oic acid (CDDO) and its methyl ester (CDDO-Me). These two synthetic triterpenoids have been demonstrated to possess multi-functional anticancer properties. On one hand, they are potent inducers of apoptosis in a variety of cancer types \textit{in vitro} and \textit{in vivo} (Kim et al. 2002; Konopleva et al. 2002; Lapillonne et al. 2003; Hyer et al. 2008). On the other hand, they regulate several key transcriptional factors that control pro-inflammatory and anti-inflammatory genes, and are believed to be the most potent anti-carcinogenic triterpenoids that have been identified so far (Liby et al. 2007). Intensive experimental studies have confirmed the potential of oleanolic acid-originated agents as new and effective treatment for various cancer types. Both CDDO and CDDO-Me are currently in phase I clinical trials for the treatment of metastatic or unresectable solid tumors or lymphoma (Petronelli et al. 2009). The successful development of CDDO and its derivatives ascertains the importance of the rich pool of triterpenoids serving as valuable sources of precursors and compounds for development into potential anticancer agents.
1.2.2 Triterpenoids from *Poria cocos*

The fungal species *Poria cocos*, also known as Hoelen or Fuling, is one of the most well-known herbs used in Traditional Chinese Medicine. Among the Chinese community, it has long been believed to possess highly nourishing value and is thus widely used as a tonic to improve the condition of internal organs (Li et al. 2005). In addition, it has been credited to have diuretic and sedative effects, which lead to its application for use in inflammatory conditions to reduce edema or phlegm. Due to its gentle physiological activities with relatively mild adverse effects, *Poria cocos* has also been a health-promoting food in common Chinese tonic soups on dinner tables. In Traditional Chinese Medicine, *Poria cocos* is one of the most commonly used components in complex herbal prescriptions. Scientific research has shown that
alcoholic extracts of *Poria cocos* possess multiple pharmacological activities that benefit human beings. Triterpenoids are the main constituents in the alcoholic extracts of *Poria cocos* and are therefore believed to be the active components responsible for the biological activities of this plant (Akihisa et al. 2007).

Most of the identified triterpenoids contained in *Poria cocos* belong to the lanostane group. These lanostane-type triterpenoids are observed to exhibit a wide range of activities including anti-rejection, anti-emetic, anti-inflammatory and anticancer effects. For instance, ethanol extract of *Poria cocos* has been reported to relieve the acute rejection of heart transplantation in a rat model (Zhang et al. 2004), indicating that triterpenoids contained in *Poria cocos* have the ability to regulate immune response. In addition, some triterpenoids have been demonstrated to inhibit emetic action induced by oral administration of copper sulfate pentahydrate to leopard frog (Tai et al. 1995). Though much preliminary and incomplete, these two findings suggest that triterpenoids from *Poria cocos* may play a protective role in a human body by relieving unfavorable responses.

Apart from the studies mentioned above, the majority of research interests have been focused on investigating the anti-inflammatory activities of *Poria cocos*-originated triterpenoids. The methanol extracts of *Poria cocos* are found to markedly inhibit the inflammatory activity provoked by 12-O-tetradecanoylphorbol-13-acetate (TPA) in mice (Yasukawa et al. 1995). In another study, triterpenoids including pachymic acid, 3-O-acetyl-16α-hydroxytrametenolic acid, dehydropachymic acid, dehydroeburiconic
acid, 3β-hydroxylanosta-7,9(11),24-trien-21-oic acid, poricoic acid A and poricoic acid B are found capable of reducing TPA-induced mouse ear edema with 50% inhibitory dose of 17-44 µg/ear (Kaminaga et al. 1996). Their anti-inflammatory effects have been observed to be comparable with that of hydrocortisone, the most widely used anti-inflammatory drug for the treatment of skin inflammation (Kaminaga et al. 1996).

A different research group had reported the isolation of several new anti-inflammatory triterpenoids from *Poria cocos* methanol extracts more than a decade ago (Nukaya et al. 1996). These compounds were identified as 3β,16α-dihydroxylanosta-7,9(11),24-trien-21-oic acid, 16α-hydroxydehydropachymic acid, 16α-hydroxytrametenolic acid and dehydrotumulosic acid. All these four triterpenoids have been demonstrated to be effective against TPA-induced mouse ear edema. Another identified triterpenoid, 3β-p-hydroxybenzoyldehydrotumulosic acid has also shown anti-inflammatory activity in a mouse model (Yasukawa et al. 1998). It is effective against ear inflammation in mice induced by either TPA or arachidonic acid, being relatively more potent in inhibiting inflammation induced by the former (50% inhibitory dose for TPA and arachidonic acid is 0.27 and 1.25 mg/ear respectively). Similarly, Cuellar *et al.* had demonstrated that alcoholic extracts from *Poria cocos* were effective against various inflammatory conditions; topical or oral application of the alcoholic extracts not only reduced acute inflammation induced by carrageenan, arachidonic acid and TPA, but also showed inhibitory effect on TPA-induced chronic
inflammation and oxazolone-induced delayed hypersensitivity in mice (Cuellar et al. 1997).

Two isolated monomeric triterpenoids from *Poria cocos*, pachymic acid and dehydrotumulosic acid have shown efficacy against TPA-induced mouse ear inflammation, but exert no effects against inflammation induced by arachidonic acid (Giner et al. 2000). In another study by the same research group, alcoholic extracts of *Poria cocos* have demonstrated effectiveness against phospholipase A2-induced mouse paw edema either application through oral or intraperitoneal route (Giner-Larza et al. 2000). A more recent paper has assessed the anti-inflammatory effects of *Poria cocos* extracts on human subjects (Fuchs et al. 2006). In this study, the anti-inflammatory effect of *Poria cocos* extracts has been evaluated in experimentally induced irritant contact dermatitis resulting from repeated exposure to sodium lauryl sulphate. Clinical assessment and skin measurement reveal that tropical application of *Poria cocos* extracts successfully prevents sodium lauryl sulphate-induced dermatitis in these subjects. In summary, these studies have demonstrated the anti-inflammatory potential of *Poria cocos*-originated triterpenoids.

Inflammation plays an important role in the transformation of normal cells into cancer cells (Federico et al. 2007). Therefore, the anti-inflammatory property of triterpenoids from *Poria cocos* indicates that these compounds may also be effective agents for cancer chemoprevention. Indeed, previous studies had explored the chemopreventive effects of these triterpenoids. Kaminaga *et al.* first demonstrated that several triterpenoids from *Poria cocos*, namely pachymic acid,
3-O-acetyl-16 alpha-hydroxytrametenolic acid, and poricoic acid B, inhibited the promoting effect of TPA on skin tumor formation following initiation with DMBA (Kaminaga et al. 1996); these triterpenoids are found to exert inhibitory effect at dosages as low as 0.2 μmol/mouse. Subsequently, Ukiya et al. demonstrated that ten triterpenoids from *Poria cocos* potently inhibited TPA-induced Epstein-Barr virus early antigen (EBV-EA) activation (Ukiya et al. 2002). These triterpenoids include poricoic acid A, poricoic acid B, poricoic acid G, poricoic acid H, tumulosic acid, dehydrotumulosic acid, 3-epidehydrotumulosic acid, polyporenic acid C, 25-hydroxy-3-epidehydrotumulosic acid and dehydroabietic acid methyl ester.

In a recent study, two new anti-tumor-promoting triterpenoids have been isolated from *Poria cocos* and identified as 16-deoxyporicoic acid B and poricoic acid C (Akihisa et al. 2007). Tropical application of either compound inhibits skin tumor formation in an in vivo two-stage carcinogenesis test using DMBA as an initiator and TPA as a promoter (Akihisa et al. 2007). In the same study, most of the other triterpenoids isolated from *Poria cocos* have also been found to inhibit EBV-EA activation induced by TPA. Since EBV-EA activation is usually regarded as an early sign of tumor-promoting activity (Ito et al. 1981) and the two-stage mouse skin carcinogenesis test (DMBA as initiator and TPA as promoter) is a well-established animal model for prediction of chemopreventive ability of a compound (Drinkwater 1990), these abovementioned studies provide evidence that triterpenoids from *Poria cocos* may have potential applications as cancer chemopreventive agents.
Besides the chemopreventive potential of these triterpenoids, several lines of evidence have suggested that triterpenoids from *Poria cocos* may have cancer therapeutic potential. Li *et al.* investigated the cytotoxicity and DNA topoisomerases inhibitory activity of triterpenoids from *Poria cocos* and found that polyporenic acid C, dehydropachymic acid, pachymic acid and tumulosic acid exhibited moderate cytotoxicity on a human colon carcinoma cell line (Li *et al.* 2004). They further showed that these compounds effectively inhibited DNA topoisomerase I and II as potently as the positive control etoposide. The inhibition of DNA topoisomerase by triterpenoids from *Poria cocos* is further substantiated by the study of another group in which dehydroebriconic acid, another lanostane-type triterpenoid from *Poria cocos*, was identified as a DNA topoisomerase inhibitor (Mizushina *et al.* 2004). In a different study by the same research group, dehydroeburiconic acid, and another *Poria cocos*-originated triterpenoid dehydrotrametenonic acid were shown to inhibit eukaryotic DNA polymerase α and β (Akihisa *et al.* 2004). In the light that DNA topoisomerases and polymerases are important cellular targets for chemotherapeutic intervention in the treatment of cancer (Spadari *et al.* 1986), findings of these studies therefore provide the basis to predict the cytotoxic potencies of such triterpenoids found in *Poria cocos*.

Moreover, a study by Kang *et al.* found that dehydrotrameteolic acid selectively inhibited the growth of H-ras transformed rat2 cells and induced apoptosis through the caspase-3 pathway, whereas it had no lethal effect on untransformed normal cells (Kang *et al.* 2006), indicating that triterpenoids from *Poria cocos* may be
developed as safe anticancer agents without eliciting toxicity to normal cells.

The ability of triterpenoids from *Poria cocos* to induce apoptosis of cancer cells was also previously demonstrated by our own research group (Gapter et al. 2005) in which pachymic acid, a lanostane-type triterpenoid and main component of *Poria cocos* alcoholic extracts, is reported to induce apoptosis in prostate cancer cells by caspase activation and reducing Akt activity. Together, these studies suggest that triterpenoids from *Poria cocos* possess beneficial biological activities that render them potential cancer therapeutic and/or preventive agents.

Despite the broad studies on the biological activities of triterpenoids from *Poria cocos*, the mechanisms underlying their anti-inflammatory and anticancer properties have not been well studied. Some researchers have proposed that triterpenoids from *Poria cocos* may exhibit their biological activities through regulation of phospholipase A2, an important enzyme involved in inflammation and carcinogenesis. For example, one research group has demonstrated the inhibitory effects of two lanostane triterpenoids, pachymic acid and dehydrotumulosic acid, on phospholipase A2 derived from snake venom (Cuella et al. 1996). Pachymic acid has been found to inhibit phospholipase A2 activity with the same potency as a positive control mepacrine, while dehydrotumulosic acid causes inhibition of phospholipase A2 with a potency 3-fold higher than mepacrine. The involvement of phospholipase A2 in anti-inflammatory activity of triterpenoids from *Poria cocos* has been further supported by *in vivo* studies. In one of these *in vivo* studies, Giner et al. testified that administration of *Poria cocos* alcoholic extracts effectively inhibited
phospholipase A2-induced mouse paw edema by either the oral or parenteral route, and hypothesized that the inhibition of phospholipase A2 enzyme by triterpenoids from *Poria cocos* was the main mechanism underlying their anti-inflammatory and anticancer activities (Giner et al. 2000).

However, these studies may have over-concluded their findings. Firstly, there are three major types of phospholipase A2 enzymes (secretary, calcium-independent and cytosolic) and each type is further sub-classified according to their structural or biological differences. However, in the above experiments, only type IA secretary phospholipase A2 from snake venom was examined. The effects of these triterpenoids on other PLA2 isoforms which are more biologically relevant to human beings have not yet been evaluated. Secondly, the reported effective concentrations of tested triterpenoids are obtained from an *in vitro* assay with IC$_{50} > 2$mM, thus the biological relevance of the observed inhibitory effect in relation to triterpenoids’ anti-inflammatory properties is questionable. As such, the exact underlying mechanisms to date remain largely unknown.
1.3 Apoptosis

1.3.1 Overview of apoptosis

Apoptosis, also called programmed cell death, is a genetically directed process of cell self-destruction that is marked by the fragmentation of nuclear DNA (Hockenbery 1995). The term apoptosis was first used by Kerr, Wyllie, and Currie in 1972 to describe a morphologically distinct form of cell death. These authors described this kind of cell death to be an active and inherently programmed phenomenon and emphasized the significance of this cell death form in removing redundant cells (Kerr et al. 1972). A large number of studies arising thereafter have since established that apoptosis is a normal physiological process for removing DNA-damaged, superfluous, or unwanted cells.

One typical example is the formation of separated fingers during fetal life due to apoptosis of cells between fingers, in which failure to do so would result in the existence of webbed fingers. Another example is the loss of a tadpole’s tail through programmed cell death during amphibian development. In addition, apoptosis is also important for the maintenance of the normal functions of immune system by removing both ineffective and potentially damaging immature T cells (Werlen et al. 2003). Therefore, apoptosis is critical to cell growth regulation, development, immune response as well as maintaining a constant net number of cells in organs. Conversely, alternations that affect apoptosis can disrupt homeostasis and have pathological effects. Insufficient apoptosis may cause disorders like hyperplasia or neoplasia, whereas excessive apoptosis can lead to abnormal cell loss, such as the loss
of CD4+ T cells seen in AIDS patients (Elmore 2007).

1.3.2 Characteristics of apoptosis

Apoptosis and necrosis are two common types of cell death. Compared to necrosis, apoptosis has some distinct characteristics, which involve a defined set of programmed morphological and biochemical changes. The differences between cells undergoing apoptosis and necrosis are summarized below in Table 1.1.

Table 1.1 Differences between apoptosis and necrosis.

<table>
<thead>
<tr>
<th>Apoptosis</th>
<th>Necrosis</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Morphological features</strong></td>
<td></td>
</tr>
<tr>
<td>Begins with shrinking of cytoplasm and</td>
<td>Begins with swelling of cytoplasm and</td>
</tr>
<tr>
<td>condensation of nucleus</td>
<td>mitochondria</td>
</tr>
<tr>
<td>Blebbing of plasma membrane without loss</td>
<td>Loss of membrane integrity</td>
</tr>
<tr>
<td>of integrity</td>
<td></td>
</tr>
<tr>
<td>Aggregation of chromatin at the nuclear</td>
<td></td>
</tr>
<tr>
<td>membrane</td>
<td></td>
</tr>
<tr>
<td>Formation of membrane bound vesicles</td>
<td>No vesicle formation, complete lysis</td>
</tr>
<tr>
<td>(apoptotic bodies)</td>
<td></td>
</tr>
<tr>
<td><strong>Biochemical features</strong></td>
<td></td>
</tr>
<tr>
<td>Tightly regulated process involving</td>
<td>Loss of regulation of ion homeostasis</td>
</tr>
<tr>
<td>activation and enzymatic steps</td>
<td></td>
</tr>
<tr>
<td>Energy (ATP)-dependent</td>
<td>No energy requirement</td>
</tr>
<tr>
<td>Non-random mono- and oligonucleosomal</td>
<td></td>
</tr>
<tr>
<td>length fragmentation of DNA (Ladder pattern</td>
<td>Random digestion of DNA (smear of</td>
</tr>
<tr>
<td>after agarose gel electrophoresis)</td>
<td>DNA after agarose gel electrophoresis)</td>
</tr>
<tr>
<td>Activation of caspase cascade</td>
<td></td>
</tr>
<tr>
<td><strong>Physiological significance</strong></td>
<td></td>
</tr>
<tr>
<td>Induced by physiological stimuli (eg. lack of</td>
<td>Evoked by non-physiological</td>
</tr>
<tr>
<td>growth factors, changes in hormonal</td>
<td>disturbances</td>
</tr>
<tr>
<td>environment)</td>
<td></td>
</tr>
<tr>
<td>Localized effect that destroys individual cells</td>
<td>Affects groups of contiguous cells</td>
</tr>
<tr>
<td>No inflammatory response</td>
<td>Significant inflammatory response</td>
</tr>
</tbody>
</table>
1.3.2.1 Morphological features

Apoptosis involves a series of sequential morphological events (Elmore 2007). These morphological features of cells undergoing apoptosis include cell shrinkage, blebbing, nuclear condensation and nuclear fragmentation (Figure 1.2A). During the early stage of apoptosis, cell shrinkage can be observed under a light microscope (Hacker 2000). Consequently, cells become smaller in size with a more compact cytoplasm. The plasma membrane retains its integrity during the process of apoptosis. The earliest detectable change in the nucleus is the condensation of nuclear chromatin along the nuclear membrane (Hacker 2000). The condensed nucleus then breaks up into similarly dense, smaller particles which are packed and taken up. Accompanying the progress of the cell shrinkage and nuclear condensation, cells start to show protrusions known as “blebs” from the plasma membrane. These blebs finally form apoptotic bodies, which are membrane-bound vesicles that vary in size and composition (Hacker 2000). First termed by Kerr et al., apoptotic bodies are one of the most striking characteristics of cells undergoing apoptosis. The apoptotic bodies can contain a mixture of various parts of cells contents, such as cytosolic components, organelles or fragmented nuclei. In tissues, apoptotic bodies are quickly engulfed and digested by neighboring macrophages or epithelial cells (Figure 1.2B). During the apoptotic process, cell contents are well enclosed by plasma membrane. Therefore, apoptosis of cells does not cause inflammation or serious damage to nearby cells since there is no leakage of cell contents (Elmore 2007).
In contrast to apoptosis, necrotic cell death is usually accompanied by development of inflammation and injury to neighboring cells (Elmore 2007). Cells undergoing necrosis are characterized by irreversible swelling of cytoplasmic compartments and distortion of organelles. These events lead to rupture of the...
Chapter 1

plasma membrane and release of cytoplasmic contents. Consequently, these cytoplasmic contents cause damage to the surrounding tissues by sending chemostatic signals with eventual recruitment of inflammatory cells.

1.3.2.2 Biochemical features

During apoptosis, cells display a series of biochemical characteristics. The typical biochemical features of apoptosis include loss of membrane asymmetry, caspase activation, cleavage of apoptosis-related proteins and DNA fragmentation (Hengartner 2000).

a. Loss of membrane asymmetry

Cells dying of apoptosis can expose ‘eat me’ signals for their recognition and subsequent removal by neighboring cells (Elmore 2007). One of these signals is the exposure of phosphatidylserine on the outer leaflet of the cell membrane bilayer. Phosphatidylserine is an anionic phospholipid normally located in the inner leaflet of the plasma membrane. During apoptosis, phosphatidylserine moves to the outer side of the membrane due to loss of membrane asymmetry (Martin et al. 1995). The mechanism that underlies the loss of membrane asymmetry remains unclear. Some studies suggested the involvement of downregulation of an ATP-dependent aminophospholipid translocase and upregulation of 'flippase' in this event (Bratton et al. 1997; Savill 1997). The exposure of phosphatidylserine may also be a result of the opening of mitochondrial 'megachannels' by the so-called permeability transition,
which may release an apoptosis-inducing protease from mitochondria into the cytoplasm (Savill 1997). Although the exact mechanism remains to be further clarified, the exposure of phosphatidylserine is a classic marker of apoptosis and this feature has been generally used for the detection of apoptosis. Annexin V is a calcium-dependent phospholipid-binding protein that has a high affinity for phosphatidylserine. Thus, annexin V labeled with a fluorophore or biotin can be used to identify apoptotic cells by binding to phosphatidylserine exposed on the outer leaflet (Aubry et al. 1999). In addition, studies have suggested that phosphatidylserine exposure is an early marker of apoptosis preceding nuclear changes (Aubry et al. 1999). These findings indicate the importance of the annexin V binding assay as a useful tool for recognition of cells undergoing early apoptosis.

b. Caspases

The process of apoptosis is characterized by the cascade of proteases that are the central executioners of the apoptotic program. These proteases are a group of cysteine proteases that cleave after an aspartate residue in their substrates and therefore named “caspases” (Alnemri et al. 1996). To date, eleven caspases have been identified in humans and about two-third of these caspases have been suggested to play important roles in apoptosis (Hengartner 2000). The apoptotic caspases can be generally categorized into two types: initiator caspases and effector caspases. Caspase-2, -8, -9 and -10 are initiator caspases and caspase-3, -6 and -7 are effector caspases (Riedl and Shi 2004). An initiator caspase usually contains one or more
adaptor domains in its extended N-terminal region, while an effector caspase has 20-30 residues in its prodomain sequence (Riedl and Shi 2004). In the apoptosis process, initiator caspases are activated by apoptotic stimuli that in turn activate the downstream effector caspases. As the executioner proteases, effector caspases cleave their target proteins, leading to the morphological changes seen in apoptotic cells.

All caspases are synthesized in cells as catalytically inertzymogens and need to be activated to exert their catalytic function in apoptosis. Generally, there are three mechanisms of caspase activation (Figure 1.3): (1) processing by an upstream caspase, (2) induced proximity and (3) association with a regulatory subunit (Hengartner 2000). Effector caspases are activated by the upstream initiator caspases through proteolytic cleavage of their zymogen domains. This mode of procaspase activation applies to caspase-3, -6 and -7. Induced proximity is the mechanism underlying activation of caspase-8. As a key initiator caspase in death receptor-mediated apoptosis, caspase-8 is cross-activated through aggregation of multiple procaspase-8 protein molecules into close proximity (Muzio et al. 1998). Since procaspase-8 has a weak intrinsic protease activity, the close proximity will allow cleavage and activation of adjacent procaspase-8 molecules. Likewise, activation of caspase-2 is also achieved through this induced proximity (Hengartner 2000). Unlike caspases described above, caspase-9 is activated by the mechanism of association with a regulatory subunit. The activation of procaspase-9 requires the assembly of the mammalian apoptosome (Cain et al. 1999). The apoptosome is a
seven-subunit complex assembled by Apaf-1 and cytochrome c. Interaction of the apoptosome with procaspase-9 causes the autocleavage and dimerization of this procaspase, which is required for caspase-9 activation (Cain et al. 1999). Therefore, as depicted by Figure 1.3, it can be summarized from the above discussion that initiator caspases are usually activated by regulated protein-protein interactions, whereas effector caspases are activated by initiator caspases.

Figure 1.3 Mechanisms of caspase activation. Caspase activation can be achieved through proteolytic cleavage by an upstream caspase (A), induced proximity (B) or holoenzyme formation (C). (Adopted from Hengartner, 2000)
c. Cleavage of apoptosis-related proteins

The cleavage of apoptosis-related proteins causes the morphological characteristics of apoptosis. These proteins are the target of executor caspases and they include nuclear lamins, poly-ADP ribose polymerase (PARP), p21-activated kinase 2 (PAK2) and inhibitor of caspase activated DNase or DNA fragmentation factor 45 (ICAD/DFF45) (Elmore 2007). Nuclear lamins and PARP are among the earliest proteins discovered to be specifically cleaved during apoptotic cell death. Lamins are nuclear proteins which are located at the inner side of the nuclear envelope and interact with other membrane-associated proteins to form the nuclear lamina. The degradation of nuclear lamins by effector caspases may contribute to the nuclear breakdown in apoptotic cells. This notion was supported by the findings that overexpression of lamin A or lamin B can prevent or delay changes in nuclear morphology. PARP is a nuclear protein that catalyses the synthesis of poly(ADP-ribose) from nicotinamide adenine dinucleotide (Satoh and Lindahl 1992). It is involved in a number of cell functions, such as DNA repair and genome integrity (Herceg and Wang 2001). Thus, the maintenance of PARP function is essential for cell survival. During apoptosis, PARP is cleaved by effector caspases such as caspase-3 from the full length (116 kDa) to a cleaved form of 89 kDa, and thereby loses its function to protect cells (Lazebnik et al. 1994). Studies have consistently showed the coincidence of cleavage of caspase-3 and PARP with the appearance of other morphological and biochemical characteristics of apoptosis (Boulares et al. 1999). Therefore, generation of the cleaved form of PARP has been regarded as a
Chapter 1

hallmark of apoptotic cells. In addition to nuclear lamins and PARP, caspases also activate PAK2, which is critical for cytoskeleton reorganization and nuclear signaling (Rudel and Bokoch 1997). In apoptotic cells, PAK2 is activated by caspase cleavage and contributes to apoptotic events leading to membrane and morphological changes. ICAD/DFF45 is a specific inhibitor of CAD/DFF40. The cleavage and inactivation of ICAD/DFF45 by caspase-3 unleashes the endonuclease activity of CAD/DFF40 (Nicholson 1999; Widlak 2000). As a DNA fragmentation factor, CAD/DFF40 leads to the characteristic changes in the nucleus of apoptotic cells (Nicholson 1999; Widlak 2000). In summary, these apoptosis-related proteins serve as targets of effector caspases and relay the apoptotic signal to result in eventual cell death.

d. DNA fragmentation

DNA fragmentation is a key feature of apoptotic cell death. In apoptotic cells, endogenous endonucleases are activated by caspases and these enzymes specifically cleave genomic DNA in the linker regions between nucleosomes (Nagata 2000). As a result, nuclear DNA is cleaved into multiples of 180 bp, where each fragment corresponds to the internucleosomal spacing. The resulting DNA fragments, when separated in agarose gel electrophoresis, take the appearance of a unique ladder pattern that is conventionally called the “DNA ladder” (Nagata et al. 2003). In contrast, necrotic cell death is accompanied by random DNA degradation and digestion of histone (Elmore 2007). When analyzed by agarose gel
electrophoresis, the degraded DNA forms a diffuse smear on gels. Therefore, assessing genomic DNA laddering can be used to distinguish cell apoptosis from cell necrosis.

1.3.3 Pathways of apoptosis

There are two main apoptotic pathways that have been described in caspase-mediated cell death (MacFarlane and Williams 2004; Riedl and Shi 2004). One is the extrinsic apoptosis pathway and the other is the intrinsic apoptosis pathway (Figure 1.4). These two pathways converge at the level of effector caspases such as caspase-3. The effector caspases then induce the downstream targets which will finally lead to programmed death and removal of apoptotic cells.
Figure 1.4 Extrinsic and intrinsic apoptotic pathways. The extrinsic pathway is triggered by members of the death receptor superfamily, such as CD95 and TNF receptor 1. Binding of corresponding ligands to their death receptors induces receptor clustering and formation of a death-inducing signaling complex (DISC). This complex recruits multiple procaspase-8 molecules, resulting in caspase-8 activation through induced proximity. In the intrinsic pathway, stress signals bring about disruption of mitochondria and subsequently cause release of proteins such as cytochrome c, from the inter-mitochondrial membrane space. Cytochrome c release is regulated by Bcl-2 family members (anti-apoptotic proteins such as Bcl-2 and Bcl-XL; pro-apoptotic proteins such as Bax, Bak and tBid). Once released, cytochrome c binds to Apaf-1 and then procaspase-9, forming apoptosome complex. As a result, procaspase-9 is activated by holoenzyme formation. The activated initiator caspases 8 and 9 then activate the effector caspase 3, 6 and 7, which are responsible for the cleavage of important cellular substances resulting in the classical biochemical and morphological changes associated with apoptosis. (Adopted from MacFarlane and Williams, 2004)

1.3.3.1 Extrinsic pathway

The extrinsic pathway, also known as the death-receptor pathway, is triggered by the engagement of death receptors on the cell surface. Death receptors are members of the tumor necrosis factor (TNF) receptor superfamily and comprise a subfamily that is characterized by a cytoplasmic domain of about 80 amino acids called the death domain (Igney and Krammer 2002). The death receptors include Fas receptor (also known as CD95), tumor necrosis factor receptor 1, death receptor 3, death receptor 4 and death receptor 5.

The events of death receptor-mediated apoptosis are characterized by the Fas ligand/Fas receptor and TNFα/ TNF receptor models (Schulze-Osthoff et al. 1998; Wang and El-Deiry 2003). In these models, binding of the ligand to the corresponding receptor leads to clustering of receptors. As a result, the death domains in the death receptor attract the intracellular adaptor protein Fas-associated death domain (FADD) or TNF receptor-associated death domain (TRADD) with
recruitment of FADD and receptor interacting protein 1 (RIP-1). FADD then associates with procaspase-8 via dimerization of the death effector domain and forms a death-inducing signaling complex (DISC). At the DISC level, procaspase-8 and procaspase-10 are cleaved through induced proximity and yield active initiator caspases. The activation of caspase-8 or caspase-10 causes the activation of effector caspases, and leads to apoptotic cell death.

Other death receptors such as those that respond to TNF-related apoptosis inducing ligand (TRAIL) are found to convey apoptotic signals in a similar way to Fas ligand (Wang and El-Deiry 2003). The induction of apoptosis by TRAIL also involves the DISC formation and ensuing caspase activation. Interestingly, as compared to normal cells, tumor cells are seemingly more sensitive to TRAIL treatment. This may be due to the decoy receptors expressed by normal cells which sequester the TRAIL signal (Zhang et al. 2000). This difference between normal cells and cancer cells renders TRAIL a possible treatment to selectively kill cancer cells, which is a desired strategy in cancer treatment.

On the other hand, several proteins can negatively regulate the death receptor pathways. For instance, a homologue of procaspase-8, FADD-like IL-1β-converting enzyme (FLICE)-inhibitory protein [c-FLIP], can bind to FADD and caspase-8, rendering them biologically inactive (Kataoka et al. 1998; Scaffidi et al. 1999). A protein called Toso also has been shown to block Fas-induced apoptosis in T cells via inhibition of caspase-8 processing (Hitoshi et al. 1998).

It has been reported that there are two types of cells that employ a different
mechanism to induce apoptosis following death receptor stimulation (Figure 1.5) (Scaffidi et al. 1999). In type I cells, activated caspase-8 is sufficient to directly activate the effector caspases and cause the following apoptotic events. In type II cells, the amount of activated caspase-8 is too small to directly induce apoptosis by itself. Instead, caspase-8 relays the apoptotic signal through the mediation of Bid, which connects the death receptor-mediated pathway with the intrinsic apoptosis pathway and thus amplifies the apoptotic signal (Li et al. 1998). Bid is a member of the Bcl-2 family and truncation of Bid by caspase-8 leads to the translocation of Bid from cytosol to mitochondria (Chou et al. 1999). As a pro-apoptotic protein, truncated Bid causes changes in mitochondrial proteins in a way favoring apoptosis. The sum of these changes leads to release of cytochrome c and subsequently drives activation of caspase-9. Caspase-9 removes the prodomain of other family members of caspases and activates the effector caspases, which then execute the task of cell death by acting on their target proteins. Therefore, in type II cells, death receptor-mediated apoptosis recruits the mitochondria to amplify the apoptotic signal initiated from death receptors.
Figure 1.5 Model of the two CD95 signaling pathways. (A) In type I cells CD95 triggering leads to strong caspase-8 activation at the DISC which bypasses mitochondria directly leading to activation of other caspases such as caspase-3 and subsequently to apoptosis. (B) In type II cells only a little DISC is formed leading to the activation of mitochondria, which in turn results in cleavage of caspase-8 and caspase-3. Therefore, blocking activation of mitochondria by Bcl-2 or Bcl-xL inhibits apoptosis only in type II cells. Red box, death domain; blue box, death effector domain. (Adopted from Scaffidi et al., 1999)

1.3.3.2 Intrinsic pathway

The intrinsic pathway is also called the mitochondria-mediated apoptotic pathway. Being different from the extrinsic pathway that is triggered upon stimulation of death receptors, intrinsic apoptosis is induced by a wide range of non-death receptor-mediated stimuli which act directly on intracellular events. The stimuli inducing intrinsic apoptosis may act in a positive or negative way (Elmore 2007). Negative signals involve the absence of certain growth factors, hormones
and cytokines which are important for maintaining cell growth. The negative signals lead to an imbalance between cell growth and cell death by loss of cell growth signal, thus activation of apoptosis. The stimuli that positively induce intrinsic apoptosis include a large variety of signals such as radiation, toxins, hypoxia, viral infection and free radicals.

Both the negative signals and positive signals cause changes in the mitochondrial membrane which include the opening of mitochondrial permeability transition pore and loss of mitochondrial membrane potential (Elmore 2007). These mitochondrial membrane changes allow release of two groups of pro-apoptotic proteins into the cytosol, and thereby activate the caspase cascade (Saelens et al. 2004). The first group of released proteins such as cytochrome c and Smac/DIABLO contributes to the following activation of caspase-9 (Du et al. 2000; Goldstein et al. 2000; Garrido et al. 2006). In the cytosol, cytochrome c associates with Apaf-1 and then with procaspase-9 to form the apoptosome. The assembly of the apoptosome leads to autocleavage and activation of caspase-9 (Chinnaiyan 1999; Hill et al. 2004).

On the other hand, Smac/DIABLO has been demonstrated to negatively regulate inhibitors of apoptosis proteins (IAP), and thereby increases the sensitivity of cells to apoptotic signals (van Loo et al. 2002; Schimmer 2004). The second group of released proteins from mitochondria to cytosol includes pro-apoptotic proteins like apoptosis inducing factor (AIF), endonuclease G and caspase-activated DNase (CAD) (Elmore 2007). These proteins contribute to mitochondria-mediated apoptosis as a
late event in apoptosis. After releasing into the cytosol, AIF can further translocate to the nucleus to cause DNA fragmentation as well as condensation of peripheral nuclear chromatin (Joza et al. 2001). This nuclear change caused by AIF is considered to be a relatively early event and hence referred by Susin et al. as “stage I” condensation (Susin et al. 2000). Endonuclease G is the enzyme responsible for the production of oligonucleosomal DNA fragments (Li et al. 2001). As implicated in its name, cleavage of CAD by caspase-3 is needed for the activation of CAD. The functional CAD exerts its enzyme activity and causes oligonucleosomal DNA fragmentation (Susin et al. 2000). The DNA fragmentation caused by CAD produces more prominent and advanced nuclear condensation. Thus, it is defined by Susin et al as “stage II” nuclear condensation (Susin et al. 2000). Together, endonuclease G and CAD contribute to DNA fragmentation and formation of DNA ladder, the key feature of apoptosis.

The mitochondrial events are largely regulated by Bcl-2 family proteins (Gross et al. 1999). The Bcl-2 family proteins can either be pro-apoptotic or anti-apoptotic. The pro-apoptotic Bcl-2 family members include Bcl-10, Bax, Bak, Bid, Bad, Bim, Bik, and Blk; while Bcl-2, Bcl-x, Bcl-XL, Bcl-XS and Bcl-w belong to anti-apoptotic Bcl-2 family proteins. As mentioned earlier, Bid can cross-link the extrinsic apoptotic pathway and the intrinsic apoptotic pathway by relaying apoptotic signal from caspase-8 to the mitochondria (Li et al. 1998).

Another pro-apoptotic protein Bad plays an important role in intrinsic apoptosis (Hengartner 2000). Phosphorylated Bad is sequestered in the cytosol by
its inhibitor protein 14-3-3. Pro-apoptotic proteins such as p53 can cause unphosphorylation of BAD and thus lead to separation of BAD and 14-3-3 (Yang et al. 1995; Zha et al. 1996). Consequently, BAD translocates from cytosol to nucleus where it neutralizes the anti-apoptotic effects of Bcl-2 and Bcl-XL. As a result, the mitochondrial membrane potential is disrupted and cytochrome \(c\) is released into the cytosol. These events trigger the activation of caspase-9 and activation of effector caspases follows.

On the other hand, Bcl-2 and Bcl-XL represent Bcl-2 family proteins that play important roles in preventing apoptotic cell death (Hengartner 2000). Studies have shown that overexpression of Bcl-2 or Bcl-XL contributes to the resistance of cancer cells to apoptosis (Gross et al. 1999; Hengartner 2000; Elmore 2007). Bcl-2 and Bcl-XL prevent the formation of mitochondrial apoptosis-induced channel (MAC) (Dejean et al. 2006). During the apoptotic events, anti-apoptotic Bcl-2 and Bcl-XL are down-regulated, while pro-apoptotic Bak and Bax are activated and associated together to form MAC in the outer mitochondrial membrane (Dejean et al. 2006). The formation of MAC mediates cytochrome \(c\) release into the cytosol, triggering the commitment step of the mitochondrial apoptotic cascade (Dejean et al. 2006). Therefore, pro-apoptotic and anti-apoptotic Bcl-2 family proteins work in coordination to regulate the function of mitochondria. Apoptosis occurs when the balance is interrupted in a way favoring pro-apoptotic signals.
1.3.4 Promoting apoptosis as strategy against cancer

To maintain tissue homeostasis, cell growth and cell death have to be well balanced. Apoptosis plays an important role in removing unwanted or damaged cells by causing self-destruction. In cancer cells, the apoptotic pathways have various defects which make the cells insensitive to apoptotic signals (Igney and Krammer 2002). Dysregulation of apoptosis causes too much cell growth and too little cell death, leading to the unlimited growth of cancer cells. Indeed, as described by Hanahan and Weinberg, evasion of programmed cell death is one of the six essential characteristics of cancer (Hanahan and Weinberg 2000). Therefore, restoration of the apoptosis machinery of cancer cells can be a useful strategy against cancer.

The death receptors of the TNF superfamily can serve as potential targets for promoting apoptosis in cancer. TNF-α is a cytokine intensely involved in the immune system and inflammation. Apart from its role in inflammation, TNF-α has been shown to activate the death-receptor mediated apoptotic pathway through its stimulation on corresponding death receptor (Wang and El-Deiry 2003). However, the apoptosis-inducing capacity of TNF-α is relatively weak, as a result of counteraction by its simultaneous activation of the anti-apoptotic protein NF-κB (Natoli et al. 1998; Rath and Aggarwal 1999). Notably, TNF-α induces the binding of TRADD to the death domain located in the intracellular TNF receptor. TRADD then binds to FADD, which causes the recruitment of procaspase-8 and leads to the cascade of apoptotic events. On the other hand, TRADD can bind to tumor necrosis factor receptor associated factor 2 (TRAF2). TRAF2 in turn activates a protein
kinase which is the upstream signal of NF-κB signaling pathway. Consequently, NF-κB enhances the transcription of anti-apoptotic proteins such as c-FLIP, Bcl-2 and cIAP1/cIAP2, which interfere with the death receptor-mediated apoptosis.

Combined treatments with TNF-α and inhibitors of NF-κB have been shown to increase the apoptosis-inducing effect of TNF-α in cancer cells (Nakanishi and Toi 2005). The clinically used anticancer drug 5-fluorouracil induces apoptosis through stimulation on death receptor CD95 (Eichhorst et al. 2001). Studies have shown that 5-fluorouracil increases CD95 expression through p53-dependent transcriptional regulation (Muller et al. 1998). Simultaneously, it leads to upregulation of CD95 ligand through SAPK/JNK pathway (Eichhorst et al. 2000; Eichhorst et al. 2001).

Another death receptor ligand TRAIL has attracted much attention due to its selective induction of apoptosis in cancer cells (Gura 1997; Sadarangani et al. 2007). Many cancer cell lines are sensitive to TRAIL treatment. In addition, TRAIL has been shown to induce apoptosis and cause tumor regression in animal models without causing significant adverse effects (Mishier et al. 1975). Combination of TRAIL with other anticancer agents such as irinotecan or paclitaxel has shown synergistic effects against cancer growth in mouse model (Singh et al. 2003; Ravi et al. 2004). Although the development of TRAIL is still in trial phases, its potential providing a more effective and safe anticancer treatment has earned itself continuous attention from scientists and clinicians.

The overexpression of anti-apoptotic Bcl-2 family proteins is a common approach employed by cancer cells to evade apoptosis (Igney and Krammer 2002).
An abnormal chromosomal translocation t(14;18) has been found in over 60% of human follicular B-cell lymphomas. This chromosomal translocation leads to overexpression of Bcl-2 protein, which largely contributes to the lymphoma development. In addition, Bcl-2 protein is found to be overexpressed in other cancer types such as lung, breast, pancreatic and colorectal cancers (Thomadaki and Scorilas 2006). Approaches targeting anti-apoptotic Bcl-2 proteins have been developed. These approaches include using small molecules that inhibit Bcl-2, developing antisense oligonucleotide against Bcl-2 and synthesizing peptides which interact with and inhibit Bcl-2 (Fesik 2005). For instance, the antisense oligonucleotide Genasense has been developed to inactivate the mRNA of Bcl-2 gene, thereby reducing Bcl-2 protein expression (Frantz 2004). Having been shown to be effective for treatment of lymphoma \textit{in vitro}, Genasense had entered clinical trials. It failed to receive FDA approval due to disappointing results from phase III trial in malignant melanoma and multiple myeloma (Bedikian et al. 2006). More recently, a phase III trial in refractory chronic lymphocytic leukemia (CLL) was conducted to seek approval of Genasense (O'Brien et al. 2007). Unfortunately, FDA did not approve this new drug application. A five-year follow-up study of the CLL trial showed a significant increase in patient survival by using Genasense (Rai et al. 2008), and therefore an amended new drug application has been submitted and currently under review by FDA. Other agents to inhibit Bcl-2 family proteins such as Gossypol, ABT-737 and GX15-070, are evaluated in phase I/II clinical trials (Kand and Reynolds 2009).
The phosphatidylinositol 3-kinase (PI3K)-Akt pathway plays an important role in cell growth and survival (Vivanco and Sawyers 2002). In many cancers, PI3K-Akt pathway is deregulated in a way that results in overgrowth of cancer cells. The activation of PI3K-Akt pathway not only renders tumor cells more competencies for growth, but also provides a mechanism for cancer cells to evade apoptosis (Hennessy et al. 2005). The function of Akt signaling is achieved by its regulation of downstream signals (Vivanco and Sawyers 2002). On one hand, Akt signaling brings about activation of anti-apoptotic proteins. For instance, Akt activates the NF-κB signaling pathway, which in turn activates the transcription of pro-survival proteins. It can also promote cell growth through activating its downstream signal, mammalian target of rapamycin (mTOR), which is a protein kinase regulating stimulation of cell proliferation. On the other hand, Akt signaling inactivates pro-apoptotic proteins. One such protein is the Bcl-2 family member protein, BAD. The unphosphorylated form of BAD can function to antagonize the anti-apoptotic Bcl-2 and Bcl-XL, thereby disrupting the mitochondria potential and initiating caspase cascade. Once phosphorylated by Akt, BAD loses its function and gets sequestered by its inhibitor 14-3-3 protein (Datta et al. 1997).

Another class of protein inactivated by Akt is the forkhead family transcription factors. These factors can induce transcriptional activation of CD95L and contribute to death receptor-mediated apoptosis (Suhara et al. 2002). Akt inhibition of these factors leads to reduced CD95L, therefore reversing the apoptosis-inducing effects of these factors. In addition, Akt can inhibit the function
of the tumor suppressor p53 by activating MDM2, which is the inhibitor of p53 protein (Mayo and Donner 2001). The evasion of cancer cell from apoptosis through enhanced Akt activity thus provides the basis that this pathway be targeted to restore tumor apoptosis.

Inhibitors of the PI3K family have been developed and tested for their apoptosis-inducing effects in cancer cells (Hennessy et al. 2005). Two PI3K inhibitors, wortmannin and LY294002 have been demonstrated to exert anticancer activity in vitro and in vivo (Schultz et al. 1995; Hu et al. 2000). In addition, these two compounds have been shown to sensitize tumors to other chemotherapeutic treatments (Hu et al. 2002). Unfortunately, these compounds are not suitable to be developed into anticancer agents since they lack selectivity against PI3K isoforms (Stein 2001). Instead, they have been widely used as pharmacological tools in the study of PI3K-Akt pathway.

Another target of this pathway for restoring tumor cell apoptosis is the downstream effector protein mTOR. Inhibitors of mTOR include macrolide rapamycin, CCI-779 and RAD001 (Eng et al. 1984; Shi et al. 1995; Dudkin et al. 2001). These compounds have shown inhibitory effects on tumor growth both in vitro and in vivo. They also synergize with other chemotherapeutic agents such as tamoxifen (deGraffenried et al. 2004). Because of its effectiveness against cancer, CCI-779 has been under clinical evaluation for treatment of patients with renal cell carcinoma and glioblastoma (Hennessy et al. 2005). Furthermore, the combination of CCI-779 with aromatase inhibitor letrozole is under a phase III clinical trial for
Chapter 1

treatment of metastatic breast cancer (Hennessy et al. 2005). However, more detailed understanding of the mTOR pathway is warranted to validate the treatments aimed to target mTOR. Advances in research to elucidate this signaling pathway will also help to develop more specific mTOR inhibitors with higher therapeutic efficacy and less adverse effects.

Tumor suppressor p53 plays a critical role in inducing cell cycle arrest and cell death in response to DNA damage (Ljungman 2000). As a result, p53 has been called the “the guardian of the genome” because of its protective role in genome mutation and maintenance of gene stability. Mutation of p53, which results in circumvention of apoptosis, occurs in around 50% of human cancers (Bullock and Fersht 2001). The loss of p53 function accelerates cancer tumorigenesis in many ways (Bullock and Fersht 2001). Firstly, mutated p53 is not able to repair DNA damage, and the consequent accumulation of DNA damage forms the genetic basis for cancer development. Secondly, p53 can induce cell cycle arrest and prevent the continuous growth of cells suffering from DNA damage through transcriptional activation of the p21 cyclin kinase inhibitor gene. This allows DNA repair to set in before cells are released out of cell cycle arrest. In cancer cells with p53 mutation, non-functioning p53 is not able to hold damaged cells for damage repair. Thirdly, p53 can induce apoptosis if DNA damage is beyond repair, thereby eliminating the chance of existence of cells with instable genome. Cancer cells have a defective p53-mediated apoptotic pathway, thereby leading to excessive cell growth but diminished cell death (Bullock and Fersht 2001). The inactivation of p53 can either
be caused by the mutation of p53 or be a result of overexpression of its negative regulator, MDM2. Therefore, the strategies restoring the function of p53 as tumor suppressor include (1) delivery of the p53 gene into cells with inactive p53 proteins and, (2) reducing the binding of p53 to overpressed MDM2 (Fesik 2005). The p53 gene therapy drugs such as INGN201 and INGN201 have been under clinical trials for their effectiveness against a variety of cancer types (Zhang 2002). Being a negative regulator of p53, MDM2 binds to p53 protein and leads to the proteasomal degradation of p53 (Shieh et al. 1997). Several types of human cancers have been reported to overexpress MDM2 (Bueso-Ramos et al. 1993; Bueso-Ramos et al. 1996; Watanabe et al. 1996). Blocking p53-MDM2 interaction is one way to prevent p53 degradation and re-establish p53 function. Using p53 analogues to prevent the binding of MDM2 to p53 has been shown to effectively sensitize cancer cells to apoptosis, especially in cells expressing wild-type p53 (Vassilev et al. 2004). Nutlin is a small-molecule MDM2 inhibitor developed based on this strategy (Vassilev et al. 2004). It causes accumulation and activation of p53 in cancer cells, and consequently induces cell cycle arrest in G1 and G2 phase. Most importantly, it inhibits tumor growth in xenograft nude mouse models without causing significant damage to normal organs (Vassilev et al. 2004). These findings highlight the importance of p53 in suppressing tumor formation and regulating apoptosis in cancer cells.

In addition to the targets discussed above, there are many other pathways that can be targeted for the induction of apoptosis in cancer cells. Promoting apoptosis
has been one of the most potent defenses against cancer. Indeed, the conventionally used chemotherapeutic agents exert their anticancer effects largely through the induction of apoptosis in tumor cells (Kaufmann and Earnshaw 2000). With the expansion of knowledge on apoptosis and its underlying mechanisms, more effective anticancer agents may be developed by targeting specific molecules of the apoptotic pathways.
1.4 Prostaglandins in cancer development

1.4.1 Overview of prostaglandins

Prostaglandins are 20-carbon atom fatty acid derivatives, which are structurally composed of hydroxylated fatty acids and a five-member carbon ring. The prostaglandins are classified into several types (A to I) according to their structural differences in the carbon ring. A number is added after the type designation to indicate the number of double bonds in the fatty acid side chains (Karmali 1983). All prostaglandins in the body are derived from polyunsaturated fatty acids (as depicted in Figure 1.6). Specifically, arachidonic acid is the precursor of prostaglandins with two double bonds in the side chain (Karmali 1983). Therefore, the production of these prostaglandins depends on the availability of arachidonic acid. There are two kinds of key enzymes involved in the production of prostaglandins. One is phospholipase A2 (PLA₂) family of enzymes, which is responsible for release of arachidonic acid from membrane phospholipids; the other one is the cyclooxygenase (COX) family of enzymes, controlling the rate-limiting conversion of arachidonic acid to prostaglandins.
Chapter 1

Figure 1.6 Prostaglandin biosynthesis cascade. Membrane phospholipids can be cleaved by PLA$_2$ family enzymes at the sn-2 position to release arachidonic acid. Arachidonic acid is subsequently converted by COX enzymes (COX-1 and -2) to prostaglandin G2 and then prostaglandin H2. Prostaglandin H2 is further converted to series-2 tissue specific prostaglandins. PG, prostaglandin; TX, thromboxane.

1.4.1.1 PLA$_2$ family enzymes

As a substrate for further conversion to prostaglandins, arachidonic acid plays a critical role in the formation of prostaglandins. In the cells, the basal levels of arachidonic acid are usually low, and thus limit the synthesis of arachidonic acid metabolites. The formation of arachidonic acid is tightly controlled by PLA$_2$ family enzymes (Murakami et al. 1997). The PLA$_2$ family comprises a group of enzymes that cleave membrane phospholipids from the sn-2 position and subsequently release fatty acids, the predominant form being arachidonic acid (Murakami et al. 1997).

The PLA$_2$ family enzymes can be generally categorized into three classes: secretary PLA$_2$ (sPLA$_2$), calcium-dependent cytosolic PLA$_2$ (cPLA$_2$) and calcium-independent PLA$_2$ (iPLA$_2$) (Six and Dennis 2000). The class of sPLA$_2$
enzymes can be further classified into ten groups (I-III, V and IX-XIV) based on their genetic sequence (Six and Dennis 2000). The sPLA₂ enzymes share several characteristics. Firstly, they are all between 14-19 kDa. Secondly, their enzymatic activation requires millimolar concentrations of calcium. Thirdly, as their names describe, they are secreted from cells and generally found in the extracellular space.

According to the classification system based on gene sequences, cPLA₂ family enzymes include mainly group IV PLA₂ enzymes which are further subclassified into type IVA, IVB AND IVC. These enzymes have a high molecular mass of 85, 114 and 61 kDa respectively. They are found in the cytosolic fraction of almost all the cell types studied. In response to increase in intracellular calcium levels, cPLA₂ enzymes translocate to membranes and subsequently hydrolyze the phospholipids on the cell membrane for the release of arachidonic acid (Durstin et al. 1994). Thus, one unique characteristic of cPLA₂ family enzymes is their expression in the membrane. The preference for phospholipid precursor of arachidonic acid is another unique characteristic of cPLA₂ enzymes (Clark et al. 1991). Additionally, cPLA₂ enzymes have been suggested to be involved in receptor-activated mitogen-activated signaling cascade (Lin et al. 1993). The phosphorylation of cPLA₂ enzymes by kinases of the mitogen-activated protein kinase cascade results in an enhancement of their enzymatic activity (Lin et al. 1993).

iPLA₂ enzymes are represented by group VI enzymes, which tend to share some similarities with both sPLA₂ and cPLA₂ (Balsinde and Dennis 1997). Similar to sPLA₂, iPLA₂ enzymes have no substrate specificity for phospholipid precursors of
arachidonic acid. The similarities between iPLA$_2$ and cPLA$_2$ include their comparable molecular mass, cellular location and some catalytic elements. A unique feature of iPLA$_2$ enzymes is that their activation does not require the involvement of calcium (Balsinde and Dennis 1997).

The hydrolysis of membrane phospholipids by PLA$_2$ family enzymes represents a critical primary step in the arachidonic acid signaling cascade and the formation of lysophospholipids. As rate-limiting enzymes controlling the release of arachidonic acid, PLA$_2$ enzymes provide the essential substrate for COX enzymes to convert into prostaglandins.

1.4.1.2 COX enzymes

Conversion of arachidonic acid to prostaglandins is essentially the main arachidonic acid metabolic pathway (Rosenfeld 1988). COX enzymes are the key enzymes responsible for the production of prostaglandins from arachidonic acid. Under the catalysis of COX enzymes, prostaglandins are formed by the cyclization of the central five carbons in the 20-carbon polyunsaturated chain of arachidonic acid (Karmali 1983). There are two steps involved in the formation of prostaglandins. The fatty acid is first oxidized to prostaglandin G2 and prostaglandin H2; prostaglandin H2 is subsequently converted to one of the series-2 products, including structurally related prostaglandins such as prostaglandin E2, D2, F2 and I2 (Karmali 1983).

To date, two isoforms of COX have been identified (FitzGerald 2003).
COX-1 is constitutively expressed in most tissues in the body, and is generally regarded as a housekeeping enzyme responsible for synthesis of physiologically relevant prostaglandins. Through the synthesis of prostaglandins, COX-1 executes functions such as protection of gastric mucosa, regulation of renal blood flow and platelet aggregation. On the other hand, COX-2 is undetectable in most tissues; expression of COX-2 is usually induced by a large variety of pro-inflammatory factors such as growth factors and cytokines. Being highly inducible, COX-2 is perceived to be the main enzyme responsible for prostaglandin formation in inflammation and cancer (FitzGerald 2003).

1.4.2 Prostaglandins and cancer

Prostaglandins have a wide range of physiological functions such as control of cell growth and differentiation (Wang and Dubois 2006). They act through their widely distributed and diverse receptors on various cell types (Wang and Dubois 2006). These receptors that are recognized by prostaglandins belong to the family of cell surface seven-transmembrane G protein-coupled receptors. The binding of receptors by the corresponding prostaglandins activates a complex series of downstream signals which subsequently exert the cellular effects.

In addition to the maintenance of normal physiological function, prostaglandins also play important roles in pathological conditions such as pain, fever, inflammation and cancer development (Wang and Dubois 2006). Among the prostaglandins, prostaglandin E2 (PGE₂) plays a predominant role in promoting
cancer progression (Wang and Dubois 2006). The central role of PGE2 in
tumorigenesis has been supported by experiments involving animals with deleted EP1
and EP4 receptors, which show findings that the occurrence of aberrant crypt foci
induced by colon carcinogen is reduced (Watanabe et al. 1999; Mutoh et al. 2002).
Disruption of another PGE2 receptor, EP2, effectively reduces the number and size of
intestinal polyps in a mouse carcinogenesis model (Sonoshita et al. 2001).
Furthermore, PGE2 has been shown to induce cancer cell growth and proliferation in
vitro (Sheng et al. 2001).

These studies indicate that PGE2 may serve as a target for cancer prevention.
Indeed, inhibition of PGE2 production has been demonstrated to be an effective
strategy to prevent cancer occurrence and development. Clinical trials have shown
that regular use of non-steroidal anti-inflammatory drugs (NSAIDs) over 10-15 years
reduces the risk of developing colorectal cancer by 40-50% (Smalley and DuBois
1997). In addition, use of NSAIDs leads to regression of pre-existing adenomas in
patients with family adenomatous polyposis (Gupta and Dubois 2001). Together
with other experiments showing the effectiveness of NSAIDs against cancer (Watson
1998; Gupta and Dubois 2001; Harris et al. 2003), these results suggest that inhibition
of PGE2 is a useful strategy for cancer prevention.

In addition, prostaglandins are found to be overexpressed in many human
cancer types. The overexpression of prostaglandins in cancer further validates the
prevention strategy involving reduction of prostaglandin production (Wang and
Dubois 2006). These findings provide a rationale for the development of anticancer
agents that exert inhibitory effects on prostaglandin formation. As key enzymes involved in prostaglandin formation, \( \text{PLA}_2 \) and COX-2 have been suggested to be highly involved in carcinogenesis and cancer development (Wang and Dubois 2006).

1.4.2.1 \( \text{PLA}_2 \) enzymes and cancer

a. s\( \text{PLA}_2 \) and cancer

All three classes of \( \text{PLA}_2 \) enzymes have been reported to be associated with cancer development. The role of s\( \text{PLA}_2 \) in cancer promotion remains controversial. s\( \text{PLA}_2 \) IIA has been suggested by some research groups to have a role in tumor development and progression (Kennedy et al. 1998; Graff et al. 2001; Jiang et al. 2002). The expressions of s\( \text{PLA}_2 \) IIA protein and its gene have been found to be elevated in human colorectal adenomas from patients with familial adenomatous polyposis (Kennedy et al. 1998). In addition, significantly higher expression of s\( \text{PLA}_2 \) IIA has been observed in prostate cancer patients, as compared to benign prostatic glands (Jiang et al. 2002). Furthermore, prostatic s\( \text{PLA}_2 \) IIA expression increases with progression to androgen independence, and is inversely related to 5-year patient survival (Graff et al. 2001).

These findings therefore lead to the hypothesis that s\( \text{PLA}_2 \) IIA enzyme may be a prognostic indicator of cancer development and progress. However, other groups of researchers have reported contradictory findings suggesting that s\( \text{PLA}_2 \) may act as a tumor suppressor. This notion is supported by hemizygous deletion of s\( \text{PLA}_2 \) IIA gene observed in human neuroblastoma and colorectal carcinomas.
(Riggins et al. 1995; Kawamata et al. 1997). Another line of evidence supporting the suppressive role of sPLA2 in cancer development is the establishment of a positive correlation between sPLA2 IIA expression and prolonged patient survival and decreased metastatic frequency in human gastric adenocarcinoma (Leung et al. 2002). In addition, sPLA2 IIA gene has been identified as a negative regulator of intestinal adenoma formation in the condition of multiple intestinal neoplasia (MacPhee et al. 1995; Cormier et al. 1997). Given the contradictory results obtained so far, the role of sPLA2 in tumor development thus remains to be further clarified.

b. cPLA2 and cancer

Elevated expression and functional activity of cPLA2 have been observed in a range of human tumor types such as colorectal, intestinal and lung cancers, which result in high levels of arachidonic acid and prostaglandins (Kawamoto et al. 1995; Wendum et al. 2003). The expression of oncogenic Ras mutation is a common genetic characteristic of several human tumor types such as colon, prostate and lung cancers (Bos 1989). In non-small cell lung cancer, cPLA2 expression is found to be correlated with the occurrence of Ras mutation (Heasley et al. 1997). Interestingly, constitutively active Ras was able to increase the expression of both cPLA2 and COX-2 in normal lung epithelial cells (Heasley et al. 1997; Blaine et al. 2001). On the other hand, studies have shown that Ras inhibition results in decreased cPLA2 expression and activity, and thereby a reduction in prostaglandin synthesis (Heasley et al. 1997). The regulation of cPLA2 activity by Ras may be mediated by the
mitogen-activated protein kinase (MAPK) signaling pathway, which induces the phosphorylation and activation of cPLA$_2$ (Lin et al. 1993; Blaine et al. 2001). Considering the frequent mutation of Ras gene in non-small cell lung cancer, these studies provide a possible mechanism for the high expression of PGE$_2$ frequently observed in this type of lung cancer.

The role of cPLA$_2$ in lung cancer development was further established by a knock-out animal study (Meyer et al. 2004). In this study, as compared to wild-type control group, deletion of the cPLA$_2$ gene significantly reduced tumor formation by 43%. The levels of prostaglandins are also lower in tumors of cPLA$_2$-null mice than those in wide-type mice. These studies suggest that cPLA$_2$ promotes pathogenesis of lung tumors through the production of prostaglandins.

cPLA$_2$ also plays an essential role in the development of intestinal tumors. In an animal model of human family adenomatous polyposis, knockout of cPLA$_2$ gene reduced the size of small intestinal polyps by approximately 11-fold, but had little effect on decreasing the number of polyps (Takaku et al. 2000). In another similar study, knockout of cPLA$_2$ not only reduced the size of tumors, but also significantly reduced the number of tumors by 80% (Hong et al. 2001). In another study using COX-2 knockout mice, a similar inhibitory effect on intestinal tumor formation is observed (Oshima et al. 1996). Altogether, these studies show a critical role of cPLA$_2$ in cancer pathogenesis, and accordingly the inhibition of cPLA$_2$ expression and activation may be a useful strategy for prevention of cancer development.
c. iPLA$_2$ and cancer

iPLA$_2$ enzymes are the most recently identified PLA2 family members. Studies suggest that these enzymes do not appear to be directly involved in stimulating arachidonic acid release (Balsinde and Dennis 1997). Instead, they are important for phospholipid fatty acid remodeling (Balsinde et al. 1995; Balsinde and Dennis 1997). Therefore, iPLA$_2$ enzymes are generally regarded as “housekeepers” within cells by controlling phospholipid levels and maintaining homeostasis. Compared to sPLA$_2$ and cPLA$_2$, the role of iPLA$_2$ in human cancers is not well investigated. Two members of the iPLA$_2$ enzymes, Group VIA and VIB PLA$_2$, have been shown to be expressed in cultured cell lines including pancreatic, kidney and brain cancer cells (Ma et al. 1998; Zhang et al. 2005; Bao et al. 2006). iPLA$_2$ enzymes have also been shown to mediate cell growth in cancer cell models (Bao et al. 2006; Saavedra et al. 2006). Although these studies may indicate the involvement of iPLA$_2$ in cancer, the exact role of iPLA$_2$ in cancer development remains largely unknown because of the lack of \textit{in vivo} studies on iPLA$_2$.

1.4.2.2 COX enzymes and cancer

COX enzymes include COX-1 and COX-2. As a housekeeping enzyme, COX-1 is constitutively expressed in tissue cells (FitzGerald 2003). Under the pathological conditions, COX-2 is the main enzyme that can be induced by a wide range of stimuli to produce prostaglandins (FitzGerald 2003). The importance of COX-2 in tumorigenesis was first noted by the efficacy of aspirin and other NSAIDs
to reduce the risk of colon cancer (Gupta and Dubois 2001). These inhibitors of COX-2 also promote tumor regression in experimental animal models of colon cancer as well as colon cancer patients (Gupta and Dubois 2001). COX-2 has been found to be overexpressed in a wide range of cancer types such as colorectal, lung, breast, prostate, bladder, stomach and pancreas cancers (Gupta and Dubois 2001; FitzGerald 2003). Several studies have suggested that COX-2 has an important role in promoting cancer development. In one study, knockout of COX-2 gene significantly reduces the number of intestinal polyps in mice (FitzGerald 2003). In other studies, overexpression of COX-2 is found to induce carcinogenesis of breast and skin in animal models (Liu et al. 2001; Muller-Decker et al. 2002). The tumor promotion effect of COX-2 has also been demonstrated in human cancer cell lines in which inhibition of COX-2 activity effectively suppresses cancer cell proliferation (Hsu et al. 2000). Therefore, COX-2 can serve as a valid target for cancer prevention. Additionally, since COX-2 is highly inducible by pro-inflammatory factors, agents that can inhibit the induction of COX-2 may be as effective in preventing tumor formation as COX-2 inhibitors.

1.4.3 Prostaglandins as target for cancer prevention

Because of the important role of prostaglandins in cancer development, a variety of approaches have been explored for the control of prostaglandin formation. The inhibition of prostaglandin formation can be achieved by reducing arachidonic acid release or by suppressing the conversion of arachidonic acid to prostaglandins.
1.4.3.1 Inhibition of arachidonic acid release

The release of arachidonic acid is tightly controlled by the PLA$_2$ family enzymes (Laye and Gill 2003). Among these enzymes, cPLA$_2$ may become a target for cancer chemoprevention because of its close association with cancer development (Nakanishi and Rosenberg 2006). Downregulation of cPLA$_2$ expression or its activity would decrease the release of arachidonic acid, thereby inhibiting cancer development. In many cancer types, dysregulation of cPLA$_2$ is often observed (Laye and Gill 2003; Nakanishi and Rosenberg 2006). The high expression and activity of cPLA$_2$ has been illustrated by the transcriptional activation of cPLA$_2$ by oncogenic Ras in non-small cell lung cancer (Heasley et al. 1997). Ras can activate MAPK signaling pathway, which subsequently leads to the activation of transcriptional factors such as SP1 and c-Jun (Blaine et al. 2001). These transcriptional factors can bind to the promoter region of cPLA$_2$ gene, thus initiate the transcription of $cPLA_2$ gene (Blaine et al. 2001). In addition, a downstream signal of c-Jun, epidermal growth factor receptor, can lead to translocation and phosphorylation of cPLA$_2$, thus increases its enzymatic activity (Sato et al. 1997). Therefore, it can be postulated that the inhibition of these pathways leading to cPLA$_2$ expression and activation may reduce cPLA$_2$-catalyzed release of arachidonic acid. Similarly, specific enzyme inhibitors of PLA$_2$ enzymes can also result in a reduction in arachidonic acid release. For instance, a cPLA$_2$ inhibitor Wyeth-1 was found to effectively inhibit tumor growth in a xenograft prostate cancer model (Patel et al. 2008). A small interfering RNA against cPLA$_2$ was also found to be effective in
reducing prostate cancer growth (Patel et al. 2008). To date, although there are no clinical trials investigating the effectiveness of cPLA$_2$ inhibitors as cancer preventive agents, inhibition of cPLA$_2$ is certainly a promising strategy to be employed for cancer prevention.

1.4.3.2 Inhibition of arachidonic acid conversion to prostaglandins

The cancer prevention strategy targeting prostaglandins initiates from the findings that NSAIDs effectively reduce cancer occurrence (Gupta and Dubois 2001). NSAIDs, as inhibitors of COX enzymes, have been used to treat inflammation, fever and pain for over a century. Unexpectedly, a combined use of two common NSAIDs (indomethacin and sulindac) for the purpose of pain relief was reported to have cured a patient with rectal polyps (Waddell and Loughry 1983). Subsequent studies have found that neoplastic lesions contain elevated levels of prostaglandins, suggesting a role of prostaglandins in tumorigenesis (Rolland et al. 1980; Rigas et al. 1993; Uefuji et al. 2000). It is thus hypothesized that reducing prostaglandins by COX inhibition may underlie the cancer preventive mechanism of NSAIDs, which to date, has been supported by multiple lines of evidence obtained from $\textit{in vitro}$ and $\textit{in vivo}$ work, as well as from clinical trials (Gupta and Dubois 2001). However, traditional nonselective COX inhibitors cause damage to the gastric mucosa that thus pose safety issues regarding the use of NSAIDs as chemopreventive agents; this side effect is found to be related to inhibition of COX-1 (Gupta and Dubois 2001). Therefore, efforts have been made to develop selective COX-2 inhibitors that retain
the anti-inflammatory and chemopreventive activity but exert less toxicity to the gastrointestinal system.

Specific COX-2 inhibitors such as celecoxib and rofecoxib have been developed. They are very effective agents in relieving inflammation and pain. In addition, celecoxib has been shown to be very effective in preventing colorectal carcinogenesis in high-risk patients (Williams et al. 2000; Phillips et al. 2002; Huls et al. 2003). However, COX-2 selective inhibitors are found to increase the risk for heart attack and stroke (Gupta and Dubois 2001; Bresalier et al. 2005; Solomon et al. 2005). As such, rofecoxib was withdrawn from the market in 2004. The mechanism underlying this possible cardiovascular side effect of COX-2 specific inhibitors is still unknown and further elucidation is required. Nevertheless, the inhibition of COX-2 has been demonstrated to be an effective strategy for cancer chemoprevention. Compounds that are able to reduce prostaglandin formation either through inhibition of COX-2 expression or COX-2 enzyme may possess chemopreventive potential against cancer.
1.5 Matrix metalloproteinases and tumor invasion

1.5.1 Overview of tumor invasion

Tumor invasion and metastasis is one of the six essential characteristics of cancer and metastasis is responsible for 90% of human cancer deaths (Hanahan and Weinberg 2000). Tumor metastasis causes the spread of cancer from its primary site to distant organs, and ultimately makes surgical resection impossible. The high mortality rate of cancer is largely associated with cancer invasion and metastasis (Bogenrieder and Herlyn 2003). The occurrence of tumor metastasis comprises a complex series of sequential and interrelated events (Chambers et al. 2002; Steeg 2006). These events include invasion of adjacent tissues, intravasion, transport through the circulatory system, arrest at a distant vascular bed, extravasation and growth as a secondary colony and angiogenesis. Disruption of the basement membrane and subsequent tumor invasion represent critical early steps required for tumor metastasis; changes in tumor cell-cell adherence and on the extracellular matrix allow the tumor cells to detach from their original sites and invade into adjacent tissues or blood vessels (Steeg 2006).

The cell-cell adhesion is regulated by a class of transmembrane proteins called cadherins through protein-protein interaction involving their extracellular domains (Cavallaro and Christofori 2004). In addition, they can interact with catenins and the actin cytoskeleton using their intracellular domain (Cavallaro and Christofori 2004). The members of cadherin proteins have distinct functions. For instance, E-cadherin is often expressed in epithelial tissues. It plays an essential
role in maintaining cell-cell adhesion and thereby blocks invasion. On the contrary, N-cadherin is normally expressed on mesenchymal cells and its function is to facilitate cell binding to stroma, thus to assist tumor invasion. Tumor invasion is often accompanied by the loss of E-cadherin expression or function and increased expression of N-cadherin (Cavallaro and Christofori 2004).

In addition to the cell-cell adhesion changes, successful tumor invasion requires remodeling of extracellular matrix which will facilitate cell migration through tissues (Steeg 2006). The remodeling of tumor microenvironment is usually achieved by proteolytic degradation of extracellular matrix. Matrix metalloproteinases (MMPs) are prime candidates for the remodeling of tumor microenvironment since they are able to digest the various structural components of the extracellular matrix (Coussens et al. 2002).

1.5.2 Matrix metalloproteinases

1.5.2.1 The MMP family

MMPs are a family of zinc-dependent proteinases capable of degrading extracellular matrix proteins; more than 20 human MMPs have been identified and the number of MMP members is still increasing (Nelson et al. 2000). Based on their preferred substrate, these MMPs can be classified into collagenases, gelatinases, stromelysins and matrilysins. Alternatively, these MMPs are named by a sequential numbering system according to their structures. All MMPs share common structural domains, such as a pre-region, a pro-region and an active catalytic region (Overall
and Lopez-Otin 2002). These domains have different function: the pre-region is to target for enzyme secretion; the pro-region is to maintain the enzyme in a latent state; the active region contains a zinc-binding site for catalytic activation.

Apart from these basic domains, many MMPs have other structural components such as the hemopexin region, fibronectin-like region and transmembrane domain (Overall and Lopez-Otin 2002). The functions of these regions include recognition of substrate, binding to inhibitors and attaching to cell surface. For instance, gelatinases A and B (MMP-2 and MMP-9, respectively) have a unique insertion of three head-to-tail cysteine-rich repeats in the catalytic region (Sternlicht and Werb 2001). Having similar structures with collagen-binding type II repeats of fibronectin, these inserts allow MMP-2 and MMP-9 to digest collagen and elastin (Murphy et al. 1994; Shipley et al. 1996).

The MMPs with transmembrane domain are called membrane-type MMPs (MT-MMPs). One function of MT-MMPs is to allow anchorage of secreted MMPs and trigger the activation of these MMPs (Nelson et al. 2000). The secreted MMPs that are known to be activated by MT-MMPs include MMP-2 and MMP-13 (Strongin et al. 1995; Kinoh et al. 1996; Knauper et al. 1996).

1.5.2.2 Regulation of MMP activity

To create an extracellular environment favoring cancer invasion, the proteolytic activity of MMP family enzymes must be activated to degrade the extracellular matrix components. The activation of MMPs is controlled at three
different levels: transcription, proenzyme activation and inhibition by endogenous MMP inhibitors (Sternlicht and Werb 2001).

a. Transcriptional regulation

The biological function of MMPs is largely controlled at the transcriptional level (Sternlicht and Werb 2001). Because of differences in their gene structures, MMP family members are regulated differently by transcriptional stimuli, which results in different expression patterns for each individual MMP member. The majority of MMP genes are usually expressed at low levels under normal quiescent conditions, but in pathological conditions such as inflammation, wound healing and cancer, copies of MMP transcripts can increase rapidly (Overall and Lopez-Otin 2002). In cancer pathogenesis, MMP genes are transcriptionally activated by various stimulatory factors such as phorbol esters, cytokines, growth factors and oncogene products (Sternlicht and Werb 2001). These stimulatory factors are usually originated from stroma, inflammatory cells or tumor cells (Sternlicht and Werb 2001).

Several signaling pathways have been proposed to mediate the induction of \(MMP\) gene transcription by these stimulatory factors. MAPK signaling pathways are highly involved in the regulation of \(MMP\) gene transcription (Sternlicht and Werb 2001). The MAPK family enzymes such as extracellular signal-regulated kinase (ERK), c-Jun NH2-terminal kinase (JNK) and p38 MAPK have all been shown to stimulate gene transcription of MMP family enzymes, and thereby lead to increased
protein expression of their protein levels (Gum et al. 1997; Simon et al. 1998; Johansson et al. 2000; Lakka et al. 2002). These MAPK signal transduction pathways converge at the transcription factor activator protein-1 (AP-1), which binds to the promoter region of most MMP genes and activate the transcriptional process (Overall and Lopez-Otin 2002). AP-1 is a heterodimer composed of the two oncoproteins, c-Jun and c-Fos. Studies have suggested that c-Fos is mainly activated by ERK signaling, while c-Jun is the downstream signal of JNK and p38 MAPK (Shaulian and Karin 2002). Several other signaling pathways are also found to be associated with MMP gene transcription. NF-κB signaling is one of the most important regulators for MMP gene transcription. It has been found to induce gene expression of MMP-1, 3, 9, 13 and 14 (Bond et al. 1999; Han et al. 2001). Other transcriptional regulators leading to MMP gene expression include signal transducers and activators of transcription (STATs), ETS family of oncoproteins, T-cell factor 4 and CAS-associated zinc-finger protein (Overall and Lopez-Otin 2002). STATs is known to mediate interferons-induced MMP gene expression; ETS oncoproteins bind to PEA3 sites located in MMP gene promoter region and consequently activate gene transcription; T-cell factor 4 and CAS-associated zinc-finger protein activates gene expression of MMP-1, 3 and 7 (Overall and Lopez-Otin 2002).

b. Proenzyme activation

MMP proteins are synthesized as the inactive zymogens or proenzymes (Nelson et al. 2000). Therefore, the conversion of MMP proenzymes into their
active forms represents a critical step in regulating MMP function. The latency of MMP proenzymes is maintained by MMP prodomain which covers and prevents hydration of the zinc-containing active site (Sternlicht and Werb 2001). The protection on the active site is achieved by an unpaired cysteine residue near the C-terminal end of the propeptide domain. To activate proenzymes of MMP, the cysteine-zinc interaction has to be destroyed or the propeptide domain has to be removed (Sternlicht and Werb 2001). The concept of cysteine-switch mechanism of MMP activation has been demonstrated by structural analysis of pro-MMP-2 and pro-MMP-3 (Becker et al. 1995; Morgunova et al. 1999). Most MMP proteins are secreted to the extracellular environment as inactive proenzymes. However, several MMP members such as MMP-27, MMP-11 and MT-MMPs are secreted as active forms (Sternlicht and Werb 2001). These enzymes have a furin-like enzyme recognition motif located between the propeptide and catalytic domains. This motif makes it possible that these enzymes be activated by intracellular furin-like proprotein convertase before they are secreted (Pei and Weiss 1995). For those MMP enzymes without the furin-like motif, the activation of proenzymes usually occurs after they are secreted to the extracellular space. In the extracellular space, they are activated by other MMP members or by serine proteinases that have the ability to remove the prodomains (Sternlicht and Werb 2001). MMP-2 is activated at the cell surface by MT-MMPs with the involvement of TIMP-2 in a multi-step process (Sternlicht and Werb 2001). The N-terminal domain of TIMP-2 can bind to MT-MMP, which is located on the membrane. The C-terminal domain of TIMP-2,
on the other hand, can bind to pro-MMP-2 and thus anchor MMP-2 enzymes on the cell surface. As a result, the adjacent MT-MMP is able to cleave and activate pro-MMP-2. A residual portion of the MMP2 prodomain is subsequently removed by another MMP-2 molecule to allow for fully activation of MMP-2 (Deryugina et al. 2001).

c. Inhibition by endogenous inhibitors

The blocking of MMP enzyme activity by endogenous inhibitors is another mechanism to regulate MMP function. The MMP inhibitors include α2-macroglobulin and tissue inhibitors of metalloproteinases (TIMPs) (Overall and Lopez-Otin 2002). α2-Macroglobulin is present in the plasma and tissue fluids, thus representing a major inhibitor of MMPs in tissue fluids. Comparatively, TIMPs are more specific inhibitors of MMPs and may perform their inhibitory effects locally. Another difference between these two kinds of inhibitors is that α2-macroglobulin often results in irreversible clearance of MMPs, while the inhibition of MMPs by TIMPs is reversible (Sottrup-Jensen and Birkedal-Hansen 1989; Sternlicht and Werb 2001). The TIMP family comprises four members, namely TIMP-1, -2, -3 and -4, where each inhibits different MMP members. They can bind MMPs tightly through non-covalent force to form a 1:1 MMP-inhibitor complex (Brew et al. 2000). As a result, the MMP-inhibitor complex is unable to bind to substrate of MMP and MMP proteolytic activity is inhibited. TIMP expression levels have been suggested to negatively correlate with tumorigenesis (Khokha et al. 1989). However, many
studies have reported an increase in TIMP expression during tumor development (Jiang et al. 2002; Overall and Lopez-Otin 2002). These findings may indicate a host-protective response during tumor progression or they may indicate that TIMPs have more functions other than inhibition of MMPs. Indeed, in the case of MMP-2, TIMP binding seems to be necessary for the conversion of pro-MMP-2 to its active form (Overall and Lopez-Otin 2002).

1.5.3 MMP-2 and MMP-9

1.5.3.1 The role of MMP-2 and MMP-9 in tumor invasion

Among the MMPs identified so far, MMP-2 and MMP-9 are believed to be the most relevant enzymes involved in tumor invasion (Brinckerhoff and Matrisian 2002). According to their substrate specificity, MMP-2 and MMP-9 are also referred as gelatinase A and gelatinase B, respectively. They play a key role in the degradation of type IV collagen and gelatin, which are the main components of extracellular matrix (Brinckerhoff and Matrisian 2002). Therefore, they are the enzymes most responsible for the remodeling of extracellular matrix and subsequent tumor invasion.

Increased expression of MMP-2 and MMP-9 has been observed in many human cancer types such as ovarian, prostate and breast cancers (Duffy et al. 2000; Egeblad and Werb 2002). In addition, expression levels of MMP-2 and MMP-9 are generally correlated with the stage of tumor progression (Egeblad and Werb 2002). For instance, MMP-2 expression is found to be associated with tumor grade in
melanomas (Vaisanen et al. 1996). In the same kind of tumor type, conversion from radial growth phase to vertical growth phase and subsequent invasion occurs with an increased expression of MMP-9 (MacDougall et al. 1995). The ratio of activated form and inactive form also seems to correlate with cancer malignancy. For example, although MMP-2 is widely expressed in breast cancers, metastatic cancers seem to have higher levels of activated MMP-2 (Brown et al. 1993). A series of *in vivo* studies has established the role of MMP-2 and MMP-9 in promoting metastatic spread of tumor cells to the lung after intravenous injection of bladder, fibrosarcoma and lung cancer cells (Bernhard et al. 1994; Kawamata et al. 1995; Tsunezuka et al. 1996). Observations from experiments investigating tumor metastasis have also ascertained the involvement of MMP-2 and MMP-9 in tumor invasion and metastasis (Bernhard et al. 1994; Kawamata et al. 1995; Hasegawa et al. 1998). On the contrary, treatment of mice with TIMP-1 has been shown to reduce the number of metastatic lung nodules after intravenous injection of melanoma cells (Khokha 1994).

*In vitro*, the negative regulation of TIMPs on tumor invasion has been demonstrated in a Matrigel basement membrane model, which mimics the tumor extracellular environment (Albini et al. 1991). In an *in vivo* tumor intravasation model using chick chorioallantoic membrane that has been developed to quantify tumor cells invading the bloodstream and circulating to other sites, MMP-9 expression is found to be indispensable for intravasation of human epidermal carcinoma (Koop et al. 1994).

These studies together suggest that MMP-2 and MMP-9 alter the extracellular environment in a way that favors tumor invasion and subsequent
metastatic spread to distant locations. Additionally, it has been reported that MMP-2 and MMP-9 not only facilitate tumor invasion by degradation of extracellular components, but also contribute to the process of angiogenesis (Handsley and Edwards 2005). For instance, decreased and delayed angiogenesis has been observed respectively in MMP-2-null and MMP-9-null mice as compared to their wild-type counterparts (Itoh et al. 1998). Since angiogenesis is a fundamental step in the metastatic spread and growth of tumors, the involvement of MMP-2 and MMP-9 in angiogenesis further highlights their importance in tumor invasion.

1.5.3.2 Regulation of MMP-2 and MMP-9

Although MMP-2 and MMP-9 share functional similarities as gelatinases, large differences exist between their structures (Overall and Lopez-Otin 2002). These structural differences underlie the different mechanisms regulating MMP-2 and MMP-9 activity. MMP-9 activity is controlled by gene transcription, proenzyme activation and inhibition by endogenous inhibitors, whereas MMP-2 activity is regulated by the two latter factors and lacks regulation at the transcriptional level (Overall and Lopez-Otin 2002).

MMP-2 is usually constitutively expressed with only modest regulation at the transcriptional level under certain conditions. The insensitivity of MMP-2 to transcriptional regulation is largely due to its gene promoter structures. It has been identified that regulatory elements located in the MMP-2 promoter region include AP2, p53, SP-1, CREB and PEA3 (Overall and Lopez-Otin 2002). These regulatory
elements do not respond to a large variety of stimulatory signals such as growth factors and cytokines, and it is only under selective conditions that can stimulate these regulatory elements, will MMP-2 gene expression be possibly induced.

Conversely, MMP-9 is highly inducible by a wide range of stimuli. The promoter region of MMP-9 contains multiple DNA-binding sites which recognize AP-1, NF-κB, SP-1, TIE, KRE, RCE and PEA3 (Overall and Lopez-Otin 2002). The regulatory units such as AP-1 and NF-κB binding sites can be activated by various inflammatory stimuli which can trigger MAPK and NF-κB signaling pathways. For instance, protein kinase C (PKC) activator phorbol ester is a strong inducer of MMP-9 expression through activating MAPK and NF-κB signaling pathways (Van den Steen et al. 2002). Cytokines such as TNF-α and IL-1β can cause similar transcriptional activation of MMP-9 through the regulatory elements located on MMP-9 promoter region. Using β-galactosidase activity as an indicator, MMP-9 promoter activity has been detected in animal models carrying a β-galactosidase reporter gene (Kupferman et al. 2000).

Both MMP-2 and MMP-9 are synthesized and secreted as proenzymes without proteolytic activity. To perform their functions, both of them need the conversion from proenzymes to active forms. As described in Section 1.5.2.2, the mechanism for pro-MMP-2 activation involves MT-MMP and TIMP-2 (Sternlicht and Werb 2001). However, the activation of pro-MMP-9 is currently not clear (Fridman et al. 2003). Several mechanisms underlying the activation of pro-MMP-9 have been proposed. It has been hypothesized that MMP-9 is activated by
interaction with cell surface components (Fridman et al. 2003). For instance, some studies suggest that MMP-9 is activated by multi-step process with the involvement of plasminogen/MMP-3 and urokinase plasminogen activator (uPA). Plasminogen is firstly cleaved by uPA to produce plasmin, and plasmin in turn activates pro-MMP-3, followed by activation of pro-MMP-9 by MMP-3.

Since uPA is bound to its cell surface receptor uPAR, the complex activation of pro-MMP-9 has been indicated to occur at the cell surface. Interestingly, knockout of uPA gene, but not MMP-3 gene, reduced MMP-9 activation in mouse models (Fridman et al. 2003). This may indicate that while plasmin is indispensible for MMP-9 activation, MMP-3 may not be the only partner mediating signaling from plasmin to MMP-9 activation.

Other mechanisms in play include the activation of pro-MMP-9 by human tumor-associated trypsin-2 or by chymotrypsin-like proteinase (Fridman et al. 2003). In addition, several purified MMPs including MMP-2, -3, -7, -10 and -13 are found to cleave the 92-kDa pro-MMP-9 protein to the 82-kDa active MMP-9 enzyme in vitro (Fridman et al. 2003). More intriguingly, pro-MMP-9 has been reported to have proteolytic activity without removal of its propeptide (Okamoto et al. 2001; Bannikov et al. 2002; Gu et al. 2002). Indeed, the presumed 82-kDa active MMP-9 has been commonly reported to be undetectable in cultured cells and cancer tissues (Fridman et al. 2003). Conversely, high expression of 92-kDa pro-MMP-9 is frequently found in invasive cancers.
These findings thus suggest that the activation of pro-MMP-9 may not be necessarily associated with a significant change in molecular size. To date, despite its clear involvement in tumor invasion, how the pro-MMP-9 is actually converted to the active form remains elusive. In addition to transcriptional regulation and zymogen activation as also mentioned in Section 1.5.2.2, MMP-2 and MMP-9 can be further regulated by endogenous inhibitors such as TIMPs and α2-macroglobulin (Overall and Lopez-Otin 2002). For instance, MMP-2 is regulated by TIMP-2, while MMP-9 can form a noncovalent complex with TIMP-1 (Fridman et al. 2003).

1.5.3.3 MMP-2 and MMP-9 as targets for control of tumor invasion

Given the importance of MMP-2 and MMP-9 in tumor invasion, strategies intended to exert control over metastatic spread of cancer cells have been undertaken to modulate MMP-2 and MMP-9 activity. One most-explored strategy is to reverse the invasion-promoting effects of these MMPs by direct inhibition of their activities (Coussens et al. 2002). The direct inhibition of enzyme activity is especially suitable for MMP-2, as the gelatinase is largely regulated at the posttranscriptional level. The endogenous MMP inhibitors have been initially considered as potential therapeutics for cancer malignancies. However, the development of TIMPs into useful drugs has been hampered by technical difficulties (Coussens et al. 2002). Efforts have been focused on development of small molecule inhibitors of MMPs. One of the successful small molecule inhibitors is MPI SC-44463, a broad MMP inhibitor. It has exhibited inhibitory effect on tumor metastasis in mouse models.
Batimastat, a broad-spectrum hydroxamate inhibitor, is found to reduce breast cancer metastasis in a mouse xenograft model (Sledge et al. 1995). However, despite the effectiveness of these MMP inhibitors in animal models, results of clinical trials evaluating these MMP inhibitors have been disappointing (Coussens et al. 2002). One of the problems is that many MMP inhibitors in clinical trials are broad-spectrum MMP inhibitors. Different MMP family members have distinct roles in tumor development and some may even have anti-tumor activity (Coussens et al. 2002); broad-spectrum inhibition of MMPs may not show beneficial effects to cancer patients. A better understanding of the molecular function of each MMP and development of more specific MMP inhibitors may improve the therapeutic value of MMP inhibitors.

Tumors are frequently found to grow in an inflammatory environment that is characterized by increased expression of cytokines, growth factors and other proinflammatory factors (Federico et al. 2007). Many of these inflammatory factors can increase MMP expression through transcriptional regulation. Due to the highly inducible nature of MMP-9, an increased expression of MMP-9 has been frequently found in invasive cancers. Therefore, downregulation of MMP-9 expression can be a plausible approach to inhibit cancer invasiveness. The effectiveness of targeting MMP-9 to inhibit tumor invasion has been demonstrated by various in vitro and in vivo studies. For instance, a hairpin RNA-triggered interference of MMP-9 synthesis not only significantly inhibits invasiveness of glioblastoma cell line in vitro, but also decreases the metastasis of gliomas in vivo (Lakka et al. 2004). A naturally
occurring active compound, curcumin, has been shown to reduce the number of metastases in an animal prostate cancer model through downregulation of MMP-9 expression and activity (Hong et al. 2006). Natural flavonoids have also been shown to inhibit metastasis of gliomas \textit{in vitro} and \textit{in vivo}, with their anti-invasive effects attributed to the modulation of MMP-9 expression (Shen et al. 2006).
1.6. Aims of study

Alcoholic extracts of *Poria cocos* have shown anti-inflammatory and cancer chemopreventive activities. However, the exact compounds responsible for these activities and the underlying mechanisms are largely undefined. Previous studies carried out in our laboratory had demonstrated that the alcoholic extracts of *Poria cocos*, with triterpenoids as the main constituents, exhibited growth inhibitory effects against a variety of cancer cells. On the basis that alcoholic extracts of *Poria cocos* potentially served as a rich source of triterpenoids with promising therapeutic benefits, this PhD project was undertaken to extend the currently limited research and understanding of triterpenoids with anticancer properties. Studies were carried out to fulfill the following aims.
Aim 1: To isolate and identify triterpenoids contained in alcoholic extracts of *Poria cocos*; and to evaluate the antiproliferative effects of the isolated triterpenoids against human-derived cancer cells

Aim 2: To investigate the apoptosis-inducing effects of triterpenoid(s) that had demonstrated effective antiproliferative activities

Aim 3: To investigate the cancer chemopreventive mechanism of PA with focus on its possible effect on prostaglandin production

Aim 4: To evaluate the *in vitro* anti-invasive effects of triterpenoids on breast cancer migration

Experimental approaches to achieve the above aims and corresponding results are presented in Chapter 2, 3, 4 and 5 respectively.
Chapter 2: Isolation and identification of triterpenoids from *Poria cocos*

**Summary**

In this chapter, the procedures used to isolate triterpenoids from *Poria cocos* are described. Isolated compounds were subjected to physical and spectroscopic analyses for elucidation of their chemical structures. In total, eight compounds were obtained and identified as (1) pachymic acid, (2) dehydropachymic acid, (3) 3-acetyloxy-16α-hydroxytramentenic acid, (4) polyporenic acid C, (5) 3-epi-dehydropachymic acid, (6) 3-epi-dehydrotumulosic acid, (7) tumulosic acid, and (8) 29-hydroxypolyporenic acid. Among these identified lanostane-type triterpenoids, compound 8 was novel, whereas the other compounds were known triterpenoids. Cell viability assays were conducted on these compounds, of which most of them showed moderate cytotoxicity on human non-small cell lung cancer A549 cells.

**2.1 Introduction**

*Poria cocos* has been widely used in Traditional Chinese Medicine for its diuretic, sedative and tonic effects (Yuan and Lin 2000). As a famous herb with a long history of use, *Poria cocos* has been acclaimed for its mild yet effective biological functions. To understand the underlying mechanisms responsible for its health-promoting effects, various studies have been carried out in an attempt to identify the main active components contained in *Poria cocos*. In the aqueous extracts of *Poria cocos*, polysaccharides are identified as the main active compounds (Lee et al. 2004; Zhang et al. 2006). The biological activities of polysaccharides include modulation of immune system and anticancer activity (Kanayama et
Polysaccharides are shown to inhibit proliferation, as well as induce differentiation of leukemic cells (Chen and Chang 2004). They are also reported to cause cell cycle arrest and induce apoptosis in breast cancer cells (Zhang et al. 2006). On the other hand, the alcoholic extracts of *Poria cocos* are found to have anti-inflammatory and anticancer activities. Chemical analyses have identified that triterpenoids are the main components in the alcoholic extracts (Zhong and Liu 2002; Setzer and Setzer 2003; Akihisa et al. 2007). A complex mixture of triterpenoids or a single purified triterpenoid from *Poria cocos* have both been shown to possess certain anticancer properties, including inhibition of DNA polymerases, inhibition of tumor cell growth induced by TPA and inhibition of DNA topoisomerase II (Kaminaga et al. 1996; Kaminaga et al. 1996; Ukiya et al. 2002; Akihisa et al. 2004; Li et al. 2004; Mizushina et al. 2004).

Earlier efforts in our laboratory had demonstrated the antiproliferative effect of alcoholic extracts of *Poria cocos* on a wide range of cultured human cancer cells. Pachymic acid, a lanostane-type triterpenoid largely contained in *Poria cocos*, was also isolated on earlier occasions in the laboratory, and subsequent study yielded a novel finding that it was able to prevent the growth of both androgen-responsive and -insensitive prostate cancer cells by inducing apoptosis in a dose- and time-dependent manner (Gapter et al. 2005). Encouraged by these preliminary findings, we sought to conduct further research on *Poria cocos* with the objective to uncover and evaluate other pachymic acid-like compounds with possible anticancer activities. The approach employed began with the isolation and purification of potential anticancer compounds from *Poria cocos* using bioassay-guided fractionation. The purified compounds were then subjected to structural identification, after
which their possible antiproliferative effects against cancer cells were evaluated *in vitro*.

### 2.2 Materials and methods

#### Materials

Dried sclerotia of *Poria cocos* were purchased from Tai Shan Medicinal Hall in Singapore, with origins from An Hui Province, China. All sorbents for chromatographic isolation, such as Silica gel (60-200 µm), Silica gel 60 (40-63 µm), LiChroprep® RP-18 (40-63 µm), and pre-coated silica gel 60 TLC plates, were purchased from Merck KGaA (Darmstadt, Germany). MTT was purchased from Sigma (St. Louis, MO).

#### Thin Layer Chromatography

Thin Layer Chromatography (TLC) was used to facilitate column chromatographic separation from several aspects. Firstly, TLC was used to select and determine suitable mobile phase and stationary phase before each separation using column chromatography. The crude extracts or fractions to be separated were tested on both silica gel (normal phase) and C18 (reverse phase) TLC plates as well as on different solvent systems. The conditions that gave the best separation on the TLC plates were adopted as the separating condition in column chromatography. Secondly, TLC was performed to assess polarities of compounds contained in collected fractions. Fractions with similar retention factor values were combined and subjected to the next separation step. Thirdly, TLC was used to determine if pure compounds were obtained. The compounds were tested on both normal phase and reverse phase TLC plates using at least three different solvent systems. The TLC plate was
inspected under UV light before using 6% sulphuric acid as a visualization agent to develop the spots. If all the plates showed the presence of one single spot, it would indicate that a pure compound was successfully isolated.

**Column chromatography**

Column chromatography was used for the separation and isolation procedures. Based on the TLC results, normal phase or reverse column chromatography was chosen for the specific separation steps. For normal phase column chromatography, silica gel with particle size of 40-63µm was used as the stationary phase in the column. The elution solvent system was used to suspend silica gel, and then the mixture of silica gel and solvent was poured into the column. After continuously flushing the silica gel with solvent, a homogeneous and compact silica gel bed was finally formed. The fractions to be separated was dissolved in the minimum amount of solvent and added drop by drop onto silica gel (particle size: 63-200 µm) so as to form an even coating of compounds on the silica gel. The silica gel coated with compounds was then loaded onto the top of the compact silica gel bed in the column.

For reverse phase column chromatography, LiChroprep® RP-18 (40-63 µm) was used as the stationary phase in the column. LiChroprep® RP-18 was suspended in methanol and poured into the column. The stationary phase was made compact by the continuous flow of solvent. The fractions to be separated was dissolved in a small amount of solvent and added directly onto the compact stationary phase.

For both methods, compounds were eluted by gradient elution. Solvent was added
continuously and the polarity of solvent system was changed gradually to allow for best separation of compounds. The fractions were collected in different volumes depending on the size of the column. The collected fractions were then evaporated under reduced pressure using a rotary evaporator to remove excess solvent. Guided by TLC results, fractions containing similar compounds were combined together for next round of separation.

Cell culture

A549 human non-small cell lung cancer cells were obtained from American Type Culture Collection (Rockville, MD) and cultured in F12 Ham Kaighn’s modification (F12K) medium supplemented with 10% fetal bovine serum (FBS; Hyclone Laboratories, Logan, UT), 10 mM HEPES (AppliChem, Darmstadt, Germany) and antibiotics (100 U/ml penicillin G and 100 µg/ml streptomycin; Invitrogen, Carlsbad, CA). The cells were maintained at 37 °C in a 5% CO₂ humidified incubator.

Cell proliferation assay

The effects of collected fractions or isolated compounds on the proliferation of A549 cells were determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. MTT is a tetrazolium salt which can be converted by mitochondrial dehydrogenases in living, metabolically active cells to dark blue formazan crystals (Denizot and Lang 1986; Carmichael et al. 1987). These formazan crystals can be solubilized by isopropanol or other solvents. The absorbance of the dissolved formazan is proportional to the number of viable cells over a wide range (Denizot and Lang 1986; Carmichael et al. 1987).
Generally, the more viable cells present, the more formazan crystals are formed, and thus the higher absorbance reading is measured (Denizot and Lang 1986; Carmichael et al. 1987). Therefore, the MTT assay offers a convenient method for quantitatively measuring cell viability.

The MTT assay was performed in 96-well microplate. $5 \times 10^3$ A549 cells suspended in 100 µl of culture medium were seeded into each well and the plate was left to incubate overnight. Dried fractions or pure compounds were reconstituted in DMSO at a stock concentration of 20 mg/ml and further diluted to the working concentrations for the experimental procedures. DMSO was employed as a negative control in all experiments. After overnight incubation, the cells were treated with fresh medium containing the desired concentrations of fractions or pure compounds. The plates were left to incubate for the indicated period of time, after which 11 µl of 5 mg/ml MTT dissolved in PBS was added into each well. The plate was left to incubate for 4 hours to develop the dark blue formazan crystals. The developed formazan crystals were then dissolved in 111 µl of solubilizing solution (stock composition: 125ml anhydrous isopropanol containing 10% Triton X-100 and 0.1N HCl). Absorbance was measured immediately at 590 nm using an automatic microplate reader (Tecan GENios, Salzburg, Austria). The effect of various treatments on cell proliferation was expressed as percent cell viability compared with DMSO treated control cells set at 100%.

MTT assays were also performed on pure compounds to determine the IC$_{50}$ values. IC$_{50}$ was obtained by plotting the percent cell viability values against a dose response curve using GraphPad Prism (GraphPad Software, San Diego, USA).
2.3 Results

2.3.1 Separation of alcoholic extracts into four fractions

Sclerotia of *Poria cocos* (4 kg) were crushed and then extracted three times with 95% ethanol (12 liter) under reflux for 3 h. The ethanol solution was combined and evaporated in vacuum to give a crude extract (18 g). The crude extract was mixed with silica gel (particle size: 63-200 µm), and fractionated on silica (particle size: 40-63 µm) column chromatography [600 × 80mm internal diameter (i.d.)] by gradient elution using CHCl₃ and CH₃OH (100:0 → 95:5 → 80:20, with methanol added at 1% gradient). Fractions were collected, combined and subjected to further chromatography on a silica gel 60 column (600 × 80 mm i.d.) by step gradients of CHCl₃–CH₃OH (100:0 → 85:15). The collected fractions were combined on the basis of their TLC characteristics to give four pooled fractions: A (1 g), B (3.6 g), C (4.12 g), and D (1.85 g), listed in increasing order of polarity.

The effects of these four fractions on proliferation of non-small cell lung cancer A549 cells were evaluated using the MTT assay. As shown in Figure 2.1, fraction B and C showed stronger inhibitory effects on A549 cell proliferation. With a concentration of 30 µg/ml, fraction B brought about a 50% decrease on cell viability of A549 cells after 72 h treatment. Fraction C inhibited A549 cell proliferation to a similar extent at the concentration of 30 µg/ml. In contrast, fraction A and D exhibited weak cytotoxicity. At 30 µg/ml, fraction A only slightly reduced A549 cell viability to 80%. Fraction D did not exert apparent cytotoxicity on A549 cells at all three concentrations tested. Based on these observations, it was concluded that fractions B and C might contain potential anticancer compounds. Therefore, fractions B and C were subjected to repeated column
chromatography to isolate the pure components in these fractions.

Figure 2.1 Effects of four pooled fractions obtained from crude extract of *Poria cocos* against A549 cell proliferation. A549 cells were treated with different concentrations (10, 30 and 100 µg/ml) of fraction A-D for 72 h, respectively. After treatment, MTT assay was performed to determine cytotoxicity of these fractions on A549 cells as detailed in Materials and methods. Columns, means of three experimental readings; bars, standard deviation.

2.3.2 Isolation of pure compounds

Fraction B (3.6 g) was subjected to reverse column chromatography on an Octadecyl Silane (ODS) column (500 g) with step gradient elution conducted with CH$_3$OH–H$_2$O (60:40 → 85:15), which gave rise to two subfractions: fraction B-a and B-b. Both sub-fractions were taken into the next phase for further separation and isolation of active pure compounds. Fraction B-a was subjected to an ODS column chromatography with step gradient elution conducted with CH$_3$OH–H$_2$O (70:30 → 85:15) to give a pure compound (compound 1: 1.5 g) and a mixture of compounds. The mixture was further fractionated on silica gel 60 column chromatography (500 × 40 mm i.d.) to give compounds 2 (43 mg) and 3 (7 mg), using isocratic elution with hexane-ethyl acetate-CH$_3$OH (70:30:5). Fraction B-b was subjected to chromatography on a silica gel 60 column (500 × 40mm i.d.), from which two pure
compounds, 4 (230 mg) and 5 (24 mg), were isolated with step gradient elution of 
CHCl$_3$–CH$_3$OH (98:2 → 93:7). Fraction C (4.12 g) was subjected to chromatography on a 
silica gel 60 column (600 × 80 mm i.d.). Step gradient elution was conducted with 
CHCl$_3$–CH$_3$OH (97:3 → 85:15) to give 2 sub-fractions, fraction C-a and C-b. Fraction C-a 
was subjected to an ODS column chromatography with step gradient elution conducted with 
CH$_3$OH–H$_2$O (55:45 → 70:30) to isolate compound 7 (80 mg). Fraction C-b was 
fractionated on silica gel 60 column chromatography (500 × 40 mm i.d.) with isocratic elution 
of CHCl$_3$–CH$_3$OH (93:7) to give compounds 6 (110 mg) and 8 (28 mg). A scheme was 
provided in Figure 2.2 to illustrate the extraction and isolation processes.

![Figure 2.2 Schematic diagram illustrating the extraction schemes leading to isolation of eight triterpenoids from *Poria cocos*](image-url)
2.3.3 Identification of purified compounds

The identification work was performed by Dr. Zhou Liang, Department of Pharmacy, NUS. The chemical structures were determined using physical and spectroscopic tools including melting point (mp) measurement, nuclear magnetic resonance (NMR), mass spectrometry (MS) and infrared spectroscopy. The eight purified compounds were identified as (1) pachymic acid, (2) dehydropachymic acid, (3) 3-acetyloxy-16α-hydroxytrametenolic acid, (4) polyporenic acid C, (5) 3-epi-dehydropachymic acid, (6) 3-epi-dehydrotumulosic acid, (7) tumulosic acid, and (8) 29-hydroxypolyporenic acid. The chemical structures of compounds 1-8 were presented in Figure 2.3. The identification of compounds 1-7 was conducted by comparison of their physical and spectroscopic data (1H-, 13C-NMR and MS) with the corresponding compounds reported in the literature (Tai et al. 1995; Tai et al. 1995; Ukiya et al. 2002; Mizushina et al. 2004). A detailed discussion on steps undertaken leading to the identification of novel compound 8 was published in the publication entitled “Cytotoxic and anti-oxidant activities of lanostane-type triterpenes isolated from *Poria cocos*” (Zhou et al. 2008).
Figure 2.3 Chemical structures of compounds 1-8
The physical and spectroscopic features of compounds 1-8 were listed below. A summary of $^{13}$C-NMR spectral data was presented in Table 2.1. The original $^{13}$C-NMR spectral of compounds 1-8 were provided in the Appendices.

**Pachymic Acid (1):** White powder, mp 293-294 °C. ESI-MS $m/z$: 529 [M + H]$^+$; $^1$H-NMR (300 MHz, Pyridine-$d_5$) δ: 0.92 (3H, s, H-28), 0.94 (3H, s, H-29), 0.97 (3H, s, H-19), 0.99, 1.01 (ea. 3H, d, $J_6.5$ Hz, H-26, H-27), 1.13 (3H, s, H-18), 1.49 (3H, s, H-30), 2.07 (3H, s, CH3CO), 2.27 (1H, m, H-25), 2.84 (1H, dd, $J = 11$, 5.5 Hz, H-17), 2.94 (1H, m, H-20), 4.54 (1H, t, $J = 6$ Hz, H-16), 4.68 (1H, dd, $J = 11$, 4 Hz, H-3), 4.86, 4.99 (ea. 1H, s, H-31); $^{13}$C-NMR (75 MHz, Pyridine-$d_5$); Table 2.1.

**Dehydropachymic Acid (2):** White powder, mp 265-267 °C. ESI-MS $m/z$: 527 [M + H]$^+$; $^1$H-NMR (300 MHz, Pyr-$d_5$) δ: 0.90 (3H, s, H-28), 0.99 (3H, s, H-29), 1.01 (3H, s, H-19), 0.99, 1.01 (ea. 3H, d, $J_6.5$ Hz, H-26, H-27), 1.05 (3H, s, H-18), 1.50 (3H, s, H-30), 2.05 (3H, s, CH3CO), 2.30 (1H, m, H-25), 2.88 (1H, dd, $J = 11$, 5.5 Hz, H-17), 2.94 (1H, m, H-20), 4.54 (1H, t, $J = 6$ Hz, H-16), 4.70 (1H, dd, $J = 11$, 4 Hz, H-3), 4.86, 4.99 (ea. 1H, s, H-31), 5.34 (1H, d, $J = 6$ Hz, H-11), 5.59 (1H, br s, H-7); $^{13}$C-NMR (75 MHz, Pyr-$d_5$); Table 2.1.

**3-Acetyloxy-16α-hydroxytrametenolic acid (3):** White powder, mp > 300 °C. ESI-MS $m/z$: 515 [M + H]$^+$; $^1$H-NMR (300 MHz, Pyr-$d_5$) δ: 0.90 (3H, s, H-28), 0.93 (3H, s, H-29), 0.96 (3H, s, H-19), 1.13 (3H, s, H-18), 1.48 (3H, s, H-30), 1.60, 1.62 (ea. 3H, s, H-26, H-27), 2.05 (3H, s, CH3CO), 2.80 (1H, dd, $J = 11$, 6 Hz, H-17), 2.94 (1H, m, H-20), 4.53 (1H, t, $J = 6$ Hz,
H-16), 4.70 (1H, dd, \( J = 11, 4 \) Hz, H-3), 5.35 (1H, br s, H-24); \(^{13}\)C-NMR (75 MHz, Pyr-d5); Table 2.1.

**Polyporenic Acid C (4):** White needles, mp 261-263 °C. ESI-MS \( m/z \): 483 [M + H]; \(^1\)H-NMR (300 MHz, Pyr-d5) \( \delta \): 1.06 (3H, s, H-28), 1.14 (3H, s, H-29), 1.14 (3H, s, H-19), 0.99, 1.01 (ea. 3H, d, \( J = 6.5 \) Hz, H-26, H-27), 1.07 (3H, s, H-18), 1.46 (3H, s, H-30), 1.64 (1H, m, H-5), 2.88 (1H, m, H-17), 2.94 (1H, m, H-20), 4.55 (1H, t, \( J = 6 \) Hz, H-16), 4.86, 4.99 (ea. 1H, s, H-31), 5.37 (1H, d, \( J = 6 \) Hz, H-11), 5.60 (1H, d, \( J = 6 \) Hz, H-7); \(^{13}\)C-NMR (75 MHz, Pyr-d5); Table 2.1.

**3-epi-Dehydropachymic Acid (5):** White powder, mp 276-278 °C. ESIMS \( m/z \): 527 [M + H]; \(^1\)H-NMR (300 MHz, Pyr-d5) \( \delta \): 0.90 (3H, s, H-28), 0.93 (3H, s, H-29), 1.03 (3H, s, H-19), 0.99, 1.01 (ea. 3H, d, \( J = 6.5 \) Hz, H-26, H-27), 1.07 (3H, s, H-18), 1.44 (3H, s, H-30), 1.75 (1H, m, H-5), 2.86 (1H, dd, \( J = 11, 6 \) Hz, H-17), 2.94 (1H, m, H-20), 4.54 (1H, t, \( J = 6 \) Hz, H-16), 4.85 (1H, br s, H-3), 4.86, 4.99 (ea. 1H, s, H-31), 5.37 (1H, d, \( J = 6 \) Hz, H-11), 5.60 (1H, br s, H-7); \(^{13}\)C-NMR (75 MHz, Pyr-d5); Table 2.1.

**3-epi-Dehydrotumulosic Acid (6):** White powder, mp 236-239 °C. ESIMS \( m/z \): 485 [M + H]; \(^1\)H-NMR (300 MHz, Pyr-d5) \( \delta \): 1.19 (3H, s, H-28), 0.98 (3H, s, H-29), 1.11 (3H, s, H-19), 0.99, 1.01 (ea. 3H, d, \( J = 6.5 \) Hz, H-26, H-27), 1.08 (3H, s, H-18), 1.43 (3H, s, H-30), 2.30 (1H, m, H-25), 2.88 (1H, m, H-17), 2.94 (1H, m, H-20), 4.52 (1H, t, \( J = 6 \) Hz, H-16), 3.63 (1H, br s, H-3), 4.85, 4.98 (ea. 1H, s, H-31), 5.47 (1H, d, \( J = 6 \) Hz, H-11), 5.64 (1H, br s, H-7);
$^{13}$C-NMR (75 MHz, Pyr-$d_5$); Table 2.1.

**Tumulosic Acid (7):** White powder, mp 253-256 °C. ESI-MS $m/z$: 487 [M + H]$^+$; $^1$H-NMR (300 MHz, Pyr-$d_5$) $\delta$: 1.01 (3H, s, H-28), 1.06 (3H, s, H-29), 1.14 (3H, s, H-19), 0.96, 0.98 (ea. 3H, d, $J = 6.5$ Hz, H-26, H-27), 1.23 (3H, s, H-18), 1.47 (3H, s, H-30), 2.27 (1H, m, H-25), 2.84 (1H, dd, $J = 11$, 5.5 Hz, H-17), 2.94 (1H, m, H-20), 4.52 (1H, t, $J = 6$ Hz, H-16), 3.43 (1H, t, $J = 7$ Hz, H-3), 4.86, 4.99 (ea. 1H, s, H-31); $^{13}$C-NMR (75 MHz, Pyr-$d_5$); Table 2.1.

**29-Hydroxypolyporenic Acid C (8):** White powder, mp 255-258 °C. UV (MeOH) $\lambda_{\text{max}}$ nm: 236, 244, 252; IR (KBr) $\nu_{\text{max}}$ cm$^{-1}$: 3400, 2960, 1710, 1675, 1642, 1380, 1100, 900; HR-ESI-MS $m/z$: 497.3265 [M + H]$^+$; $^1$H-NMR (300 MHz, Pyr-$d_5$) $\delta$: 0.99, 1.01 (ea. 3H, d, $J = 6.5$ Hz, H-26, H-27), 1.07 (6H, s, H-18, H-28), 1.14 (3H, s, H-19), 1.42 (3H, s, H-30), 1.64 (1H, m, H-5), 2.25 (1H, m, H-25), 2.86 (1H, m, H-17), 2.92 (1H, m, H-20), 3.59, 4.17 (each 1H, d, $J = 10.5$ Hz, H-29), 4.53 (1H, t, $J = 6$ Hz, H-16), 4.86, 4.99 (ea. 1H, s, H-31), 5.44 (1H, d, $J = 6$ Hz, H-11), 5.59 (1H, br s, H-7); $^{13}$CNMR (75 MHz, Pyr-$d_5$); Table 2.1.
Table 2.1 13C-NMR spectral data for compound 1-8 (75 MHz in C5D5N; δ in ppm)

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a, b) Assignments in the same column may be interchanged; ppm; peak hidden in pyrrole.

2.3.4 Cytotoxicity test

The effects of the eight isolated compounds on A549 cell growth were evaluated using MTT assay. As shown in Figure 2.4, all these compounds exhibited cytotoxicity on A549 cells after treatment for 72 h. However, the cytotoxicity of dehydropachymic acid was relatively weak. Although dehydropachymic acid at 20 µM caused reduction in cell viability to about 70%, further elevation of concentration did not manifest stronger cytotoxicity on A549 cells. Even a high dose of 400 µM failed to reduce the viability of A549 cells below 50%. As compared to dydropachymic acid, other seven compounds showed stronger antiproliferative effects, in particular polyporenic acid C exhibited the
greatest growth inhibitory effect. These compounds generally inhibited A549 cell growth in a dose-dependent manner, with the exception of 3-epi-dehydrotumulosic acid.

Figure 2.4 Effects of compounds 1-8 on A549 cell proliferation. In a 96-well plate, A549 cells were exposed to compounds 1-8 with indicated concentrations for 72 h. Relative cell viability was then determined by MTT assay as detailed in materials and methods. Columns, means of two independent experiments repeated in triplicate; bars, standard deviation.
From the MTT data illustrated in Figure 2.4, IC\textsubscript{50} values of these compounds were calculated using GraphPad software. Table 2.2 lists the IC\textsubscript{50} values of the eight isolated triterpenoids.

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC\textsubscript{50} (μM) ± S.D.</th>
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<tbody>
<tr>
<td>1 Pachymic acid</td>
<td>30.5 ± 9.7</td>
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<tr>
<td>2 Dehydropachymic acid</td>
<td>194.3 ± 247.3</td>
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<tr>
<td>3 3-Acetyloxy-16α-hydroxytrametenolic acid</td>
<td>44.3 ± 39.1</td>
</tr>
<tr>
<td>4 Polyporenic Acid C</td>
<td>19.8 ± 5.2</td>
</tr>
<tr>
<td>5 3-epi-Dehydropachymic Acid</td>
<td>24.0 ± 12.8</td>
</tr>
<tr>
<td>6 3-epi-Dehydrotumulosic Acid</td>
<td>25.3 ± 9.2</td>
</tr>
<tr>
<td>7 Tumulosic acid</td>
<td>44.0 ± 1.2</td>
</tr>
<tr>
<td>8 29-Hydroxypolyporenic acid C</td>
<td>38.1 ± 19.9</td>
</tr>
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</table>

Table 2.2 IC\textsubscript{50} values of compounds 1-8 against A549 cell growth. IC\textsubscript{50} values were calculated from 72 h-drug exposure MTT assays and expressed as mean ± standard deviation from two independent experiments repeated in triplicate.

2.4 Discussion

Previous studies on \textit{Poria cocos}-originated triterpenoids had encouraged us to search for potential anticancer agents among these triterpenoids. Since triterpenoids are mainly contained in the alcoholic extracts of \textit{Poria cocos}, the sclerotia of \textit{Poria cocos} was extracted with ethanol to obtain a mixture of triterpenoids. The alcoholic extracts were subsequently separated into four fractions based on their TLC characteristics. Fraction A and D did not
possess considerable cytotoxic effects on lung cancer A549 cells, which implied that these fractions might not be good sources for isolation of active anticancer agents. Moreover, these two fractions also lacked cytotoxic effects on prostate cancer DU145 cells [as previously determined by Dr. Leslie Gapter, Department of Pharmacy, NUS; (Zhou et al. 2008)]. By contrast, fraction B and C display stronger cytotoxic effects on A549 cells. Similar effects were also observed on DU145 cells in experiments performed by Dr. Gapter (Zhou et al. 2008). These results suggested that fractions B and C might contain compounds with active anticancer properties. Isolation work performed on fractions B and C resulted in the purification of a total of eight compounds, among which 29-dehydroxypolyporenic acid C was a novel lanostane-type triterpenoid. Among these compounds, pachymic acid had the highest yield (1.5 g) and 3-acetyloxy-16α-hydroxytrametenolic acid had the lowest yield (7 mg). Considering the significantly higher yield of pachymic acid, it was deduced that it is the main triterpenoid present in *Poria cocos*. The eight compounds isolated belonged to the lanostane type of triterpenoids and therefore shared structural resemblance to one another. These findings further supported the notion that lanostane-type triterpenoids are the main triterpenoid type in *Poria cocos* (Tai et al. 1995; Kaminaga et al. 1996; Zheng and Yang 2008; Zheng and Yang 2008). Based on previous reports, the number of triterpenoids identified to be present in *Poria cocos* was more than eight. However, due to limited time and resources, our isolation work had only focused on the compounds with relatively high abundance. Some minor spots did appear on TLC during the isolation process, but due to low abundance, were not further purified and identified. As such, the possibility of discovering novel triterpenoids responsible for the minor spots might be missed. In addition, some compounds
might possess highly similar polarity which would not allow them to be easily differentiated on TLC. Therefore, the efficiency of separation and purification of these compounds with the use of column chromatography would be compromised. With the advance in chromatographic techniques, it is reasonable to expect that more novel triterpenoids will be separated and identified from *Poria cocos*.

With the exception of dehydropachymic acid, all the isolated compounds display moderate cytotoxic effects on A549 cells with IC$_{50}$ values ranging from 20 to 45 µM. The equipotencies in cytotoxicity registered by these compounds would indicate that the basic lanostane-type structure possibly contributed mainly to the anticancer activity of these compounds. However, it remained unexplained for the much weaker antiproliferative activity of dehydropachymic acid. Interestingly, the weak anticancer activity of dehydropachymic acid was also observed by Dr. Gapter on prostate cancer DU145 cells (Zhou et al. 2008). Although much is still unknown about the structural-activity relationship of these lanostane-type triterpenoids, results demonstrating that these triterpenoids possess moderate cytotoxicity on human cancer cells had indeed suggested the anticancer potential of these triterpenoids. The following chapters in this PhD thesis therefore focused on discussing in further detail the anticancer activity and mechanism of these *Poria cocos*-originated triterpenoids.
Chapter 3: Polyporenic acid C induces caspase-8-mediated apoptosis in human lung cancer A549 cells

Summary

In this chapter, we examined the apoptosis-inducing effect of polyporenic acid C (PPAC), one of the eight triterpenoids isolated from *Poria cocos*, on lung cancer cells. PPAC was found to inhibit proliferation of small cell lung cancer H82 and H187 cells. In addition, PPAC inhibited anchorage-dependent and –independent growth of non-small cell lung cancer A549 cells, which was associated with increase in sub-G1 cell population, positive annexin V staining, and increase in cleavage of procaspase-8, -3 and PARP. Apoptosis induced by PPAC was not accompanied by disruption of mitochondrial membrane potential nor an increase in procaspase-9 cleavage. Moreover, PPAC-induced apoptosis was inhibited by specific caspase-8 inhibitor z-IETD-fmk and pan caspase inhibitor z-VAD-fmk, and not by specific caspase-9 inhibitor z-LEHD-fmk. Taken together, the obtained results suggested that PPAC induced apoptosis through the death receptor-mediated apoptotic pathway where the activation of caspase-8 by PPAC had led to the direct cleavage of execution caspases without the involvement of mitochondria. Furthermore, PPAC was shown to suppress PI3-kinase/AKT signal pathway and enhance p53 activation, implying the involvement of an additional mechanism by which apoptosis was induced by the triterpenoid.
3.1 Introduction

Lung cancer is one of the most frequent and malignant cancers in the world. In the United States, it has been estimated that there will be 219,440 new cases and 159,390 deaths from lung cancer in 2009 (Jemal et al. 2009). Lung cancer is ranked as the second most common cancer among both men (15%) and women (14%) in the United States (Jemal et al. 2009). It is also the leading cause of cancer mortality for both men (30%) and women (26%) (Jemal et al. 2009). According to histological characteristics, lung cancer can be classified into non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). NSCLC, which represents the vast majority of lung cancers, accounts for 80% of all lung cancer cases (Travis et al. 1995; Felip et al. 2007). The prognosis for both types of lung carcinoma is poor, with overall 5 year survival rates varying from 8.9% in developing countries to 15% in the United States (Parkin et al. 2005). The prevailing morbidity and poor prognosis of lung cancer highlight the importance of searching for new and effective agents against lung cancer.

In the previous chapter, the isolation and identification of eight lanostane-type triterpenoids from Poria cocos was reported. The anti-cancer activity of these compounds was tested on NSCLC A549 cells using MTT assay. Among these compounds, PPAC showed the greatest cytotoxicity on A549 cells with an IC_{50} of 20 µM, thus indicating the potential of PPAC as an anticancer agent. Although PPAC has been previously shown to possess anti-bacterial activity (Marcus 1952; Keller et al. 1996) and inhibit EBV-EA activation by TPA (Ukiya et al. 2002), its cytotoxic effects against cancer cells has not been well studied. One study has suggested that PPAC inhibits DNA topoisomerases (Li et al. 2004), however, its anticancer potency and mechanism of antitumor action remain elusive.
Therefore, in this chapter, the apoptosis-inducing effect of PPAC on A549 cells was determined. Further studies conducted to achieve understanding of the mechanisms involved revealed the effect of PPAC on PI3-Kinase/AKT pathway and p53 protein.

3.2 Materials and methods

Reagents

Vybrant Apoptosis Assay Kit and 3,3'-dihexyloxacarbocyanine iodide [DiOC₆(3)] were purchased from Molecular Probes (Eugene, OR). Antibody for actin (I-19) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies specific for phospho-p53 (Ser15), phospho-AKT (Ser473), cleaved caspase-3 (Asp175), cleaved caspase-8 (Asp374), caspase-9, cleaved PARP, phospho-JNK (Thr183/Tyr185), goat anti-rabbit IgG-conjugated to horseradish peroxidase (HRP), and goat anti-mouse IgG-conjugated to HRP were purchased from Cell Signaling Technology (Beverly, MA). Pan caspase inhibitor (z-VAD-fmk), caspase-8 inhibitor (z-IETD-fmk) and caspase-9 inhibitor (z-LEHD-fmk) were purchased from R&D Systems (Minneapolis, MN). JNK-specific inhibitor, SP600125, was purchased from Biomol International LP (Plymouth Meeting, PA).

Cell culture and treatment

A549 human NSCLC cells were obtained from American Type Culture Collection (Rockville, MD) and cultured in F12 Ham Kaighn’s modification (F12K) medium supplemented with 10% fetal bovine serum (FBS; Hyclone Laboratories, Logan, UT), 10 mM HEPES (AppliChem, Darmstadt, Germany) and antibiotics (100 U/ml penicillin G and 100
µg/ml streptomycin; Invitrogen, Carlsbad, CA). Human SCLC cell lines H82 and H187 were purchased from American Type Culture Collection and were maintained in RPMI-1640 Medium from Sigma Aldrich supplemented with 10% fetal bovine serum, 10mM HEPES and antibiotics. The cells were maintained at 37°C in a 5% CO₂ humidified incubator. PPAC was reconstituted in DMSO at a stock concentration of 20 mg/ml and further diluted with DMSO to the working concentrations for the experimental procedures. DMSO was employed as a negative control at a final concentration ≤ 0.5% in all experiments.

Cell proliferation assay

The effect of PPAC on the proliferation of lung cancer cells (H82, H187 and A549 cells) was determined using MTT assay as described in the “Materials and methods” section of chapter 2. Briefly, 1 × 10⁴ (for H82 and H187) or 5 × 10³ (for A549) cells per well were plated in 96-well culture plates. After overnight incubation, the cells were treated for different time points with fresh medium containing the desired concentrations of PPAC. Absorbance was measured at 590 nm using a microplate reader (Tecan GENios, Salzburg, Austria). The effect of PPAC on cell viability was expressed as percent cell viability compared with vehicle treated control cells set at 100%.

Soft agar assay

The soft agar assay was performed to evaluate the ability of cells to grow in an anchorage-independent environment. Agarose (ultra pure or low-melt) was autoclaved as a 1.25% stock solution. For the base layer, 0.5% agarose medium (Ham’s F12K medium
containing 10% FCS and 0.5% ultra pure agarose) was dispensed into each well of a 6-well plate and allowed to solidify. The function of the base layer of agar medium was to prevent the attachment of cells to the culture plate. To make the cell layer, A549 cells (1.5 × 10^4) were suspended in 0.33% agarose medium (Ham’s F12K medium containing 10% FCS and 0.33% low-melt agarose) and mixed with DMSO or PPAC. This mixture was then added over the base layer in each well. The plates were incubated at 37°C under an atmosphere of 5% CO2 for 3 weeks without further medium feeding. To limit evaporative loss of water, the plates were wrapped loosely with parafilm. Colonies ≥ 0.1 mm were enumerated under a light microscope at 20 x objective (Olympus CKX41, Tokyo, Japan). Images were obtained using the Nikon Eclipse TE2000-U microscope (Nikon Corp., Tokyo, Japan).

**Propidium iodide staining assay**

To quantify PPAC-induced apoptosis, A549 cells were stained with propidium iodide (PI) and subjected to flow cytometric analysis. The cells were treated with various concentrations of PPAC for the indicated time periods. The floating and trypsinized adherent cells were pooled and the collected cell pellet was washed twice with PBS. The cells were then fixed using ice-cold ethanol at -20°C for at least 30 min, followed by incubation with 0.5 mL of PI staining solution [0.1% (v/v) Triton X-100 in PBS containing 200 µg/ml of RNase and 20 µg/ml of PI] at room temperature for 30 min. Cell cycle distribution was analyzed by a CYAN-LX flow cytometer (DakoCytomation, Fort Collins, CO) equipped with Summit v4.3 software (DakoCytomation). Cells with sub-G1 DNA (as designated by R1 in the histogram) were classified as apoptotic cells.
Apoptosis assay

A549 cells were analyzed for presence of apoptosis by annexin V-FITC labeling according to the manufacturer’s instructions (Vybrant Apoptosis Assay Kit [Molecular Probes]). 1.5 × 10^5 A549 cells in 2 ml media were seeded in each well of 6-well plates and maintained at 37°C in 5% CO₂ humidified incubator. Upon adherence, cells were incubated in fresh media with different concentrations of PPAC for the indicated time periods. The floating and trypsinized adherent cells were pooled, pelleted and washed twice with PBS. The cell pellets were suspended in 100 µl of Annexin Binding Buffer provided by the manufacturer, and stained with 5 µl of Alexa Fluor® 488 annexin V and 1 µl of 100 µg/ml PI. After incubation at room temperature for 15 min, the cells were gently mixed with 400 µl of Annexin Binding Buffer and analyzed immediately by flow cytometry as described above in “Propidium iodide staining assay”. The early apoptotic cells, which bind to annexin V-FITC and give a green fluorescence, are represented in the lower right quadrant of the histogram. The late apoptotic cells, which bind to both FITC and PI, give a red-green fluorescence and are represented in the upper right quadrant of the histogram. For experiments involving caspase inhibitors, A549 cells were pretreated with the desired inhibitor for 1 h prior to treatment with PPAC.

Analysis of mitochondrial membrane potential

The mitochondrial membrane potential (ΔΨ_m) was analyzed by labeling A549 cells with DiOC₆(3). DiOC₆(3) is a cationic lipophilic dye that can diffuse through the plasma membrane, enter the cytoplasm and finally accumulate inside the mitochondria matrix
The accumulation of DiOC<sub>6</sub>(3) is driven by the ΔΨ<sub>m</sub> and loss of this potential will lead to decreased retention of DiOC<sub>6</sub>(3) in the mitochondria (Korchak et al. 1982). Therefore, cells can be stained with DiOC<sub>6</sub>(3) to monitor the change in ΔΨ<sub>m</sub>. In our study, A549 cells were grown to semi-confluence in cell culture flasks and treated with DMSO or PPAC in fresh medium for the indicated time periods. During the final 30 min of treatment, 40 nM of DiOC<sub>6</sub>(3) was added into the culture medium. Cells were then washed twice in PBS containing 0.1% BSA (w/v), spun to a pellet, suspended in 500 µl of PBS/0.1% BSA, and analyzed by flow cytometry (DakoCytomation).

**Preparation of cell lysates**

Following desired treatment, medium containing detached cells was collected. Attached cells were collected by scraping using a rubber policeman. Both attached and floating cells were pooled and pelleted by centrifugation. The pellet was washed twice with cold PBS, and resuspended in an appropriate volume (0.1-0.5 ml, depending on size of cell pellet) of ice-cold lysis buffer (Cell Signaling Technology) containing freshly added protease inhibitors. The lysis buffer was composed of 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na<sub>2</sub>EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na<sub>3</sub>VO<sub>4</sub> and 1 µg/ml leupeptin. The protease inhibitors added were 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin and 5 µg/ml pepstatin A. After brief sonication on ice, cell lysates were centrifuged at 14, 000 × g for 15 min at 4°C to remove cell debris. The supernatant was collected and protein content was quantified using a bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL) according to the
manufacturer’s instructions. This protein assay was conducted using a 96-well plate. 200 µl of BCA reaction solution was added to each well containing 20 µl of protein samples or different concentrations of BSA. After incubation of the plate in 37°C for 30 min, absorbance values at 562 nm were measured using a microplate reader. Protein concentration of cell lysates was calculated against BSA standard calibration curve.

**Western blot analysis**

Protein mixture in cell lysates was separated using SDS-PAGE based on their molecular size. 10% Resolving gel (solution formula for two gels: 9.6 ml distilled water, 5 ml 40% acrylamide, 5 ml 1.5 M Tris pH 8.8, 200 µl 10% SDS, 200 µl 10% ammonium persulfate and 8 µl TEMED) and 5% stacking gel (solution formula for two gels: 7.25 ml distilled water, 1.25 ml 40% acrylamide, 1.25 ml 1 M Tris pH 6.3, 100 µl 10% SDS, 100 µl 10% ammonium persulfate and 8 µl TEMED) were used to cast gels for the separation process. A 10 or 15-well comb was used to create wells for sample loading. Cell lysates containing equal amount of proteins were diluted with 1:1 Laemmli sample buffer [62.5 mM Tris HCl (pH 6.8), 2% SDS, 25% glycerol, 0.01% (w/v) bromophenol blue, 5% 2-mercaptoethanol], and boiled at 100°C for 10 min. The samples were then loaded into wells in the stacking gel. The proteins were separated by 10% resolving gel under constant current of 40 mA using running buffer containing 25 mM Tris, 192 mM glycine and 0.1% SDS.

After separation of proteins by SDS-PAGE, the proteins were transferred to a polyvinylidene fluoride membrane (Millipore, Bedford, MA) under constant voltage of 25 V
at 4°C for overnight using blotting buffer containing 25 mM Tris, 192 mM glycine, 0.1% SDS and 20% methanol. The transblotted membrane was blocked with 5% (w/v) non-fat milk in TBS-T (113 mM NaCl, 25 mM Tris HCl with 0.05% Tween 20) at room temperature for 1 h, and then incubated with the primary antibody for 2 h at room temperature. After washing the membrane with TBS-T for three times, appropriate secondary antibody was added onto the membrane to incubate at room temperature for 1 h. Prior to antibody visualization using the enhanced chemiluminescence system, the membrane was rinsed in TBS-T for three times to remove excess secondary antibody. The antibody/protein complexes were visualized by enhanced chemiluminescence (Perkin-Elmer, Boston, MA) and digital images were obtained by scanning X-ray film (Eastman Kodak, Rochester, NY) or were captured directly using the FluorChem™ 9900 (Alpha Innotech, San Leandro, CA).

**Statistical analysis**

Statistical significance between treatment and control groups were analyzed using a two-tailed Student’s t-test. Values of \( p < 0.05 \) were considered statistically significant.
3.3 Results

3.3.1 Inhibitory effect of PPAC on the proliferation of lung cancer cells

In the previous chapter, PPAC was shown to inhibit A549 cell growth after 72 h treatment. To further evaluate the antiproliferative effect of PPAC, MTT assay was conducted to determine the viability of A549 cells at three different time points of PPAC treatment (24, 48 and 72 h). As shown in Figure 3.1, treatment of A549 cells with 0-200 µM of PPAC resulted in dose-dependent inhibition of cell growth at all three time points. Significant inhibition was observed with 20 µM PPAC treatment, and at 60 µM, relative cell viability had decreased to below 40% at all time points (Figure 3.1). These data thus indicated that PPAC effectively inhibited NSCLC A549 cell proliferation.

Figure 3.1 PPAC decreased the cell viability of human NSCLC A549 cells in a dose-dependent manner. Following PPAC treatment, A549 cell viability was determined by MTT assay as detailed in Materials and Methods. Columns, means of three independent experiments repeated in triplicate; bars, standard deviation. * p < 0.05; ** p < 0.01

To examine whether the growth inhibitory effect of PPAC was selective to a specific cell line, its possible antiproliferative effect was tested on two other lung cancer cell lines. H82 and H187 SCLC cells were selected for investigation. As shown in Figure 3.2,
treatment of H82 with PPAC for 72 h resulted in decrease in cell viability at high concentrations of $\geq 60$ µM, whereas viability of H187 cells began to decrease dose-dependently at a PPAC concentration of 6 µM. These results indicated that H187 cells were more sensitive to PPAC treatment than H82 cells. In addition, the growth inhibitory effect on H187 cells by PPAC was observed to be comparable to that exerted on A549 cells. These results therefore showed that PPAC was not only effective in inhibiting NSCLC A549 cell growth, but also effective against small cell lung cancer proliferation.

![Figure 3.2](image-url)

Figure 3.2 Effect of PPAC on the proliferation of SCLC H82 and H187 cells. Following treatment of H82 or H187 cells with PPAC for 72 h, cell viability was determined using MTT assay as detailed in Materials and Methods. *Columns*, means of two independent experiments repeated in triplicate; *bars*, standard deviation. *p < 0.05
3.3.2 Inhibitory effect of PPAC on colony formation of A549 cells

Cancer cells have a unique ability to form colony in anchorage-independent conditions (Hanahan and Weinberg 2000). Comparing with monolayer cell growth studies such as MTT assay, the three-dimensional anchorage-independent growth of cancer cells mimics more closely the \textit{in vivo} growth of tumor cells in a solid tumor (Agre and Williams 1983). Soft agar assay, which can be used to study anchorage-independent cell growth, therefore provides a more biologically relevant method to assess the anticancer activity of a compound (Agre and Williams 1983). The possible effect of PPAC against colony formation was investigated using soft agar assay. As shown in Figure 3.3A, 6 and 20 µM of PPAC significantly reduced the number of colonies formed by A549 cells from 70 (untreated) to 43 and 16 respectively. In the soft agar plates treated with higher doses of 60 or 200 µM PPAC, no colonies larger than 0.1 mm could be observed, suggesting that PPAC completely inhibited A549 colony formation in these concentrations. Notably, PPAC treatment not only effectively decreased the number of formed colonies, but also reduced the colony size. The colonies formed in PPAC-treated plates were generally smaller than those in control plates. Figure 3.3B shows a typical image obtained from plates treated with PPAC or DMSO control. The remarkable inhibitory effect of PPAC on A549 colony formation further affirmed the effect of PPAC against lung cancer growth.
Figure 3.3 PPAC reduced colony formation of A549 cells. (A) Colony formation ability of A549 cells was determined using soft agar assay as described in Materials and methods. Columns, means of number of colonies ≥ 0.1 mm from two independent experiments; bars, standard deviation. (B) Representative image of colony formation of A549 cells to illustrate the reduction in colony size by PPAC.

3.3.3 Induction of apoptosis in A549 cells by PPAC

To determine if PPAC suppressed A549 cell proliferation through induction of apoptosis, PPAC-treated cells were examined using two approaches. In the first approach, PPAC-treated cells were incubated with PI and analyzed for sub-G1 staining. For cells undergoing apoptosis, the DNA content is low due to loss of small fragmented DNA (Darzynkiewicz et al. 1992). By labeling DNA with PI, the low DNA content can be detected using flow cytometric analysis. Sub-G1 population represents cells with decreased PI staining and thus represents apoptotic cells. As shown in Figure 3.4A, the cell population in sub-G1 (R1) increased time-dependently from 2% to 47% within 48 h following 60 µM
PPAC treatment. PPAC also induced apoptosis after 48 h treatment in a dose-dependent manner, as shown by the increased fraction of sub-G1 cells from 5% to 51% when cells were exposed to 20 and 80 µM PPAC respectively (Figure 3.4B).

Figure 3.4 PPAC induced apoptosis in A549 cells as evaluated by sub-G1 analysis. A549 cells were stained with PI and analyzed by flow cytometry after treatment with 60 µM of PPAC for 0, 10, 24 or 48 h (A) or with 0, 20, 60 or 80 µM of PPAC for 48 h (B). Sub-G1 values in (A) and (B) were represented by the percentage of cells in R1 region. Data is representative of a single experiment repeated on three different occasions with similar results.

To ascertain that PPAC indeed induced apoptosis, an alternative approach was adopted whereby A549 cells were stained with annexin V-FITC and PI using an apoptosis assay kit. In apoptotic cells, the phospholipid asymmetry of the plasma membrane is lost and phosphatidylserine (PS) is translocated from the inner to the outer cell membrane (Bratton et al. 1997). The exposed PS is recognized and bound by FITC-conjugated annexin
V. Apoptotic cells were counted as early and late apoptotic cells, which are detected respectively in the lower right (R5) and upper right region (R3) of the histograms (Figure 3.5A and 3.5B). As depicted in Figure 3.5A, 24 h treatment with PPAC resulted in a dose-dependent increase in the number of apoptotic cells in both the early and late stages of apoptosis: vehicle control (5.6%), 60 µM (20.8%), 100 µM (33.8%). Prolonged treatment involving 48 h treatment with 60 and 100 µM PPAC increased apoptotic fractions to 30.9% and 93.9% respectively (Figure 3.5B). Taken together, the obtained results suggested that induction of apoptosis by PPAC was responsible for its growth inhibitory effect on A549 cells.
Figure 3.5 PPAC induced apoptosis in A549 cells as evaluated by annexin V labeling assay. A549 cells were exposed to 0, 60 or 100 µM of PPAC for 24 h (A) or 48 h (B) and stained with the reagents in the Vybrant Apoptosis Assay Kit, followed by flow cytometric analysis. The percentage of early apoptotic or late apoptotic cells is designated by the value at the lower right or upper right quadrant. Data is representative of a single experiment repeated on three different occasions with similar results.

3.3.4 Activation of the caspase-8-mediated apoptotic pathway by PPAC

To investigate the involvement of caspases in PPAC-induced apoptosis, experiments were carried out to assess whether PPAC treatment could activate initiator caspase-8 and -9, and the executioner, caspase-3. As shown in Figure 3.6, treatment of A549 cells with 60 µM of PPAC resulted in caspase-8 and caspase-3 cleavage, but not that of caspase-9. Cleavage of the caspase-3 substrate, PARP, also correlated with caspase-3 activation (Figure 3.6). These results implied that PPAC was likely to induce apoptosis in A549 cells through activation of caspase-8.

Figure 3.6 PA induced cleavage of caspase-8, caspase-3 and PARP. Following 24 h treatment of A549 cells with 60 µM PPAC or vehicle control, total cell protein was extracted, resolved using SDS-PAGE, and immunoblotted with the indicated antibodies. The analysis was repeated three times with similar results and a representative immunoblot is shown.
To further confirm the essential role of caspase activation in PPAC-induced apoptosis, caspase inhibitors were used to determine whether cells could be protected from apoptosis. The results presented in Figure 3.7 lent support to a caspase-8-dependent apoptotic signaling pathway: the apoptotic fractions (including early and late apoptosis) were 4.3% and 25.5% in the control and PPAC-treated cells respectively, as compared to an apoptotic cell population of 5.1% and 6.4% in the group of cells coincubated with PPAC and pan caspase inhibitor z-VAD-fmk, or PPAC and caspase-8 inhibitor z-IETD-fmk. In contrast, the caspase-9 inhibitor, z-LEHD-fmk, failed to block PPAC-induced apoptosis, where the apoptotic cell population of 28.9% was comparable to that of PPAC only-treated cells (Figure 3.7). Consistent with the flow cytometry data, pretreatment with the caspase-8 inhibitor z-IETD-fmk, but not the caspase-9 inhibitor z-LEHD-fmk, prevented the cleavage of PARP by PPAC as determined using immunoblot analysis (Figure 3.8). Collectively, these findings suggest that induction of apoptosis by PPAC is dependent on and initiated by caspase-8.
Figure 3.7 Effect of caspase inhibitors on PPAC-induced apoptosis. A549 cells were treated with vehicle control, 60 µM of PPAC for 24 h, or pretreated with 50 µM of pan caspase inhibitor z-VAD-fmk, 50 µM of caspase-8 inhibitor z-IETD-fmk or 50 µM of caspase-9 inhibitor z-LEHD-fmk for 1 h prior to treatment with 60 µM PPAC for 24 h. Treated cells were stained with the reagents in the Vybrant Apoptosis Assay Kit, followed by flow cytometric analysis. The percentage of early apoptotic and late apoptotic cells is designated by the value at the lower right and upper right quadrant respectively. The analysis was repeated in three times with similar results and a representative flow cytometric data is shown.
Chapter 3

Figure 3.8 Effect of caspase inhibitors or JNK inhibitor on PPAC-induced PARP cleavage. A549 cells were pretreated with 50 µM of caspase-8 inhibitor z-IETD-fmk, 50 µM of caspase-9 inhibitor z-LEHD-fmk or 20 µM of JNK inhibitor SP600125 for 1 h, followed by treatment with 60 µM PPAC for another 24 h. Total cellular protein was extracted, resolved using SDS-PAGE, and immunoblotted with the indicated antibodies. The analysis was repeated three times with similar results and a representative immunoblot is shown.

3.3.5 Mitochondrial membrane potential ($\Delta \Psi_m$) was not disrupted by PPAC

Caspase-8 has been demonstrated to activate caspase-3 either directly or indirectly through a mitochondria-mediated pathway. In the latter case, Bid is truncated by caspase-8, leading to the translocation of Bid from the cytosol to the mitochondria where it disrupts the $\Delta \Psi_m$ via binding to other Bcl-2 family members and triggers cytochrome c, Apaf-1 and pro-caspase-9 (Li et al. 1998). Therefore, to determine whether PPAC induced apoptosis in a mitochondrial-dependent or independent manner following caspase-8 activation, the effect of PPAC on the $\Delta \Psi_m$ was evaluated. In the experiment, A549 cells were labeled with the cationic lipophilic dye DiOC$_6$(3), which accumulates within mitochondria in a potential-dependent manner (Korchak et al. 1982). Upon disruption of the $\Delta \Psi_m$, the intracellular DiOC$_6$(3) fluorescence signal would decrease due to the impaired function of cells to retain DiOC$_6$(3). Treatment of A549 cells with PPAC for both 24 h and 48 h (20, 60 and 100 µM) did not result in any decrease in the $\Delta \Psi_m$ (Figure 3.9). Interestingly, pachymic
acid, a structurally related lanostane-type triterpenoid from *Poria cocos* that was among the eight purified and isolated triterpenoids, caused a dose- and time-dependent decrease in DiOC₆(3) fluorescence intensity in A549 cells (Figure 3.9). The results concluded that unlike pachymic acid, PPAC induced apoptosis via a mechanism independent of the mitochondria and therefore did not bring about a disruption of the ΔΨₘ. This finding was consistent with the observations that PPAC did not activate caspase-9 and inhibition of caspase-9 using caspase-9 inhibitor z-LEHD-fmk failed to protect A549 cells from PPAC-induced apoptosis (Figure 3.6, 3.7 and 3.8). Taken together, the data indicated that PPAC induced apoptosis in NSCLC cells through caspase-8-driven proteolysis and direct activation of execution caspases.
Figure 3.9 PPAC failed to affect \( \Delta \Psi_m \) while pachymic acid caused disruption of \( \Delta \Psi_m \) in a dose- and time-dependent manner. A549 cells were stained with DiOC\(_6\)(3) and subjected to flow cytometric analysis after exposure to 0, 20, 60 or 100 \( \mu \)M of PPAC or pachymic acid, respectively for 24 h and 48 h as detailed in Materials and Methods. Measured relative DiOC\(_6\)(3) dye intensity in A549 cells after treatment with PPAC or pachymic acid for 24 h and 48 h was presented in the corresponding bar chart. * \( p < 0.05 \), versus vehicle-treated control group

3.3.6 PPAC-induced apoptosis was not blocked by JNK inhibitor SP600125

Recent studies suggest that activation of JNK may lead to caspase-8 activation (Deng et al. 2003; Zou et al. 2004). To investigate the involvement of JNK in PPAC-induced...
apoptosis, protein expression of the active form of JNK (phosphorylated JNK) was analyzed by western blot. Sustained activation of JNK in A549 cells following PPAC treatment was observed (Figure 3.10). To investigate whether JNK activation is necessary for PPAC-induced apoptosis, the effect of PPAC on PARP cleavage in presence of the JNK-specific inhibitor SP600125 was examined. As shown in Figure 3.8, SP600125 did not inhibit PPAC-induced cleavage of PARP. The results therefore indicated that inhibition of JNK activity failed to prevent PPAC-induced apoptosis of A549 cells.

![Figure 3.10 Effect of PPAC on JNK activation. A549 cells were treated with 60 µM PPAC for 0, 3, 6 and 24 h and immunoblotted with antibody specific for phosphorylated JNK.](image)

3.3.7 Inhibition of Akt activation by PPAC

The PI3-kinase/Akt signal pathway plays a critical role in controlling cell survival and apoptosis. Activation of Akt may promote cancer cell survival by inhibiting apoptosis through its phosphorylation on multiple downstream targets (Vivanco and Sawyers 2002). The tumor suppressor, PTEN, has been shown to negatively regulate the PI3-kinase/Akt signal pathway (Maehama and Dixon 1998; Vivanco and Sawyers 2002). To determine the
potential involvement of the Akt pathway in PPAC-induced apoptosis, the phosphorylation status of Akt in PPAC-treated A549 cells was analyzed by western blot. As shown in Figure 3.11, Akt phosphorylation level was dramatically reduced by PPAC treatment, whereas total Akt protein expression remained unchanged. Furthermore, PPAC treatment enhanced the phosphorylation of PTEN (Figure 3.11), which is believed to stabilize PTEN and increase its biological activity (Torres and Pulido 2001). These results indicated that PPAC might induce apoptosis in A549 cells through downregulation of Akt activity.

3.3.8 Modulation of p53 activation by PPAC

Tumor suppressor protein p53 is activated by cellular stress induced by a variety of stimuli including chemotherapeutic drugs (Ljungman 2000). A549 cells are known to express wide-type p53 (Lehman et al. 1991). To determine whether exposure of A549 NSCLC cells to PPAC triggered p53 activation, we analyzed the phosphorylation status of this protein at serine 15. As shown in Figure 3.11, PPAC enhanced p53 activation in A549 cells as evident from the observed increase in band intensity of phosphorylated p53. These results therefore indicated that PPAC treatment brought about activation of the p53 stress response program in A549 cells. The increased activation of p53 might also contribute to PPAC-induced apoptosis.
Figure 3.11 PPAC treatment suppressed Akt activation and increased the activation of p53. A549 cells were treated with 60 µM PPAC for 24 h and immunoblotted with antibodies specific for phosphorylated PTEN, total Akt, phosphorylated Akt and phosphorylated p53, respectively. The analysis was repeated three times with similar results, and a representative immunoblot for each protein is shown.

3.4 Discussion

In this chapter, PPAC was demonstrated to be a potent inhibitor of human lung cancer proliferation. PPAC not only reduced the viability of NSCLC A549 cells, but also inhibited SCLC cell proliferation of H82 cells and H187 cells. The results had thus indicated that PPAC possessed antiproliferative activity on both NSCLC and SCLC types of lung cancer. More importantly, a decrease in the colony-forming ability of A549 cells was observed following PPAC treatment in the soft agar assay, implying that PPAC inhibited anchorage-independent growth of A549 cells over a long period of time (three weeks). The three dimensional growth and relatively long period of culture spanning over weeks allow the soft agar assay to mimic more closely the in vivo growth of cancer cells in a solid tumor as compared to cell viability MTT assay that is employed to assess growth of monolayer of
cancer cells (Agre and Williams 1983). Indeed, the sensitivities of the colony formation
assay to certain anticancer drugs have been found to agree with the drug sensitivities of the
same tumors in vivo (Agre and Williams 1983). Therefore, based on the results obtained
from the soft agar assay, it would be reasonable to postulate that PPAC might be effective for
lung cancers in vivo.

Resistance to apoptosis is implicated in the etiology of many cancer types, including
lung cancer (Shivapurkar et al. 2003). Drugs which promote apoptosis in cancer cells may
be effective for cancer treatment (Fesik 2005). PPAC treatment was observed to cause cell
shrinkage and rounding under the microscope, indicating occurrence of apoptosis in A549
cells. Moreover, PPAC treatment increased the fraction of sub-G1 cells and binding of
annexin V to treated cells, which strongly implied that PPAC-induced growth inhibition of
A549 cells was mainly due to enhanced apoptosis.

The activation of the caspase family of proteolytic enzymes plays an important role in
apoptosis triggered by pro-apoptotic stimuli (Thornberry and Lazebnik 1998). To
investigate if the caspase cascade was indeed involved in PPAC-induced apoptosis, the
activation of caspases was examined by monitoring their cleavage using immunoblotting
analysis. Results showed that PPAC treatment cleaved caspase-3 into the active enzyme
fragments (19 kDa and 17 kDa). As a critical executioner of apoptosis, capsase-3 is either
partially or totally responsible for the proteolytic cleavage of many key proteins such as the
nuclear enzyme PARP (Fernandes-Alnemri et al. 1994; Affar et al. 2001). The
accompanying appearance of a typical 89 kDa PARP cleavage product, a hallmark of
apoptosis (Lazebnik et al. 1994), further confirmed the proteolytic activity of caspase-3. To
further confirm the dependence of caspase activation in PPAC-induced apoptosis, cells coincubated with a pan-caspase inhibitor and PPAC were assessed for degree of apoptosis. Results obtained had indeed demonstrated the effective suppression of PPAC-induced apoptosis by z-VAD-fmk (pan-caspase inhibitor), thus ascertaining the induction of apoptosis in A549 cells via caspase activation by PPAC.

As discussed in Chapter 1, the induction of apoptosis can be mediated through two different pathways: the death receptor-mediated extrinsic pathway and the mitochondria-mediated intrinsic pathway (Hengartner 2000). Results obtained from flow cytometric and western blot analysis showed that PPAC induced apoptosis through activation of caspase-8, indicating the involvement of death receptor-mediated extrinsic apoptotic pathway. Activation of the death receptor-mediated apoptotic signaling pathway by triterpenoids is not uncommon. The naturally occurring triterpenoid, acetyl-keto-β-boswellic acid (Lu et al. 2008), and the synthetic triterpenoid CDDO-Me (Zou et al. 2004) have been previously reported to induce apoptosis through the extrinsic apoptotic pathway.

Apoptosis can be promoted by caspase-8 through direct proteolytic cleavage of execution caspases or indirect mediation of mitochondrial damage (Schulze-Osthoff et al. 1998). Based on the previous finding that pachymic acid, also a lanostane-type triterpenoid from Poria cocos, had induced apoptosis in prostate cancer cells through the mitochondria-mediated pathway (Gapter et al. 2005), it was hypothesized that mitochondrial damage might be involved following activation of caspase-8 by PPAC in A549 cells. Surprisingly, in contrast to pachymic acid, PPAC did not bring about disruption of the
mitochondrial membrane potential. Therefore, our results indicated that PPAC-induced activation of caspase-8 had directly led to the activation of effector caspase caspase-3 without the relay of the mitochondria. The findings clearly showed that PPAC and pachymic acid prevented cell growth and induced apoptosis by distinct mechanisms despite their similar chemical structures. Due to the current limited understanding of structure-activity relationship involving this class of triterpenoids, it remained unclear how PPAC, which differs from pachymic acid by having an extra double bond in the planar ring system and an oxo instead of an acetyloxy group at the C3 position, could elicit such a different mechanism of induction of apoptosis.

The JNK cascade is activated in response to a variety of cellular stresses including ultraviolet radiation, cytotoxic drugs and pro-inflammatory cytokines (Deng et al. 2003). The role of JNK in apoptosis remains controversial. Several reports indicate that activation of JNK contributes to death receptor-mediated apoptosis (Deng et al. 2003; Zou et al. 2004; Lu et al. 2008), whereas other studies suggest that JNK is dispensable (Tournier et al. 2000; Stadheim et al. 2002). Given that some triterpenoids might induce apoptosis in human cancer cells through JNK-mediated up-regulation of death receptors (Stadheim et al. 2002; Zou et al. 2004), it was questioned whether JNK was activated by PPAC and contributed to apoptosis. Although JNK was activated following PPAC treatment, the specific JNK inhibitor, SP600125, did not prevent PPAC-induced apoptosis. It therefore appeared that JNK activation was not required for PPAC-induced apoptosis in A549 cells.

Apoptosis is tightly controlled by anti-apoptotic and pro-apoptotic molecules. PI3-kinase/Akt signaling is one of the most important pathways in regulating cancer cell
survival (Vivanco and Sawyers 2002). Activated Akt signaling in cancer cells inhibits apoptosis by phosphorylation of downstream substrates, such as BAD and Bcl-2, which are involved in the regulation of the intrinsic apoptotic pathway (Vivanco and Sawyers 2002). Furthermore, Akt also inhibits the death receptor-mediated apoptosis pathway through up-regulation of c-FLIP expression (Panka et al. 2001). As an anti-apoptotic protein, c-FLIP blocks apoptosis induced by the oligomerization of the adapter protein and therefore functions as a caspase-8 dominant negative (Panka et al. 2001). Targeting the PI3-kinase/Akt signaling pathway has been reported to be an effective strategy for the treatment of lung cancer (Crowell and Steele 2003). In the experiments, PPAC treatment decreased activation of Akt and increased the stability of PTEN in A549 cells. Based on these results, it was therefore postulated that the down-regulation of Akt activity might decrease c-FLIP expression, which would in turn cause the activation of caspase-8. The suppression of Akt activity by PPAC might be one of the essential mechanisms of PPAC-induced apoptosis. However, it remains to be elucidated whether Akt inhibition would solely or in combination with other factors contribute towards PPAC-induced apoptosis.

Apart from the down-regulation of pro-survival Akt, an up-regulation of pro-apoptotic p53 by PPAC was also observed. The p53 tumor suppressor protein mediates the response to various stress signals to suppress cell growth, either through cell cycle arrest or induction of apoptosis (Ljungman 2000). An increase in phosphorylated p53 (ser 15) protein by PPAC treatment in A549 cells was detected. Phosphorylation of p53 at serine 15 is known to up-regulate the level of p53 by reducing the interaction between p53 and its
negative regulator, MDM2 (Shieh et al. 1997). Therefore, increased phosphorylation of p53 might favor cellular apoptosis. However, further studies would be needed to clarify the precise contribution of p53 in PPAC-induced apoptosis.

In conclusion, PPAC was demonstrated to suppress lung cancer cell proliferation and reduce colony formation of A549 cells. In addition, PPAC induced apoptosis in A549 cells via activation of caspase-8. The involvement of PPAC-induced inhibition of Akt activation and enhancement of p53 function might also contribute to the series of apoptotic events.
Chapter 4 Inhibition of A549 cell growth and modulation of arachidonic acid metabolism by pachymic acid

Summary

In this chapter, the anticancer activity of pachymic acid (PA) against lung cancer was evaluated by examining its antiproliferative and apoptosis-inducing effect on NSCLC A549 cells. The effects of non-toxic doses of PA on arachidonic acid metabolism in A549 cells were also investigated. Results showed that PA (1) inhibited anchorage-dependent and -independent A549 cell growth in a concentration-dependent manner, (2) induced apoptosis and disrupted mitochondrial membrane potential in A549 cells, and at non-lethal levels (3) decreased IL-1β-induced activation of cPLA₂ and COX-2, (4) suppressed IL-1β-induced activation of MAPKs, as well as (5) inhibited IL-1β-stimulated NF-κB signaling pathways.

Based on the obtained results, it was postulated that inhibition of arachidonic acid metabolism by PA was mediated in part by its inhibition of MAPKs and NF-κB signaling pathways. The findings revealed a multi-factorial anticancer property of PA toward lung cancers: at high concentrations, PA exerted cytotoxicity on cancer cells by inducing apoptosis through mitochondria-mediated pathway; whereas at nontoxic concentrations, it reduced prostaglandin formation by inhibiting both arachidonic acid release and its subsequent conversion to prostaglandin E2. In view that arachidonic acid metabolism plays an important role in promoting cancer development, these findings highlighted the chemopreventive potential of PA.
4.1 Introduction

As described in Chapter 2, among the triterpenoids isolated from *Poria cocos*, pachymic acid (PA) was purified with the greatest yield of 1.5 g. Thus, it was concluded that PA could be the main triterpenoid contained in *Poria cocos*. Previous work from the laboratory has shown that PA is an effective inducer of apoptosis in prostate cancer cells (Gapter et al. 2005). Together with the finding that PA also prevents skin cancer formation in a two-stage carcinogenesis mouse model (Kaminaga et al. 1996), it was hypothesized that PA might exhibit anticancer effects against other cancer types. Therefore, our study on PA was extended to examination of its effect against human lung cancer.

Several enzymes involved in eicosanoid production including COX-2 and some PLA2 have been linked to the carcinogenesis of lung and other cancers (Marks et al. 2000; Meyer et al. 2004; Mao et al. 2005). There are three major classes of PLA2, including sPLA2, iPLA2 and cPLA2. PLA2 family of enzymes catalyze the rate-limiting hydrolysis of arachidonic acid, and released arachidonic acid is subsequently converted by COX enzymes (COX-1 and COX-2) to prostaglandins (Balsinde et al. 2002). In human and animal studies, several lines of evidence indicate that cPLA2 and COX-2 could be involved in lung tumor development. Firstly, production of arachidonic acid metabolites is higher in cancerous lung tissue relative to normal tissue (Hida et al. 1998; Hosomi et al. 2000). Secondly, decreased lung tumorigenesis is observed in mice genetically deficient in cPLA2. Thirdly, high expression levels of COX-2 are frequently associated with lung cancer development (Hosomi et al. 2000; Campa et al. 2004). Fourthly, treatment with COX-2 inhibitors (NSAIDs such as aspirin and indomethacin), which inhibit prostaglandin E2 (PGE2) synthesis by COX-2, has been shown
to inhibit lung carcinogenesis in animal lung tumor models (Campa et al. 2004). Taken together, these findings suggest that modulation of AA metabolism by inhibiting cPLA2 and COX-2 enzymes may be an effective strategy for chemoprevention and/or chemotherapy of lung and other cancers.

PA has been shown to inhibit TPA-induced tumor promotion in a two-stage carcinogenesis model in mouse skin (Kaminaga et al. 1996). However, the mechanism underlying this observed chemopreventive effect of PA is not known. PA has also been demonstrated to possess anti-inflammatory effects by several independent studies (Giner et al. 2000; Akihisa et al. 2007). In particular, Cuella et al had demonstrated the inhibitory effect of PA against PLA2 (Cuella et al. 1996). However, a shortcoming of this study is that the data was obtained from a cell-free assay using snake venom-originated PLA2, and the inhibition was only observed at high PA doses. As such, the effect of PA on human PLA2 (that is cPLA2) at biologically relevant levels has not been evaluated. Furthermore, how PA modulates downstream signals of arachidonic acid release also remains largely unknown. In this chapter, the efficacy of PA against lung cancer and its associated anticancer mechanism(s) was investigated using human-derived NSCLC A549 cells. Most importantly, the possible effects and mechanism of action of PA on arachidonic acid metabolism was evaluated in a cell-based assay using A549 cells.
4.2 Materials and methods

Reagents

The cPLA₂ assay kit, general PLA₂ inhibitor (7,7-dimethyl-5,8-eicosadienoic acid), sPLA₂ inhibitor (thioetheramide phosphatidylecholine) and PGE₂ EIA kit were obtained from Cayman Chemical Co. (Ann Arbor, MI). SP600125, a JNK-specific inhibitor, was purchased from Biomol International LP (Plymouth Meeting, PA). Bay 11-7082, a NF-κB inhibitor, was purchased from Calbiochem (San Diego, CA). Antibodies for actin and COX-2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). MEK1/2 inhibitor (U0126) and antibodies specific for cleaved PARP, cPLA₂, phospho-cPLA₂ (Ser505), phospho-ERK1/2 (Thr202/Tyr204), ERK1/2, phospho-JNK (Thr183/Tyr185), phospho-p38 MAPK (Thr180/Tyr182), NF-κB p65, IκBα, phospho-NF-κB p65 (Ser536), phospho-IKKα/β (Ser176/180), goat anti-rabbit IgG-conjugated to horseradish peroxidase and horse anti-mouse IgG-conjugated to horseradish peroxidase were purchased from Cell Signaling Technology (Beverly, MA). Unless otherwise stated, all chemicals that were not specifically indicated were purchased from Sigma (St. Louis, MO).

Cell culture and treatment

Culturing and maintenance of A549 cells was described in Chapter 2. PA was reconstituted in DMSO at a stock concentration of 20 mM and subsequently diluted to the working concentrations for experimental procedures. For experiments using IL-1β, A549 cells were grown in serum-free medium for 24 h and treated as described in the figure legends.
Trypan blue exclusion assay

Trypan blue exclusion assay was performed in 6-well culture plates. 1.5 x 10^5 A549 cells were seeded into each well and incubated overnight. The cells were then incubated in fresh medium containing either DMSO or different concentrations of PA for 24 h. Both floating and adherent cells were pooled, and a 20 µl aliquot was removed from the cell suspension and mixed with an equal volume of 0.2% trypan blue dye solution. Cell counting was performed using a hemocytometer. Clear cells that were not trypan blue-stained were counted as viable cells and the number of viable cells from each well was calculated accordingly. Results are expressed as percent cell viability compared with DMSO control set at 100%.

Cell proliferation assay

The effect of PA on cell viability of A549 cells was determined by two methods: MTT assay and an assay using a cell counting kit-8 (CCK-8, Dojindo Laboratories, Kumamoto, Japan). MTT assay was carried out as described in Chapter 2.

The CCK-8 employs the use of a water-soluble tetrazolium salt 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST-8), which can be reduced by dehydrogenases in cells to produce a yellow color formazan. This yellow color formazan is water soluble and absorbance can be measured directly using a microplate reader. The amount of the formazan dye generated by the activity of dehydrogenases in cells is directly proportional to the number of living cells.

Briefly, A549 cells were seeded in 96-well culture plates (5 x 10^3 cells / well) and
grown overnight. PA was diluted in fresh medium to the desired concentration and added to cells in each well. Following drug treatment for a designated period (24 or 48 h), 10 µl of CCK-8 solution was added into the culture medium of each well. The 96-well plate was incubated for 4 h in the cell culture incubator to allow metabolic conversion. Absorbance was subsequently measured at 450 nm using a microplate reader (Tecan GENios, Salzburg, Austria). The effect of PA on cell viability was expressed as percent cell viability compared with vehicle treated control cells set at 100%.

LDH release assay

Lactate dehydrogenase (LDH) is a soluble cytosolic enzyme that is released into the culture medium following loss of membrane integrity resulting from either apoptosis or necrosis (Cuella et al. 1996). As an additional method to determine the extent of cell death, A549 cells were seeded in 6-well culture plates, allowed to adhere overnight, then treated with the desired concentration of PA, and the LDH activity in the culture media was measured using a commercially prepared kit (LDH assay kit, Nanjing Jiancheng Bioengineering Institute, China) according to the manufacturer's instructions. The detection of LDH activity is based on the ability of LDH to catalyze the synthesis of pyruvic acid. Pyruvic acid can react with 2,4-dinitrophenyl hydrazine to form a derivative of phenylhydrazone, which has a brownish red color in basic solution. By measuring absorbance of the reaction product at 440 nm, LDH activity in the media sample can be determined. The activity of LDH was expressed in U/L where 1 U is defined as the activity that produces 1 µmol pyruvic acid from lactic acid in the assay system.
Soft agar assay

The soft agar assay was performed as described in Chapter 3. Briefly, after making the medium-containing agar base layer, cell layer containing A549 cells (1.5 × 10^4) and DMSO or PA was added over the base layer. The plates were incubated in the cell culture incubator for 3 weeks without further feeding. Colonies ≥ 0.1 mm were enumerated under a light microscope.

Propidium iodide staining assay

To quantify PA-induced apoptosis, cells were stained with propidium iodide (PI) and subjected to flow cytometric analysis as described previously in Chapter 3. Cells with sub-G1 DNA were considered as apoptotic cells.

Analysis of mitochondrial membrane potential

The mitochondrial membrane potential (ΔΨ_m) was analyzed by staining A549 cells with DiOC_6(3) as described previously in Chapter 3.

Measurement of cPLA₂ activity

cPLA₂ activity in A549 cells was measured using a commercially available assay kit (cPLA₂ assay kit, Cayman Chemical Co.) following the manufacturer’s instructions. The cells were seeded in tissue culture plates and incubated overnight for attachment. The cells were then incubated in serum-free medium for 24 h before PA and IL-1β treatment. After desired treatment, the cells were harvested and prepared in homogenization buffer (50 mM
Hepes pH 7.4, 1 mM EDTA). Following brief sonication, the lysed cells were centrifuged at 14,000 x g for 15 min at 4°C. The supernatant was collected and the protein content was quantified by the BCA colorimetric detection method according to the manufacturer’s protocol (Pierce, Rockford, IL). The samples were then incubated with arachidonoyl thio-phosphatidylcholine, a synthetic substrate of cPLA₂. Release of free thiol due to hydrolysis of arachidonoyl thio-phosphatidylcholine by cPLA₂ would in turn reduce DTNB to NTB. The NTB concentration was determined by spectrophotometric analysis at 405 nm (Tecan Infinite M200, Grodig, Austria). To ensure that only cPLA₂ activity was measured by the assay, lysate samples were pre-treated with iPLA₂-specific inhibitor (bromoenol lactone, 5 µM) and sPLA₂-specific inhibitor (thioetheramide-PC, 5 µM) for 20 min at 25°C as recommended by the manufacturer. cPLA₂ activity was calculated and expressed in nmol/min/ml.

Analysis of PGE₂

A549 cells were seeded and cultured in 6-well plates till 80% confluent. The cells were then maintained in serum-free medium for 24 h before PA and IL-1β treatment. After the indicated treatment, the conditioned medium was collected and processed for PGE₂ quantification using a specific enzyme immunoassay kit (PGE₂ EIA kit – Monoclonal, Cayman Chemical Co.) following the manufacturer’s instructions.

This assay is based on the competition between PGE₂ and a PGE₂ tracer for a limited amount of PGE₂ monoclonal antibody. The PGE₂ assay plate was precoated with a secondary antibody recognizing the PGE₂ monoclonal antibody. A mixture of conditioned
medium sample (or PGE₁ standard), PGE₂ tracer and PGE₂ monoclonal antibody was loaded into the PGE₂ assay plate. Due to their competitive binding to PGE₂ antibody, the amount of PGE₂ tracer binding to the well is inversely related to PGE₂ amount containing in the medium sample. The amount of PGE₂ tracer can be determined by adding its substrate to produce a yellow color product, whose absorbance is to be measured at 412 nm. The PGE₂ concentration of the medium sample can be determined against a PGE₂ standard curve.

RNA extraction and reverse transcription - PCR (RT-PCR)

A549 cells were seeded and cultured in 6-well plates to 80% confluency. The cells were then maintained in serum-free medium for 24 h before PA and IL-1β treatment. After the indicated treatment, total RNA from each sample was isolated using an RNeasy® Mini Kit (Qiagen, Hilden, Germany). Following the manufacturer’s instructions, cells were scraped in lysis buffer provided in this kit, and homogenized with a QIAshredder homogenizer. Ethanol (70%) was mixed with homogenized lysate, and the mixture was applied on an RNeasy spin column to remove ethanol. After washing RNeasy spin column with wash buffers, RNA was finally eluted in RNase-free water. The concentration of extracted RNA was determined by measurement of absorbance at 260 nm on an UV spectrophotometer.

Single-stranded cDNA was synthesized from 2 µg of RNA by reverse transcription using a RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, Hanover, MD). An appropriate volume containing 2 µg of total RNA was mixed with 1 µl Oligo(dT)₁₈ primer and made up finally to 12 µl with nuclease-free water. The mixture was incubated in 70 °C for 5
min, and then mixed with reaction buffer, RiboLock™ Ribonuclease inhibitor and dNTP for 5 min at 37 °C. Afterward, ReverAid™ M-MuLV Reverse Transcriptase was next added to the above mixture and incubated at 42 °C for 60 min for reverse transcription to occur. The reverse transcription was stopped by heating at 70 °C for 10 min. The resulting cDNA was stored at -20 °C until use.

Fragments specific to examined genes were amplified in reaction solution (20 µl) containing 2 µl generated cDNA, 2 µl deoxyribonucleotide triphosphate (2 mM), 0.2 µl Taq DNA Polymerase (New England Biolabs) and 1 µl of each specific sense and anti-sense primers (5 nM). PCR was run for 30 cycles of 94 °C for 15 s (denaturation), 55 °C for 15 s (annealing) and 72 °C for 30 s (extension). The primer sequences used for COX-2 and cPLA₂ and their product sizes were as followed - COX-2 (561 bp): forward 5'-GAATGGGGTGATGAGCAGTT -3', reverse 5'-CAGAAGGGCAGGATACAGC-3' (Lister et al. 1989); cPLA₂ (430 bp): forward 5'-GGATTCTCTGGTGTGATGAAGG-3', reverse 5'-CCCAATCTGCAAACATCAGC-3' (Khanapure et al. 2007). Samples of each single-stranded cDNA were also treated with primers that amplify a 452 bp fragment of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) house keeping gene (forward 5'-ACCACAGTCCATGCCATC-3, reverse 5'-TCCACCACCTGTTGCTGTA-3' (Favata et al. 1998; Yang et al. 2002)) to allow for quantitative comparison of loading. The PCR products were subjected to electrophoresis on 1.2% agarose gels containing ethidium bromide and images were captured under UV light in a Bio-Rad ChemiDoc imaging system (Hercules, CA).
Preparation of cell lysates, cytosolic and nuclear fractions

Whole cell lysates were prepared as previously described in chapter 3. For the separation of cytosolic and nuclear fractions, cells were collected, washed twice with cold PBS twice and then re-suspended in ice-cold Buffer A (10 mM HEPES pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT, 0.5 mM PMSF, 1 mg/ml leupeptin and 1 mg/ml aprotinin). After incubation on ice for 15 min, igepal (final concentration 0.5%) was added to the cell suspension and mixed gently. The cytosolic extracts were collected after cells were centrifuged at 14,000 x g for 30 sec at 4°C. The remaining nuclear pellets were then re-suspended in Buffer B (20 mM HEPES pH 7.9, 1.5 mM MgCl₂, 450 mM NaCl, 25% glycerol, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 1 mg/ml leupeptin, 1 mg/ml aprotinin), and incubated on ice for 30 min with gentle mixing once every 5 min. The final nuclear extracts were collected as supernatants after centrifugation (14,000 x g for 15 min, 4°C) and the protein concentration was quantified by the BCA colorimetric detection method as detailed in Chapter 3.

Western blot analysis

The detailed procedures on western blot analysis were described in Chapter 3.

Statistical analysis

Statistical tools employed for analysis were similar to those described in Chapter 3.
4.3 Results

4.3.1 Incompatibility of PA with MTT assay

In Chapter 2, purified and isolated triterpenoids were screened for antiproliferative activities using MTT assay. After 72 h, PA had been observed to inhibit A549 cell growth in a dose-dependent manner. As shown in Figure 4.1, treatment of A549 cells with PA for 24 h resulted in a similar trend of decrease in cell viability. According to the 24 h MTT data, 5 µM of PA brought about an apparent decrease in A549 cell viability. At 20 µM, PA has significantly reduced the viability to 50%. However, under the microscope, no difference in cell density could be observed between DMSO control and PA treatment (≤ 30 µM). To determine whether PA truly reduced cell viability at the tested concentrations, A549 cells were exposed to similar PA doses and their viability was measured using the trypan blue exclusion assay. Interestingly, as shown in Figure 4.1, PA did not reduce cell viability at concentrations of 0.2, 5, 10 and 20 µM, and only a dose of 30 µM PA was able to cause a significant reduction in viable cells (86% relative to control). The trypan blue assay data therefore indicated that only a PA concentration higher than 20 µM could affect A549 cell viability. Given that trypan blue exclusion assay was a more direct method to quantify viable cells, it was deduced that the detected decrease in absorbance in a MTT assay might falsely report a reduction in viable cells.
Figure 4.1 Discrepancy between cell viability results obtained from the MTT assay and Trypan blue exclusion assay. Following treatment of A549 cells with indicated concentrations of PA for 24 h, cell viability was determined using MTT assay or trypan blue exclusion assay as described in Materials and methods. Columns, means of three independent experiments; bars, standard deviation. * $p < 0.05$

It seemed that PA decreased the conversion of MTT to insoluble purple formazan without decreasing the number of viable cells. After adding MTT into culture medium, plates were observed under microscope every 30 min. It was noticed that in control wells, grainy formazan crystals were formed around cell nuclei with an intense dark blue color (Figure 4.2). In contrast, in PA-treated cells, long needle-like formazan crystals were formed, with a less intense color (Figure 4.2). This difference in formazan formation, but not cell numbers, would likely be the reason accounting for absorbance differences between
the control and PA treatment groups. Indeed, cholesterol has been reported to cause a similar phenomenon in A549 cells; in a MTT assay, A549 cells treated with non-toxic cholesterol register a significant lower absorbance value (Ahmad et al. 2006). PA has a similar chemical structure with cholesterol, and it was proposed that the unique chemical structure of PA might interfere with MTT metabolism. However, in A549 cells treated with PPAC, such an interference phenomenon was not observed. The exact mechanism underlying this phenomenon was not fully understood. Nevertheless, this finding indicated that at least in A549 cells, the MTT assay would not be a suitable method to evaluate PA’s antiproliferative effect.

Figure 4.2 Differences in formazan formation in a MTT assay between cells treated or untreated with PA. After treatment of A549 cells with DMSO (left) or 10 µM PA (right) for 24 h, 11 µl of MTT was added into culture medium. Pictures were taken 2 h after MTT addition using a Nikon Eclipse TE2000-U microscope (Nikon Corp., Tokyo, Japan).

4.3.2 Inhibition of anchorage-dependent and anchorage-independent growth of NSCLC A549 cells by PA

CCK-8 was next used instead of MTT assay to assess PA’s inhibitory effect on A549
cell growth. As CCK-8 involved the production of a water-soluble product, the source of interference due to differences in the formation of insoluble formazan would likely be absent. Between CCK-8 assay and trypan blue exclusion assay, PA's antiproliferative effects on A549 cells were shown to be comparatively similar (Figure 4.3, Figure 4.1). In the CCK-8 assay, 20 and 30 µM PA was observed to reduce A549 cell viability to 94% and 83% respectively, and in the trypan blue assay, similar doses of PA diminished viability to 96% and 86% respectively. Therefore, it could be concluded based on the obtained results that CCK-8 would be a more reliable and appropriate assay to evaluate PA's antiproliferative effect.

![Figure 4.3 Effect of PA on A549 cell viability as evaluated by CCK-8.](image)

As evaluated by the CCK-8 assay, PA caused a dose-dependent decrease in proliferation of A549 cells. As early as 24 h of drug treatment, PA ≥ 30 µM caused a
significant cytotoxic effect (Figure 4.3). These observations were confirmed using an alternative method that measured LDH release by A549 cells following PA treatment (Figure 4.4). A decrease in cell viability is usually accompanied by a damaged cell membrane, which causes the release of large molecules such as LDH from the cell into the medium (Bonfoco et al. 1995). In corroboration with the CCK-8 results, PA was found to exert cytotoxicity and caused marked LDH release at concentrations greater than 30 µM (Figure 4.4).

![Figure 4.4 Effect of PA treatment on LDH release of A549 cells into culture medium.](image)

After 24 h treatment of A549 cells with different concentrations of PA, LDH activity in culture medium was measured using a LDH assay kit as detailed in Materials and methods. Columns, means of three independent experiments; bars, standard deviation. * p < 0.05.

A phenotypic hallmark of cancer cells is their ability to survive and grow under non-adhesive or anchorage-independent conditions (Hanahan and Weinberg 2000). We next determined if PA could reduce the efficiency of colony initiation and anchorage-independent growth of A549 cells. As presented in Figure 4.5, PA treatment caused a decrease in A549 colony formation in a dose-dependent manner. The effective dose to inhibit colony
formation was as low as 10 µM to 20 µM (Figure 4.5). This result suggested that PA possessed inhibitory effects on both anchorage-dependent and –independent growth of A549 cells.

Figure 4.5 PA inhibited anchorage-independent growth of A549 cells. The effect of PA on anchorage-independent growth of A549 cells was determined by soft agar assay as detailed in Materials and methods. Columns, mean number of colonies ≥ 0.1 mm from three independent experiments; bars, standard deviation. * p < 0.05.

4.3.3 Induction of apoptosis and disruption of mitochondrial membrane potential by PA

To determine if PA suppressed A549 cell proliferation through the induction of apoptosis, PA-treated cells were incubated with PI and analyzed for sub-G1 staining. PA treatment increased the percentage of apoptotic cells in a concentration- and time-dependent manner (Figure 4.6A and B). PA-induced apoptosis was further substantiated by the demonstration of PARP cleavage (Figure 4.7).
Figure 4.6 PA induced apoptosis in A549 cells.  (A) Dose and time-dependent increase of sub-G1 cells by PA treatment.  After 24 h or 48 h treatment with either DMSO or different concentrations of PA, A549 cells were stained with PI and analyzed by flow cytometry.  Sub-G1 value corresponds to the percentage of cells in R1 region.  Flow cytometric graph is representative of three independent experiments with similar results.  (B) Quantification of sub-G1 cells after 24 h or 48 h PA treatment.  Columns, means of three independent experiments; bars, standard deviation.  * $p < 0.05$. 
A previous study had shown that PA induced apoptosis in prostate cancer cells through the intrinsic mitochondria-mediated pathway (Gapter et al. 2005). To determine whether the mitochondria were involved in PA-induced apoptosis of NSCLC cells, A549 cells were labeled with DiOC₆(3), which would accumulate within the mitochondria in a potential-dependent manner. It was found that PA caused a dose- and time-dependent diminution of ΔΨₘ in A549 cells as evident from the decreased ability of cells to retain DiOC₆(3) following PA treatment (Figure 4.8). Given that loss of ΔΨₘ would precede the pre-apoptotic process, results obtained therefore implied that mitochondrial membrane perturbation might at least in part account for PA-mediated induction of apoptosis.
Figure 4.8 Dose and time-dependent disruption of ΔΨm by PA treatment. PA-treated A549 cells were labeled with DiOC₆(3) during the final 30 min of PA treatment and analyzed using flow cytometry. The number in the figure corresponds to the percentage of cells in R2 region. Flow cytometric graph is representative of three independent experiments with similar results.

4.3.4 Inhibition of IL-1β-induced cPLA₂ activation by PA at non-lethal concentrations

Aberrant PLA₂ activity and arachidonic acid metabolism is known to correlate with carcinogenesis of lung and other cancers. We first investigated whether the inhibitory effects of PA on snake venom PLA₂ reported previously (Cuella et al. 1996) could be replicated using human PLA₂ isoforms at non-lethal PA concentrations as determined by CCK-8 and LDH assay (i.e., ≤ 20 µM). To test the hypothesis in a biologically relevant setting, we pre-treated A549 cells with IL-1β, a critical signal known to initiate inflammation, induce activation of cPLA₂, and stimulate arachidonic acid release in a cellular system (Croxtal et al. 1996; Choudhury et al. 2000). Consistent with previous studies, IL-1β
increased the protein expression of cPLA₂ (both total cPLA₂ and its activated phosphorylated form) in a time-dependent manner, with the maximum induction occurring after 8 h of exposure to IL-1β (Figure 4.9A). In contrast, 10 µM PA significantly suppressed the total and activated protein expression of cPLA₂ by IL-1β (Figure 4.9B).

![Figure 4.9 PA inhibited IL-1β-induced cPLA₂ protein activation in A549 cells.](image)

(A) Time-dependent increase of total cPLA₂ and phosphorylated cPLA₂ protein expression by IL-1β treatment. A549 cells were incubated in serum-free medium for 24 h and then treated with IL-1β (1 ng/ml) for different time periods. (B) Suppression of PA on IL-1β-induced cPLA₂ phosphorylation and total cPLA₂ protein expression. A549 cells were pretreated with DMSO or 10 µM PA for 1 h, and then challenged with IL-1β (1 ng/ml) for 8 h. Cell lysates were immunoblotted using antibodies against total cPLA₂ or phosphorylated cPLA₂. Images are representative of three independent experiments with similar results.
To determine if inhibition of cPLA<sub>2</sub> expression by PA occurred at the transcriptional level, RT-PCR analysis was performed. Pretreatment of A549 cells with 10 µM PA was able to suppress the IL-1β-induced cPLA<sub>2</sub> mRNA expression (Figure 4.10). Furthermore, PA pretreatment also decreased cPLA<sub>2</sub> enzyme activity in A549 cells (Figure 4.11). In contrast to control cells treated with IL-1β alone, the cPLA<sub>2</sub> enzyme activity in cells treated with both IL-1β and 10 µM PA possessed less enzyme activity (Figure 4.11). Compared to a general PLA<sub>2</sub> inhibitor, 7,7-dimethyl-5,8-eicosadienoic acid (DEDA) (Lister et al. 1989), PA decreased cPLA<sub>2</sub> activity to a lesser extent (Figure 4.11). Taken together, these results indicated that PA modulated IL-1β-induced cPLA<sub>2</sub> activation in A549 cells via suppression of cPLA<sub>2</sub> gene expression and/or activation.

\[\begin{array}{c|ccc}
 & cPLA_2 & GAPDH \\
\hline
DMSO & \text{---} & \text{---} \\
DMSO PA 10 \mu M & \text{---} & \text{---} \\
IL-1\beta (1 \text{ ng/ml}) & \text{---} & \text{---} \\
\end{array}\]

**Figure 4.10** PA inhibited IL-1β-induced *cPLA<sub>2</sub>* gene activation. A549 cells were pretreated with DMSO or 10 µM PA for 1 h, and then challenged with IL-1β (1 ng/ml) for 3 h. Total mRNA was extracted, reverse transcribed, and amplified by PCR as detailed in Materials and methods. Images are representative of three independent experiments with similar results.
Figure 4.11 PA suppressed IL-1β-enhanced cPLA2 enzyme activity. A549 cells were treated with 10 µM PA or 10 µM general PLA2 inhibitor DEDA or vehicle control (DMSO) for 1 h and then stimulated with IL-1β (1ng/ml) for 5 h. cPLA2 enzyme activity was determined using a cPLA2 assay kit as detailed in materials and methods. Columns, means of two independent experiments repeated in duplicate; bars, standard deviation. †p < 0.05 versus DMSO control; *p < 0.05 versus DMSO + IL-1β.

4.3.5 Inhibition of IL-1β-induced COX-2 mRNA and protein expression by PA at non-lethal concentrations

Prostaglandin production is regulated by both the substrate availability of arachidonic acid and activity of COX-2. As described in Section 4.3.4, PA brought about negative modulation of cPLA2 expression. Therefore, it was hypothesized that PA treatment could reduce downstream conversion of arachidonic acid to prostaglandins. Indeed, experiments performed demonstrated that PA effectively decreased IL-1β-induced PGE2 production in A549 cells in a concentration-dependent manner, with PGE2 levels reduced by around 40 % at 10 µM as compared to IL-1β only-treated cells (Figure 4.12).
Figure 4.12 PA reduced IL-1β-stimulated PGE\(_2\) production. A549 cells were pretreated with different concentrations of PA or U0126 for 1h, and then challenged with IL-1β (1 ng/ml) for 24 h. The medium was collected and subjected to PGE\(_2\) analysis using a PGE\(_2\) assay kit as detailed in Materials and Methods. Columns, mean of three independent experiments repeated in duplicate; bars, standard deviation. † \(p <0.05\) versus DMSO control; * \(p <0.05\) versus DMSO + IL-1β.

Although PA was found to be not as effective as U0126 (Figure 4.12), a MEK1/2 inhibitor reported to inhibit IL-1β-induced COX-2 expression and PGE\(_2\) production (Favata et al. 1998; Yang et al. 2002), the results implied that PA might regulate PGE\(_2\) production by reducing COX-2 expression. To investigate this possibility, the effect of PA treatment on endogenous COX-2 expression in A549 cells exposed to IL-1β was evaluated. As shown in Figure 4.13A, the expression of COX-2 protein increased in a time-dependent manner following exposure of A549 cells to IL-1β for 24 h. Expectedly, pretreatment of A549 cells with non-lethal concentrations of PA reduced IL-1β-induced COX-2 protein expression in a concentration-dependent manner (Figure 4.13B). This inhibition in COX-2 expression also occurred at the transcriptional level as pretreatment of A549 cells with 10 µM of PA caused partial suppression of COX-2 mRNA expression (Figure 4.13C).
Figure 4.13 PA inhibited IL-1β-induced COX-2 mRNA and protein expression.

(A) Time-dependent increase of COX-2 protein expression by IL-1β treatment. A549 cells were starved in serum-free medium for 24 h and then treated with IL-1β (1 ng/ml) for different time periods. Cell lysates were immunoblotted with antibody specific for COX-2 proteins. (B) Dose-dependent inhibition of PA on IL-1β-induced COX-2 protein expression. A549 cells were pretreated with different concentrations of PA for 1 h, and then challenged with IL-1β (1 ng/ml) for 24 h. Cell lysates were immunoblotted using antibodies against COX-2. (D) Inhibitory effect of PA on IL-1β-induced COX-2 mRNA transcription. A549 cells were pretreated with DMSO or 10 µM PA for 1 h, and then challenged with IL-1β (1 ng/ml) for 3 h. Total mRNA was extracted, reverse transcribed, and amplified by PCR as detailed in Materials and methods. Images are representative of three independent experiments with similar results.
4.3.6 The effect of PA on IL-1β-induced MAPKs signaling pathway

Results thus far had positively shown that PA exerted inhibition on IL-1β-induced protein expression and activity of cPLA₂ and COX-2. To probe further, the possible involvement of upstream signal transduction pathways responsible for this inhibition was next investigated. In view that MAPKs have been shown to regulate cPLA₂ and COX-2 activation (Lin et al. 1993; Choudhury et al. 2000; Chun and Surh 2004), the effect of PA on MAPKs signaling pathways (including ERK, JNK and p38) was explored herein. Firstly, it was verified that IL-1β treatment indeed resulted in activation of ERK, JNK and p38 MAPK, as shown by the enhancement in phosphorylation of each respective protein (Figure 4.14). Pre-incubation of A549 cells with non-lethal concentrations of PA was able to suppress IL-1β-induced phosphorylation of ERK, JNK and p-38 (Figures 4.14).

![Image of immunoblot showing phosphorylation of p-p38, p-JNK, p-ERK, and Actin with DMSO, PA at 1 µM, 3 µM, 10 µM, and IL-1β (1 ng/ml)](image)

**Figure 4.14 PA inhibited IL-1β-induced MAPKs activation in A549 cells.** After pretreatment of A549 cells with PA (1 µM, 3 µM, 10 µM) for 1 h, A549 cells were challenged with 1 ng/ml of IL-1β for 30 min. Cell lysates were subjected to immunoblotting analysis using corresponding antibodies. The analyses were repeated three times with similar results and a representative immunoblot is shown.
To examine the role of MAPKs in the activation of cPLA2 and COX-2, specific MAPK inhibitors, namely U0126 (MEK1/2 inhibitor) and SP600125 (JNK inhibitor), were used to investigate the relationship. As shown in Figure 4.15A and 4.15B, both U0126 and SP600125 suppressed the phosphorylation of cPLA2 and protein expression of COX-2. Taken together, the results suggested that PA suppressed the MAPK signal transduction pathway to inhibit IL-1β-induced activation of cPLA2 and COX-2.

Figure 4.15 Effect of specific MAPK inhibitors on IL-1β-induced phosphorylation of cPLA2 and COX-2 protein expression. After pretreatment with U0126 (A) or SP600125 (B) for 1 h, A549 cells were challenged with 1 ng/ml of IL-1β for 24 h. Cell lysates were subjected to immunoblotting analysis using corresponding antibodies. The analyses were repeated three times with similar results and a representative immunoblot is shown.

4.3.7 The effect of PA on IL-1β-induced NF-κB signaling pathway

NF-κB signaling pathway has been shown to be involved in the activation of both cPLA2 and COX-2 by various stimuli including IL-1β (Catley et al. 2003; Luo et al. 2008). To examine whether PA inhibited cPLA2 and COX-2 activation through NF-κB signaling pathway, the effect of PA on NF-κB nuclear translocation was studied. As shown in Figure 4.16A, treatment of A549 cells with IL-1β resulted in a marked translocation of NF-κB p65 catalytic subunit from the cytoplasm to the nucleus as well as a reduction in the steady state
levels of inhibitor of κBα (IκBα) in the cytosol, whereas PA (10 µM) pretreatment effectively impeded both the reduction of IκBα protein and p65 nuclear translocation induced by IL-1β (Figure 4.16A).

To investigate whether the effect of PA on IκBα was through the upstream signal, IκB kinase (IKK), all lysates were analyzed using a specific antibody against the phosphorylated (active) form of IKK. As shown in Figure 4.16B, IKKβ phosphorylation increased after IL-1β-treatment and this effect was suppressed by PA in a dose-dependent manner. These results suggested that PA brought about inhibition of IL-1β-induced NF-κB nuclear translocation by suppressing IKK activation. Furthermore, a dose-dependent decrease in the protein expression of phosphorylated NF-κB p65 by PA pretreatment was observed (Figure 4.16B). Since p65 phosphorylation at Ser536 regulates NF-κB activation, nuclear localization and transcriptional activity (Buss et al. 2004), data from Figure 4.16B further lent support to the suppressive effect of PA on the NF-κB signaling pathway.

To examine the role of NF-κB in PA’s inhibition of cPLA₂ and COX-2, Bay 11-7082 (a known inhibitor of IκB phosphorylation) was also used in the experiments. cPLA₂ protein expression induced by IL-1β was found to be almost completely prevented by pretreatment of A549 cells with 5 µM of Bay 11-7082, while COX-2 protein induction was partially reversed by Bay 11-7082 (Figure 4.16C); these effects of Bay 11-7082 on cPLA₂ and COX-2 were similar to that exerted by PA.

Therefore, taken together, results obtained indicated that inhibition of the NF-κB signaling pathway by PA would contribute at least in part to its inhibitory effect on IL-1β-induced cPLA₂ and COX-2 activation.
Figure 4.16 PA inhibited IL-1β-induced NF-κB activation in A549 cells. (A) Inhibition of PA on IL-1β-induced NF-κB p65 subunit nuclear translocation. A549 cells were pretreated with PA (10 µM) for 1 h and then challenged with IL-1β (1 ng/ml) for 30 min. Cytosolic lysates were immunoblotted against IκBα antibody. Nuclear lysates were immunoblotted with NF-κB p65 antibody. (B) Blockade of IKKβ activation and inhibition of NF-κB p65 phosphorylation by PA treatment. A549 cells were pretreated with PA for 1 h and then challenged with IL-1β (1 ng/ml) for 30 min. Whole cell lysates were immunoblotted against p-IKKα/β or p-p65 antibody. (C) Suppression of NF-κB inhibitor, Bay 11-7082, on IL-1β-induced cPLA2 and COX-2 protein expression. A549 cells were pretreated with Bay 11-7082, followed by treatment with IL-1β (1 ng/ml) for 24 h. Whole cell lysates were collected and subjected to western blot analysis. These analyses were repeated three times with similar results and a representative immunoblot is shown.
4.4 Discussion

In this study, an interesting finding was made about PA’s interference with the MTT assay where the antiproliferative activity of PA was falsely exemplified. MTT assay has been widely used to assess cytotoxicity because it is the simplest, most rapid as well as a sensitive method for estimation of cell viability. As reported in the previous chapter (Chapter 3), in the treatment of A549 cells with PPAC, a general consistent trend of change between the visual confluence of cells and the data obtained from assay was observed. In addition, the MTT assay also applied well to anticancer drugs such as 5-fluouracil and doxorubicin, as previously demonstrated in the laboratory. As such, this would mean that PA might have a special incompatibility with the MTT assay. It has been reported that cholesterol interferes with MTT assay in A549 cells by enhancing exocytosis of formazan crystals (Ahmad et al. 2006). Having a similar chemical structure with cholesterol, PA thus might interfere with the MTT assay in a similar mechanism. Indeed, a highly similar morphological change in A549 cells treated with PA was observed as that reported in the literature (Ahmad et al. 2006).

Other cell viability assays like the CCK-8 and LDH assays were undertaken and results obtained, being consistent with trypan blue staining assay, confirmed that only PA at concentrations higher than 30 µM exhibited cytotoxicity on A549 cells. Similar with its reported effects on prostate cancer cells, PA induced mitochondria-mediated apoptosis in lung cancer A549 cells.

On the other hand, the effect of PA on arachidonic acid metabolism was evaluated using non-lethal concentrations. Studies have shown that PA is capable of inhibiting PLA₂
isolated from snake venom and this inhibition has been linked to its anti-inflammatory and anticancer effect (Cuella et al. 1996). However, attributing these effects of PA to its inhibition on PLA2 enzyme activity remains to be established for two reasons. Firstly, the effective concentration of PA shown to inhibit PLA2 enzyme activity (IC50 = 2.89 mM) is too high to be achievable in vivo. Secondly, the inhibition of PLA2 by PA is examined in a cell-free assay involving a direct chemical reaction using snake venom-originated PLA2, which may not correlate with endogenous arachidonic acid signaling pathways in humans.

In contrast, in this study, activity of cPLA2, the enzyme considered to be primarily responsible for the liberation of arachidonic acid from membrane phospholipids in a wide variety of cells, was measured in a cell-based assay using A549 cells.

It has been shown that both cPLA2 and COX-2 are activated by proinflammatory factors, such as IL-1β (Croxtal et al. 1996), an important “alarm cytokine” secreted by macrophages that initiates inflammation. In this study, it was observed that the induction of cPLA2 by IL-1β was suppressed at both the mRNA and protein level by non-lethal levels of PA. Furthermore, the phosphorylation of cPLA2 by IL-1β was also reversed by PA treatment. This downregulation of cPLA2 by PA also correlated with reduced cPLA2 enzyme activity in A549 cells. Taken together, these results indicated a suppressive role of PA on IL-1β-induced cPLA2 activation. Notably, PA was shown to cause inhibition of both IL-1β-induced COX-2 expression and PGE2 production in A549 cells in a dose-dependent fashion, suggesting that PA might potentially inhibit inflammation and carcinogenesis by blocking arachidonic acid release and its subsequent conversion to PGE2.

MAPKs have been reported to play a pivotal role in the phosphorylation and
activation of cPLA₂ as well as induction of COX-2 (Choudhury et al. 2000; Chun and Surh 2004). Immunoblot analysis revealed that treatment of IL-1β-induced A549 cells with PA produced a decrease in the level of phosphorylated ERK, JNK and p38 MAPK. Since each of these three MAPKs has been previously reported to phosphorylate cPLA₂ induced by various stimuli, it was postulated that the inhibition of MAPKs by PA would at least in part contribute to the observed suppression of cPLA₂ phosphorylation. Apart from their kinase activities, these MAPKs have been shown to activate transcription of genes including cPLA₂ and COX-2 through regulation of AP-1 activation. For instance, JNK induces the expression and phosphorylation of c-Jun; ERK simulates AP-1 activity by inducing c-Fos, which heterodimerizes with c-Jun; p38 MAPK induces AP-1 through phosphorylation of ATF-2 (Whitmarsh and Davis 1996). It was observed that PA suppressed IL-1β-induced gene transcription of both cPLA₂ and COX-2. Based on published reports that cPLA₂ and COX-2 genes share several identical promoter elements, it was hypothesized that PA might suppress the transcription of these genes through regulation of one or more of these promoter elements. Therefore, it would be reasonable to relate that the transcription of cPLA₂ and COX-2 genes might be suppressed by PA, at least partially, through inhibition of AP-1 activity. Taken together, it was rationalized that the inhibition of cPLA₂ and COX-2 in A549 cells by PA might be mediated in part through inhibition of ERK, JNK and p38 signal transduction pathways.

NF-κB is a key mediator of inducible transcription initiated by various stimuli, including IL-1β (Luo et al. 2008). Pertaining to the experimental system employed herein, the link between NF-κB signaling and induction of cPLA₂ and COX-2 expression had been
demonstrated using a NF-κB-specific inhibitor. Results also showed that PA pretreatment brought about suppression of IL-1β-induced nuclear translocation of NF-κB, which would expectedly result in reduced p65 binding to the promoter region of cPLA₂ and COX-2 genes. Further analysis revealed that NF-κB nuclear translocation was likely suppressed due to the inhibitory effect of PA on IKK activation; PA was found to prevent IKK-induced phosphorylation and reduction of IκBα that would lead to reduced NF-κB translocation into the nucleus. As such, it could be concluded that NF-κB would serves as a molecular target for PA.

In summary, the findings presented in this chapter demonstrate the chemotherapeutic and chemopreventive properties of PA against lung cancer. Importantly, PA at non-lethal concentrations effectively suppressed IL-1β-induced cPLA₂ and COX-2 expression and activation, thus leading to reduced prostaglandin formation. This suppression appeared to be mediated through the inhibition of the MAPK family and NF-κB signaling pathways. Collectively, data presented in this chapter had highlighted PA as a potential cancer therapeutic or preventive agent.
Chapter 5: Investigation of lanostane-type triterpenoids against breast cancer cell invasion

Summary

In this chapter, the potential of several *Poria cocos*-originated triterpenoids against breast cancer invasion was investigated. Using human breast cancer MDA-MB-231 cells whose invasiveness was stimulated by phorbol ester, the possible anti-invasive activities of PA, PPAC and dehydropachymic acid were investigated. Gelatin zymography was used as a convenient screening method for anti-invasive capacity of these triterpenoids. It was found that PA, PPAC and dehydropachymic acid inhibited MMP-9 secretion with different capacities. Since PA exhibited the strongest anti-invasive effect at non-lethal concentrations, it was selected for more detailed mechanistic study. PA at non-lethal concentrations significantly inhibited TPA-stimulated migration of MDA-MB-231 cells in an *in vitro* Matrigel invasion assay. In addition, PA’s downregulation of MMP-9 secretion was found to be due to its inhibitory effect on MMP-9 synthesis at the transcriptional level. The mechanism for this inhibition of PA on MMP-9 transcription was elucidated through PA’s inhibition on TPA-induced transcriptional activity of NF-κB, but not that of AP-1. The inhibition of PA on NF-κB transcriptional activity was further attributed to PA-mediated diminution in TPA-induced degradation of IκBα through preventing phosphorylation of the upstream signal IKK. A decrease in p65 nuclear translocation was resulted, which led to attenuation of NF-κB transactivation. These findings suggest that by targeting NF-κB signaling, PA inhibited MDA-MB-231 cell invasion through decreasing MMP-9 expression.
5.1 Introduction

Tumor metastasis is the leading cause of mortality in cancer patients (Hanahan and Weinberg 2000). It involves a complex series of events, of which degradation of extracellular matrix by proteolytic enzymes and subsequent cancer invasion are the essential early steps (Steeg 2006). MMPs, a family of zinc-dependent endoproteinases, have been suggested to play a central role in the degradation of extracellular matrix (Coussens et al. 2002). Among them, MMP-2 (gelatinase-A) and MMP-9 (gelatinase-B) are key enzymes for degradation of type IV collagen and thus are believed to be the most relevant enzymes for tumor invasion (Brinckerhoff and Matrisian 2002). The regulation of MMPs can occur at multiple levels, including gene transcription, proenzyme activation and inhibition by endogenous inhibitors (Sternlicht and Werb 2001). While MMP-2 is usually constitutively expressed and mainly regulated by its inhibitors, MMP-9 is highly inducible by various stimuli including growth factors, cytokines, UV and TPA (Sternlicht and Werb 2001). It has been revealed that the MMP-9 promoter region contains multiple DNA binding sites for transcription factors, such as AP-1 (-533 bp, -79 bp), NF-κB (-600 bp) and stimulatory protein-1 (SP-1; -588 bp) (Sato and Seiki 1993). Upon stimulation, one or more transcription factors are activated and they bind to the promoter region to regulate MMP-9 transcription (Sato and Seiki 1993). Therefore, the down-regulation of MMP-9 expression may be a useful strategy for intervention of tumor metastasis.

Studies have shown that *Poria cocos*-originated triterpenoids possess remarkable anti-inflammatory effects in animal models and human subjects (Kaminaga et al. 1996; Cuellar et al. 1997; Giner-Larza et al. 2000; Giner et al. 2000; Fuchs et al. 2006). However
to date, the role of triterpenoids from *Poria cocos* against tumor invasion and metastasis has not been reported. Since tumor invasion is usually accompanied by a pro-inflammatory environment (Federico et al. 2007), it was proposed that suppression of inflammatory stimulation in the cellular environment may alleviate cancer invasion by preventing inflammatory factor-induced expression of key enzymes involved in tumor invasion. Therefore, it was our objective to evaluate whether these triterpenoids could inhibit cancer invasion by reducing MMP-9 activity. TPA, also commonly known as phorbol 12-myristate 13-acetate (PMA), is a classic inducer of inflammation as well as a potent tumor promoter. As such, it was used as a model agent to stimulate MMPs expression and invasiveness in this study. PA, PPAC and dehydropachymic acid were chosen due to their relatively larger yield and availability (refer to results represented in Chapter 2). The compounds were first screened for MMP-9 extracellular secretion by human breast cancer MDA-MB-231 cells using gelatin zymography. PA, the most effective compound was selected and subjected to further studies to characterize its anti-invasive property. As a result, it was found that PA, at non-lethal concentrations, inhibited TPA-stimulated invasiveness of MDA-MB-231 cells through reduction of MMP-9 gene and protein expression, which in turn resulted in a decline in MMP-9 secretion. In addition, it was demonstrated that this down-regulation of MMP-9 was attributed to the inhibitory effect of PA on NF-κB signaling.
5.2 Materials and methods

Reagents

Reagents also used in Chapter 3 and 4 had been described in the previous chapters. TPA and gelatin were purchased from Sigma (St. Louis, MO). BD BioCoat™ Matrigel™ Invasion Chambers were obtained from BD Biosciences (San Jose, CA). SB203580 (p38 inhibitor) was from Calbiochem (San Diego, CA). Antibodies specific for JNK, p38, NF-κB p65, phospho-IκBα (Ser32), IKKα and IKKβ were purchased from Cell Signaling Technology (Beverly, MA). Antibodies for MMP-9, histone H1, goat anti-rabbit IgG-conjugated to horseradish peroxidase, horse anti-mouse IgG-conjugated to horseradish peroxidase and rabbit anti-goat IgG-conjugated to horseradish peroxidase were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). GeneJuice® Transfection Reagent was from Novagen (Nottingham, UK).

Cell culture and treatment

The MDA-MB-231 human breast cancer cell line was purchased from American Type Culture Collection (Manassas, VA) and propagated at 37 °C in a 5% CO₂ humidified incubator in RPMI medium (Sigma Aldrich, St. Louis, MO) containing 10% fetal bovine serum, 10 mM HEPES and antibiotics. Tested compounds were reconstituted in DMSO at a stock concentration of 20 mM and subsequently diluted to the working concentrations for experimental procedures. For experiments using TPA, cells were always maintained in serum-free medium before, and during triterpenoids and TPA treatment.
Cell viability assay

The antiproliferative effect of PA on MDA-MB-231 cells was evaluated using CCK-8 as described in Chapter 4.

Gelatin Zymography

Gelatin zymography was performed according to published procedures with slight modifications (Liotta and Stetler-Stevenson 1990). MDA-MB-231 cells were seeded onto six-well plates in RPMI with 10% FBS, and allowed to propagate to 80% confluence. The cells were then maintained in serum-free medium for at least 12 h prior to designated treatments with triterpenoids and TPA. Following 20 h incubation of cells with TPA, conditioned medium was collected and centrifuged at 400 \times g to remove cells and debris. Equal volumes of conditioned medium were mixed with non-reducing Laemmli sample buffer [62.5 mM Tris HCl (pH 6.8), 2% SDS, 25% glycerol, 0.01% (w/v) bromophenol blue], placed at room temperature for 10 min, and subjected to electrophoresis on a 10% SDS-PAGE gel containing 0.1% (w/v) gelatin. After electrophoresis, the gels were washed in renaturing buffer (2.5% Triton X-100) for 30 min and then equilibrated for 30 min in developing buffer (50 mM Tris–HCl, pH 7.5, 0.2 M NaCl, 10 mM CaCl₂, and 1 mM ZnCl₂). The gels were subsequently incubated in fresh developing buffer at 37 °C for 24 h to allow gelatin digestion. The gelatinolytic activity of MMPs was visualized by staining the gels with 0.5% Coomassie blue R-250 in 45% methanol, 10% acetic acid (v/v) and destained with 45% methanol, 10% acetic acid (v/v) until clear bands suggestive of gelatin digestion were present. For the experiments evaluating direct effect of PA on MMP-9 activity, the collected
TPA-conditioned medium was incubated directly with different concentrations of PA at 37 °C for 10 min. Equal volumes of incubated medium were then subjected to gelatin zymography analysis as detailed above.

**Cell invasion assay**

The cell invasion assay was conducted using BD BioCoat™ Matrigel™ Invasion Chambers according to the manufacturer’s instructions. 5 × 10⁴ MDA-MB-231 cells suspended in 500 µl of serum-free medium were seeded into the upper chamber of Matrigel-coated filter inserts. After treatment with different concentrations of PA or DMSO for 1 h, 750 µL of serum-free medium containing 80 nM of TPA was added to the bottom wells as a chemoattractant. The Matrigel Invasion Chambers were incubated at 37 °C in a 5% CO₂ humidified incubator for another 20 h. After incubation, the filter inserts were removed from the wells and the cells on the upper side of the membrane were removed using cotton swabs. Cells that had invaded to the lower surface of the membrane were fixed with methanol for 15 minutes, and stained with 2% ethanol containing 0.2% crystal violet powder for 15 minutes. The inserts were washed with water, allowed to dry, and the invaded cells were detected and enumerated under a light microscope.

**RNA extraction and reverse transcription - PCR (RT-PCR)**

RNA extraction and RT-PCR were performed as described in Chapter 4. The primer sequences used for MMP-9 and GAPDH, and their product sizes were as follows - MMP-9 (620 bp): forward 5'-CGATGACGAGTTGGTCCGGGC-3’, reverse,
Chapter 5

5'-AATGATCTAAGCCCAGCGCGTGGC-3' (Zhang et al. 2006); GAPDH (325 bp): forward, 5'-TGAAGGTCGGAGTCAACGGATTTGGT-3', reverse, 5'-AAATGAGCCCCAGCCTTCTCCATG-3'.

**AP-1 and NF-κB-dependent luciferase reporter assay**

MDA-MB-231 cells were seeded in 24-well culture plates. At approximately 50% confluency, cells were cotransfected with 0.7 μg pAP-1-Luc or pNF-κB-Luc plasmids (PathDetect luciferase cis-reporting system containing 7 x AP-1 and 5 x NF-κB enhancer elements respectively; purchased from Stratagene, La Jolla, CA) and 14 ng pRL-CMV (Promega, Madison, WI) per well for 24 h. After transfection, the cells were maintained in serum-free medium, pretreated with PA for 1 h and followed by TPA stimulation for an additional 20 h. Firefly and renilla luciferase activities were assayed using the Dual Luciferase Assay System (Promega, Madison, WI), and results were expressed as firefly luciferase activity normalized to renilla luciferase activity.

**Preparation of whole cell lysates and nuclear fractions**

As described in Chapter 3 and 4.

**Western blot**

Western blot analysis was carried out as described in Chapter 3. For detection of MMP-9 protein level in the medium, conditioned medium was collected and centrifuged at 400 × g to remove cells and debris. Equal volumes of conditioned medium were mixed with
4 × Laemmli Sample Buffer [250 mM Tris-HCl (pH 6.8), 8% w/v SDS, 40% glycerol, 0.02% (w/v) bromophenol blue, 20% 2-mercaptoethanol], heated for 5 min at 100 °C, and subjected to electrophoresis on a 10% SDS-PAGE. After transferring resolved proteins to nitrocellulose membranes, the levels of MMP-9 protein were determined using a specific antibody against MMP-9.

Statistical analysis

As described in Chapter 3.

5.3 Results

5.3.1 Effects of PA, PPAC and dehydropachymic acid on TPA-stimulated MMP-9 secretion in MDA-MB-231 cells

Three triterpenoids from *Poria cocos*, namely PA, PPAC and dehydropachymic acid were evaluated for their potential anti-invasive effects against human breast cancer cells. MDA-MB-231, a breast cancer cell line with high invasive capacity, were pretreated with the compounds for 1 h, followed by TPA stimulation for another 20 h. Conditioned medium was collected and subjected to gelatin zymographic analysis for detection of secreted MMP-9. Consistent with previous reports (Zhang et al. 2006; Liang et al. 2009), treatment of MDA-MB-231 cells with TPA (80 nM) resulted in a striking increase in the proteolytic band intensity corresponding to the molecular size of pro-MMP-9 (92 kDa) (Figure 5.1). Pretreatment of MDA-MB-231 cells with each of the three tested compounds decreased MMP-9 band intensity in a dose-dependent manner (Figure 5.1), indicating that these
triterpenoids reduced MMP-9 extracellular expression. These compounds clearly possessed different potencies in reducing MMP-9 activity. For instance, slight reduction in MMP-9 proteolytic activity was initiated at 10 µM of PA; PPAC demonstrated suppression of MMP-9 activity at ≥ 40 µM; dehydropachymic acid only reduced MMP-9 activity at 60 µM (Figure 5.1). Considering the better efficacy in reducing MMP-9 gelatinolytic activity, PA was selected for further evaluation of its anti-invasive capacity.

Figure 5.1 Dose-dependent decrease in MMP-9 gelatinolytic activity mediated by PA, PPAC and dehydropachymic acid. MDA-MB-231 cells were pretreated for 1 h, and then challenged with PMA for 20 h. The conditioned medium was analyzed by gelatin zymography for MMP detection as described in Materials and methods.

The medium samples of cells co-incubated with PA and TPA were also analyzed by western blotting, which showed a dose-dependent decrease in MMP-9 protein levels (Figure 5.2A). This result suggested that PA caused an inhibition of MMP-9 cellular secretion. To
further investigate whether the observed decrease in MMP-9 gelatinolytic activity was due to inhibitory interaction between PA and MMP-9, TPA-conditioned medium was collected from control cells (non PA-treated), then mixed with PA (final concentrations 2.5, 5, 10, 20 µM) and finally subjected to gelatin zymography analysis. Results, as shown in Figure 5.2B, showed that there was no significant difference between PA-treated and –untreated group, indicating that PA failed to inhibit directly MMP-9 gelatinolytic activity. Therefore, it was concluded that PA caused reduction in MMP-9 gelatinolytic activity through inhibiting MMP-9 secretion, but not through direct inhibition of MMP-9’s enzymatic activity.

A

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Figure 5.2B PA reduced extracellular expression of MMP-9. (A) Reduction of TPA-induced MMP-9 secretion by PA. After pretreatment of cells with PA, and then TPA for 20 h, conditioned medium was subjected to western blot analysis for MMP-9 detection as detailed in Materials and methods. (B) Lack of direct inhibition by PA on MMP-9 gelatinolytic ability. MDA-MB-231 cells were treated with 80 nM TPA for 20 h, and conditioned medium was subsequently incubated with PA for 10 min. Samples were analyzed using gelatin zymography.
To rule out the possibility that this MMP-9 inhibition by PA was due to its cytotoxicity, CCK-8 cell viability assay was carried out to evaluate PA’s antiproliferative effects on MDA-MB-231 cells. As shown in Figure 5.3, following 24 h drug exposure, PA did not alter cell viability at concentrations \( \leq 20 \) µM. PA at 30 µM reduced cell viability to 90%, but this effect was not statistically significant. At 40 µM, PA exhibited notable cytotoxicity \( (p < 0.05) \) on MDA-MB-231 cells, reducing viability to 80% (Figure 5.3). These results, taken together, showed that the reduction of MMP-9 activity mediated by PA was independent of its cytotoxic effect.

![Graph showing cell viability at different PA concentrations.](image)

**Figure 5.3 Cytotoxicity profile of PA on MDA-MB-231 cells.** Cells were exposed to PA for 24 h and cell viability was determined by CCK-8. Columns, mean of three independent experiments repeated in triplicate; bars, standard deviation; * \( p < 0.05 \).

5.3.2 Inhibition of TPA-stimulated invasion of MDA-MB-231 cells by PA

To investigate whether PA’s reduction of MMP-9 extracellular secretion indeed led to reduced invasiveness of cancer cells, an invasion assay using Matrigel-coated transwell plates to measure cell migration following TPA stimulation was carried out. As shown in Figure
5.4A, the invasive capacity of MDA-MB-231 cells was enhanced by 4.5-fold upon TPA
treatment. Pretreatment of cells with 10 µM of PA significantly decreased TPA-induced
invasiveness from 4.5-fold to 2.8-fold (Figure 5.4A). Further increase of PA to 20 µM
decreased the invasion to 1.4-fold as compared to basal cell invasion without TPA stimulation
(Figure 5.4A). These results clearly demonstrated that PA was able to impede invasion of
breast cancer cells through the gelatin-rich extracellular matrix. This data further supported
the notion that reduction of MMP-9 secretion might decrease cancer invasive capacity and
indicated that PA inhibited breast cancer cell invasion through reducing MMP-9 extracellular
secretion.
Figure 5.4 PA inhibited TPA-induced invasion of MDA-MB-231 cells. Cells suspended in serum free medium were seeded onto upper chamber of Matrigel-coated filter inserts. Following PA treatment of cells for 1 h, TPA was added to the bottom chamber for another 20 h to stimulate cell invasion. Upon staining with crystal violet, invaded cells were counted under microscope as detailed in Materials and methods. Results were expressed as relative percent of cell invasion to that of basal invasion of TPA-untreated cells (A). Columns, means of three independent experiments; bars, SD; † p < 0.05 versus DMSO control; ** p < 0.01 versus TPA only-treated group. (B) Representative images showing the inhibitory effect of PA on TPA-induced cell invasion.
5.3.3 Inhibitory effect of PA on TPA-stimulated MMP-9 protein and gene expression

MMP-9 is known to be synthesized intracellularly and then secreted into the extracellular environment. Since PA had been found to reduce extracellular MMP-9 protein expression (Figure 5.2A), it was deduced that this reduction might be a result of decreased MMP-9 intracellular synthesis. Therefore, the intracellular expression of MMP-9 following treatments with TPA and PA was investigated. As shown in Figure 5.5A, treatment of MDA-MB-231 cells with PA decreased TPA-provoked MMP-9 protein expression. It should be pointed out that the two bands observed were all latent MMP-9 enzymes, the upper band (92 kDa) corresponding to a mature form and the lower band (85 kDa) representing a glycosylated form (Toth et al. 1997). The results were in agreement with the observed reduction in MMP-9 gelatinolytic activity and secreted levels in conditioned medium as presented in Figure 5.1 and 5.2A respectively. To further evaluate whether this inhibition of PA on MMP-9 protein expression in MDA-MB-231 cells occurred at the transcriptional level, semiquantitative RT-PCR was next performed to monitor MMP-9 gene expression. As shown in Figure 5.5B, while the expression of house-keeping gene GAPDH remained unaffected, TPA-induced MMP-9 mRNA expression was dose-dependently prevented by PA treatment. These results thus indicated that PA-mediated regulation of MMP-9 expression occurred at the transcriptional level.
Figure 5.5 PA inhibited TPA-induced MMP-9 protein and gene expression. (A) Inhibition of PA on TPA-induced MMP-9 protein expression. MDA-MB-231 cells were incubated with PA for 1 h, followed by TPA treatment for another 20 h. Whole cell lysates were analyzed by western blotting. Upper and lower bands represented latent mature form and precursor form of latent MMP-9 respectively. (B) Dose-dependent decrease of TPA-induced MMP-9 gene expression by PA treatment. MDA-MB-231 cells were incubated with PA for 1 h, followed by TPA treatment for another 20 h. MMP-9 gene expression was detected using RT-PCR analysis.
5.3.4 Lack of effect of PA on the AP-1 signaling pathway

The promoter region of the *MMP-9* gene contains binding sites for multiple transcription factors, including AP-1 and NF-κB. To elucidate the mechanism responsible for the inhibition of PA on MMP-9 transactivation, the effect of PA on AP-1 signaling pathway was examined. MDA-MB-231 cells transiently transfected with AP-1 luciferase reporter plasmids were exposed to PA and TPA for 20 h. Luciferase reporter assays performed showed that AP-1 transcriptional activity was significantly induced by TPA treatment. However, this induction was not affected by PA at concentrations of 10 µM or 20 µM (Figure 5.6A), suggesting that AP-1 would unlikely be involved in the inhibitory effect of PA on TPA-induced MMP-9 expression. MAPKs, the main signaling molecules responsible for AP-1 activation (Shaulian and Karin 2002), were also examined. Consistent with the observation obtained from the luciferase-based reporter assay, PA treatment failed to inhibit TPA-induced phosphorylation of MAPKs including ERK, JNK and p38 kinase (Figure 5.6B). Together, these results showed that the inhibition of PA on TPA-induced MMP-9 expression was not due to an effect on the AP-1 signaling pathway.

![Graph showing AP-1 Luciferase Activity](image)
Figure 5.6 PA lacked effect on TPA-activated AP-1 signaling. (A) Firefly and renilla luciferase activity of cells cotransfected with pAP-1-Luc and pRL-CMV and incubated in the presence or absence of TPA and PA was measured (details as described in Materials and methods). AP-1 firefly luciferase activity was normalized to renilla luciferase activity for each drug treatment, and the fold induction brought about was expressed relative to untreated control. Columns, means of three independent experiments; bars, standard deviation; † p <0.05 versus untreated control. (B) Effect of PA on TPA-induced MAPKs phosphorylation. Cells were incubated with PA 1 h prior to TPA addition for another 1 h. Whole cell lysates were obtained and analyzed by western blotting using antibodies specific for total or phosphorylated ERK, JNK and p38 kinase. Images are representative of three independent experiments.

5.3.5 Suppression of the NF-κB signaling pathway by PA

The possible effect of PA on the NF-κB transcriptional activity on MMP-9 gene was next investigated. MDA-MB-231 cells transfected with NF-κB reporter construct were
treated with TPA with or without presence of PA. Subsequent measurement of NF-κB-dependent luciferase activity revealed that transcriptional activity was induced to 3.2-fold by TPA and this induction was effectively suppressed dose-dependently by PA (Figure 5.7). The results therefore implied the involvement of the NF-κB signaling pathway in PA’s inhibitory effect on TPA-induced MMP-9 expression.

**Figure 5.7 PA suppressed TPA-induced NF-κB transactivation.** Firefly and renilla luciferase activity of cells cotransfected with pNF-κB-Luc and pRL-CMV and incubated in the presence or absence of TPA and PA was measured. NF-κB firefly luciferase activity was normalized to renilla luciferase activity for each drug treatment, and the fold induction brought about was expressed relative to untreated control. **Columns,** means of three independent experiments; **bars,** standard deviation; † *p* <0.05 versus untreated control; **p** *p* < 0.01 versus TPA only-treated group.

In agreement with its inhibition on NF-κB transcriptional activity, PA blocked TPA-induced nuclear accumulation of p65 (Figure 5.8A) and prevented TPA-induced degradation of IκBα protein (Figure 5.8B). As degradation of IκBα is primarily a result of IκBα phosphorylation at serine 32 and serine 36 (Ghosh and Baltimore 1990), it was
postulated that this prevention by PA might be due to its inhibition on IκBα phosphorylation. Indeed, PA induced a dose-dependent decrease in TPA-induced IκBα phosphorylation at serine 32 (Figure 5.8B). The effect of PA on the upstream signal IKK was further determined. PA suppressed IKKα/β phosphorylation in a dose-dependent manner without affecting total protein level of IKKα (Figure 5.8C). Taken together, the results indicated that the inhibitory effect of PA on NF-κB signaling could be attributed to its inhibition on IKK phosphorylation, which in turn led to a consequential decrease in p65 nuclear translocation and suppression of transcriptional activity.

\begin{center}
\begin{tabular}{c|c|c|c|c}
 & TPA (80 nM) & + & + & + \\
 & PA (μM) & - & - & 10 & 20 \\
p65 & & & & \\
Histone H1 & & & &
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Figure 5.8 PA suppressed TPA-induced signaling molecules involved in the NF-κB transactivation cascade. (A) Prevention by PA on TPA-induced NF-κB p65 nuclear translocation. Cells were incubated with PA 1 h prior to TPA addition for another 1 h. Nuclear fractions extracted from cells were analyzed by western blotting for detection of nuclear p65 protein. (B) Dose-dependent effect of PA on TPA-induced IκBα phosphorylation and steady state levels of IκBα. Cells were incubated with PA 1 h prior to TPA addition for another 1 h. Whole cell lysates collected were analyzed by western blotting using antibodies specific for total or phosphorylated IκBα. (C) Inhibitory effect of PA on TPA-induced IKK phosphorylation. Cells were incubated with PA 1 h prior to TPA addition for another 1 h. Whole cell lysates collected were analyzed by western blotting using antibodies specific for total IKKa or phosphorylated IKKa/β. Images are representative of three independent experiments.
5.3.6 Reduction of TPA-stimulated MMP-9 expression by NF-κB inhibitor

To address the causal relationship between NF-κB signaling and MMP-9 expression in MDA-MB-231 cells, the effect of NF-κB inhibitor Bay 11-7082 on MMP-9 expression was investigated. The inhibitory effect of Bay 11-7082 as a specific inhibitor of IκBα phosphorylation was first confirmed by the demonstration that TPA-induced IκBα phosphorylation was effectively prevented, which in turn resulted in the reversal of TPA-induced IκBα degradation (Figure 5.9A). The effect of Bay 11-7082 on TPA-mediated MMP-9 induction was next examined. In a similar manner as of PA, MDA-MB-231 cells were subjected to treatment of 5 µM Bay 11-7082 1 h prior to TPA (80 nM) addition for a duration of 20 h. Notably, a reduction in TPA-induced MMP-9 protein expression was detected in the treated cells (Figure 5.9B). The result was in corroboration with analysis of the corresponding conditioned medium by gelatin zymography and immunoblotting, which showed a respective decrease in MMP-9 gelatinolytic activity and extracellular secretion (Figure 5.9B). Combined together, these results clearly showed that NF-κB signaling was involved in MMP-9 induction.
Figure 5.9 Specific NF-κB inhibitor Bay 11-7082 reduced TPA-induced MMP-9 expression and activity.  (A) Effect of Bay 11-7082 on TPA-induced IκBα phosphorylation and steady state levels of total IκBα. Cells were incubated with 5 µM Bay 11-7082 for 1 h prior to TPA addition for another 1 h. Whole cell lysates were collected and subjected to western blot analysis using antibodies specific for total or phosphorylated IκBα. (B) Decrease of MMP-9 expression and activity by Bay 11-7082. MDA-MB-231 cells were incubated with 5 µM Bay 11-7082 for 1 h prior to TPA treatment for another 20 h. Conditioned medium was collected and subjected to gelatin zymography and western blot analysis (lower panel). Whole cell lysates were also collected and subjected to western blot analysis using MMP-9 antibody (upper panel). The analyses were performed in three separate experiments and a representative image is presented.
5.4 Discussion

In this study, the effects of *Poria cocos*-derived triterpenoids against *in vitro* breast cancer invasion were investigated. All three selected compounds PA, PPAC and dehydropachymic acid, were found to reduce TPA-stimulated MMP-9 extracellular expression, among which PA exhibited the greatest efficacy. PA was further demonstrated to inhibit *MMP-9* gene transcription at non-lethal concentrations. The anti-invasive mechanism was determined to be mediated through PA’s inhibitory effect on the NF-κB signaling pathway.

Both of MMP-2 and MMP-9 digest collagen IV in the basement membrane and are believed to be the most important enzymes responsible for tumor invasion (Brinckerhoff and Matrisian 2002). In addition, accumulating evidence suggests that MMP-2 and MMP-9 expression are highly correlated with breast cancer cell invasion (Brown et al. 1993; Duffy et al. 2000). On the other hand, agents that can downregulate MMP-2 or MMP-9 have been demonstrated to inhibit tumor invasion (Overall and Lopez-Otin 2002; Park et al. 2007; Lin et al. 2008). TPA is a commonly used pro-inflammatory stimulus as well as a tumor promoter. Previous studies have shown that TPA promotes cancer invasion and metastasis, and thus can be used to investigate anti-invasive capacity of tested compounds (Gomez et al. 1999; Shimao et al. 1999). Here, TPA treatment was found to markedly increase MMP-9 gelatinolytic activity, but not that of MMP-2. This result was consistent with similar observations made in a number of previous studies where MMP-9 was found to be highly inducible but not MMP-2 (Woo et al. 2004; Zhang et al. 2006; Lin et al. 2008; Weng et al. 2008; Liang et al. 2009; Park et al. 2009). It is known that the MMP-9 promoter region contains DNA-binding
sites for AP-1 and NF-κB, while MMP-2 does not contain these regulating elements (Overall and Lopez-Otin 2002). Therefore, the difference observed in their responses to TPA can be explained by the structural differences in the regulatory sequences of their genes.

However, it should be noted that latent MMP pro-enzymes can be activated during zymography, and thus digestive effect observed by gelatin zymography may not represent the natural enzyme activity of the active form of MMP-9 (Hibbs et al. 1985; Toth et al. 1997; Fridman et al. 2003). Interestingly, in this study, the extracellular secretion of the 92 kDa pro-MMP-9, but not the presumably active MMP-9 (which has a molecular weight of 82 kDa) was observed. Similar findings were reported in previous studies (Toth et al. 1997; Woo et al. 2004; Liang et al. 2009). Indeed, there has been a consistent inability to detect active MMP-9 (82 kDa) in cultured cells (Fridman et al. 2003). To date, how the extracellular 92 kDa MMP-9 protein leads to enhanced MMP-9 functional ability and subsequent tumor invasion remains elusive. An alternative mechanism for pro-MMP-9 activation in which the propeptide domain be disengaged from the active site without removal of the inhibitory propeptide domain, if confirmed, might explain at least in part the MMP-9 enzymatic activity in the absence of a change in molecular mass (Fridman et al. 2003). Nonetheless, this study demonstrated a clear down-regulation of extracellular MMP-9 protein by PA.

In the promoter region of the *MMP-9* gene, there are multiple *cis*-acting regulatory elements, including two AP-1 binding sites and one NF-κB binding site (Sato and Seiki 1993; Overall and Lopez-Otin 2002). Previous studies suggested that AP-1 and NF-κB were intensively involved in TPA-stimulated MMP-9 expression (Huang et al. 2004; Woo et al. 2004; Takada et al. 2006; Lin et al. 2008; Park et al. 2009). Here, in agreement with
previous studies, TPA was shown to increase transcriptional activity of both AP-1 and NF-κB. Importantly, PA selectively inhibited TPA-induced NF-κB transcriptional activity, but had no effect on AP-1 transcription. In addition, activation of MAPKs by TPA treatment was observed, further supporting the activation of AP-1 signaling pathway by TPA treatment. In accordance with the obtained data from the luciferase reporter assays, PA did not change protein levels of TPA-activated MAPKs. These results underpinned NF-κB signaling as the molecular target for PA's inhibitory action on MMP-9 expression.

NF-κB is an important transcription factor in a large variety of genes related to inflammation, cancer development and tumor invasion. It has been suggested to be a target for many biologically active phytochemicals such as curcumin, resveratrol and epigallocatechin gallate (Nomura et al. 2000; Chun et al. 2003; Woo et al. 2004). In most resting cells, NF-κB is sequestered in the cytoplasm with IκBα and remains inactive. Exposure of cells to inflammatory stimuli such as TPA leads to activation of IKK complex, which in turn phosphorylates IκBα. Subsequently, phosphorylated IκBα is targeted and degraded by the ubiquitin-proteasome machinery. The degradation of IκBα allows NF-κB p65 subunit to translocate freely to the nucleus and bind to the MMP-9 promoter to initiate MMP-9 gene transcription (Overall and Lopez-Otin 2002). Here, PA was found to bring about a reduction in TPA-induced p65 nuclear translocation through its inhibition on IKKα/β phosphorylation, followed by a decrease in IκBα phosphorylation and prevention of IκBα proteasomal degradation. Moreover, it was ascertained that a specific NF-κB inhibitor Bay 11-7082 could effectively decrease MMP-9 expression, thus validating the role of NF-κB in PA's inhibition on MMP-9.
In conclusion, in this chapter, it was found that PA at sub-lethal concentrations inhibited TPA-induced MDA-MB-231 breast cancer cell invasion through down-regulation of MMP-9 expression. Furthermore, it was revealed that the molecular mechanism by which PA inhibited MMP-9 expression was mediated through its suppressive effect on NF-κB activation. In the light that tumor metastasis is often associated with poor prognosis and high mortality among cancer patients, the need to discover and develop new therapeutic strategies that target early tumor invasiveness and/or metastasis is crucial. PA, having found to impair invasiveness of cultured breast cancer cells, would serve as a potential antimetastatic agent.
Chapter 6: General discussion, conclusion and future work

6.1 General discussion

6.1.1 Cancer therapeutic potential of triterpenoids from *Poria cocos*

Among the eight triterpenoids isolated from *Poria cocos*, most of them showed moderate cytotoxicity against human cancer cells. In this project, PA and PPAC were evaluated for their apoptosis-inducing effects in human lung cancer cells. PA induced apoptosis through a pathway mediated by mitochondria, whereas PPAC induced apoptosis without involvement of mitochondria. Despite these mechanistic differences, both of these compounds effectively inhibited lung cancer cell proliferation by inducing apoptotic cell death. The apoptosis-inducing effect of PA was also demonstrated in prostate cancer cells by our previous lab members (Gapter et al. 2005). In addition, dehydrotrametenolic acid, one natural triterpenoid isolated from *Poria cocos* has been demonstrated to induce apoptosis in H-ras transformed rat2 cells (Kang et al. 2006). Therefore, it could be inferred that triterpenoids from *Poria cocos* exerted cytotoxicity through inducing apoptotic death of cancer cells. However, the cytotoxicity exerted by these triterpenoids was not comparable to that of clinically used chemotherapeutic drugs. Most of the reported triterpenoids from *Poria cocos* exhibit modest cytotoxicity on cancer cells with IC$_{50}$ value larger than 20 µM (Li et al. 2004; Mizushina et al. 2004; Gapter et al. 2005; Kang et al. 2006).

Due to limited studies conducted on these triterpenoids, the *in vivo* bioavailability profiles of these compounds remain unknown. Whether an effective concentration can be achieved *in vivo* needs to be determined before these compounds can be considered as
possible anticancer agents. It is possible that the relatively high concentrations needed for their chemotherapeutic effectiveness may hinder these compounds to become useful anticancer agents themselves. However, considering the successful synthesis of CDDO from oleanolic acid (a natural pentacyclic triterpenoid with weak anticancer activity), chemical modification performed on triterpenoids from *Poria cocos* may yield more potent cancer therapeutic agents.

### 6.1.2 Cancer chemopreventive potential of triterpenoids from *Poria cocos*

Triterpenoids purified from *Poria cocos* have been reported by different research groups to inhibit skin tumor formation in a two-stage carcinogenesis mouse model. Moreover, EBV-EA activation by TPA has been shown to be inhibited by most of the tested triterpenoids from *Poria cocos* (Kaminaga et al. 1996; Kaminaga et al. 1996; Akihisa et al. 2007). In summary, these findings have highlighted the chemopreventive activity of *Poria cocos*-originated triterpenoids. However, the mechanism(s) underlying their chemopreventive effects has not been well clarified. Notably, there is a discrepancy between the observed preventive efficacy of these compounds as evaluated by carcinogenesis model and the proposed mechanism in which their chemopreventive effects are attributed to their inhibition of PLA2 enzyme activity. According to the study by Kaminaga et al, PA significantly inhibited TPA-stimulated skin tumor formation in a two-stage carcinogenesis model (Kaminaga et al. 1996). In that study, 0.2 µmol of PA dissolved in 100 µl vehicle was spread onto mouse skin (final concentration 2 µM). After 20 weeks, PA reduced occurrence of skin tumor from 73% to 27% and reduced the average number of papillomas per mouse
from 7.2 to 1.2. This chemopreventive effect of PA is comparable to that of resveratrol, a grape-originated stilbene compound intensively studied for its anticancer activity (Jang et al. 1997).

On the other hand, the attribution of biological effects of *Poria cocos*-originated triterpenoids to PLA₂ enzyme inhibition to date is based on a report showing the inhibitory effect of PA and dehydrotumulosic acid to the enzymatic activity of PLA₂ from snake venom (Cuella et al. 1996). However, the IC₅₀ (2 mM) required to inhibit PLA₂ enzymatic activity is too high to be achievable *in vivo*. Clearly, this enzymatic inhibitory efficacy by PA does not agree with PA's chemopreventive activity observed in the study by Kaminaga et al. Although *in vivo* dosages cannot be directly compared with concentrations obtained in *in vitro* experiments, the differences in effective concentrations (2 mM versus 2 µM) between the studies are too vast to establish a good correlation. In our study, we found that PA modulated arachidonic acid metabolism in A549 cells at non-lethal concentrations. PA effectively inhibited IL-1β-induced arachidonic acid release and PGE₂ formation. Considering the role of arachidonic acid and prostaglandins in cancer, it would be reasonable to infer that PA might inhibit promotion of pro-inflammatory factors on cancer development through downregulation of key enzymes involved in arachidonic acid metabolism.

In addition, we observed significant reduction of arachidonic acid metabolism by PA at 3 µM, which is biologically more relevant to the dosages used in the animal study by Kaminaga et al. As such, it would be likely that the chemopreventive effect of PA was due to reduced arachidonic acid metabolism. Although PA did not completely inhibit PGE₂ production at tested concentrations (≤ 10 µM), repeated application of PA might result in the
constant suppression of PGE$_2$ release, and thus eventually lead to prevention of tumor formation. From a general prospective, triterpenoids from *Poria cocos* seemingly possess an ability to alleviate or reduce the harmful response elicited by foreign stimuli. This ability is also reflected by the anti-inflammatory and anti-rejection effects of these triterpenoids as demonstrated in previous studies (Nukaya et al. 1996; Yasukawa et al. 1998; Giner-Larza et al. 2000; Schinella et al. 2002; Zhang et al. 2004; Fuchs et al. 2006). Interestingly, these findings share much similarity with the so-called “sedative” effect of *Poria cocos* in the principles of Traditional Chinese Medicine.

### 6.1.3 Anti-metastatic potential

Tumor invasion and metastasis is an advanced stage of cancer development, which has accounted for the majority of cancer-related mortalities. In this project, we examined the effects of triterpenoids isolated from *Poria cocos* on MMP-9 expression and tumor invasion. PA was found to inhibit tumor invasion, presumably through its downregulation of MMP-9. MMP-9 is a key enzyme involved in degradation of extracellular matrix. The downregulation of MMP-9 would result in an extracellular environment unfavorable for cancer cells to invade into nearby tissues or blood vessels. In addition, MMP-9 has been found to play an important role in angiogenesis, which is essential for metastatic tumor to survive at metastatic sites (Egeblad and Werb 2002). Our findings that PA reduced MMP-9 expression therefore might also indicate an inhibitory effect of PA on angiogenesis. On this basis, it was postulated that PA could act as an effective anti-metastatic agent by remodeling extracellular matrix and inhibiting tumor neovascularization. In this PhD study, to exclude
the interference of PA's antiproliferative effect on tumor invasiveness, non-lethal concentrations of PA were used for the experiments performed. It is anticipated that combination of PA’s cytotoxicity and inhibitory effect on tumor invasion could result in enhanced anticancer effects when lethal doses were to be used. In addition, other triterpenoids originated from Poria cocos may have similar effects, which warrants the in vitro and in vivo evaluation of their anti-metastatic potential.

6.1.4 NF-κB signaling pathway as the main molecular target

In our investigation of PA’s modulation of arachidonic acid metabolism, we found that inhibition of the NF-κB and MAPK signaling pathways were the underlying mechanisms of PA’s biological effects. In the study examining PA’s anti-invasive effect, we found that NF-κB signaling pathway, but not AP-1 signaling, was targeted by PA. This discrepancy may be due to utilization of different stimuli and different cell lines in these two studies. In the study investigating PA’s effect on arachidonic acid metabolism, IL-1β was employed as the stimulant and this study was carried out in human lung cancer A549 cells. In the anti-invasive study, PA’s effect was evaluated by TPA stimulation of a highly invasive human breast cancer cell line MDA-MB-231. Although both IL-1β and TPA bring about activation of MAPK and NF-κB signaling, mediation of the two signal transduction pathways are distinctly different. IL-1β binds to IL-1 receptor, and activates MAPK and NF-κB signaling pathways through mediator proteins such as IL1RAP, IRAK, TRAF6 and MYD88 (Kuno and Matsushima 1994; Jiang et al. 2002). TPA, a strong PKC activator, has been reported to induce activation of MAPK and NF-κB signaling pathways through mediation of PKC
isotypes or proteins such like CaMKII (Lallena et al. 1999; Hughes et al. 2001). The
different activation mechanisms by IL-1β and TPA could explain at least in part PA’s different
effects on MAPK activation in these two experimental systems. The discrepancy might also
be due to the different cell lines used in these two studies. Nonetheless, we had observed
PA’s inhibitory effects on NF-κB signaling in both studies, thus indicating NF-κB signaling as
the main target of PA.

As an important transcriptional factor, NF-κB has been found to be highly involved in
a variety of inflammatory conditions (Karin 2008). It responds to most of the inflammatory
stimuli and activates downstream transcription of inflammatory proteins. Correspondingly,
inhibition of NF-κB will reduce the inflammatory response elicited by a variety of stimuli
(Karin 2008). Therefore, our findings demonstrating triterpenoids’ inhibition of the NF-κB
activation cascade would provide possible explanation to their anti-inflammatory effects as
reported by numerous studies (Kaminaga et al. 1996; Nukaya et al. 1996; Cuellar et al. 1997;
Giner-Larza et al. 2000; Giner et al. 2000). However, the exact molecular target inhibited by
these triterpenoids remains elusive. Based on the observations made in this study that IKK
phosphorylation was downregulated by PA, we proposed that PA’s molecular target could be
an upstream signal of IKK.

6.2 Conclusion

From the above discussion, we could conclude that triterpenoids from *Poria cocos*
exerted moderate cytotoxicity against cancer cells. We had focused on PA and PPAC, and
found that they might inhibit cancer growth by causing apoptotic death of cancer cells. On
the other hand, triterpenoids from *Poria cocos* might exert their chemopreventive effects through downregulation of key enzymes involved in arachidonic acid metabolism, which led to reduced prostaglandin production. In addition to their cytotoxicity and chemopreventive capacity, these triterpenoids might also possess anti-metastatic activity through their downregulation of MMP-9. Our results had suggested that inhibition of the NF-κB signaling pathway would account for their chemopreventive and anti-metastatic effects. The findings in this project will not only expand our understanding of the anticancer potential of triterpenoids from *Poria cocos*, but also encourage more studies examining anticancer activities of these triterpenoids.
6.3 Future work

6.3.1 Structure-activity relationship study

In this PhD project, we have shown that PA and PPAC exhibit different mechanisms of induction of apoptosis in cancer cells. PA activates intrinsic apoptotic pathway which needs the mediation of mitochondria; PPAC triggers extrinsic pathway and activates caspase cascade without involvement of mitochondria. Considering their structural similarities but distinct acting mechanisms, these two compounds are worthwhile for more in-depth structure-activity relationship study. In addition, *Poria cocos* is a rich source of lanostane-type triterpenoids sharing chemical similarities. For instance, the eight triterpenoids obtained in this project are structurally similar to one another, with only minor differences on some chemical groups. This makes it possible to analyze the function of certain chemical group(s) to gain insight of structure-activity relationship of lanostane-type triterpenoids. Elucidation of the structure-activity relationship will allow the synthesis and development of novel anticancer compounds with more superior chemotherapeutic potencies.

6.3.2 Elucidation of the exact molecular target(s) of PA

NF-κB signaling pathway has been demonstrated to be the main target of PA for its chemopreventive and anti-invasive activity. However, the exact cellular protein(s) inhibited by PA remain elusive. Although we had shown that IKK phosphorylation was blocked by PA treatment, the precise mechanism underlying this blockade is not known. NF-κB signaling has been intensively investigated and found to be the target for many anti-inflammatory or anti-cancer compounds (Garg and Aggarwal 2002; Karin 2008).
However, the molecular mechanism of IKK activation has not been thoroughly studied (Ghosh and Karin 2002). Clarification of the exact molecular target of PA will not only help understand PA’s anticancer mechanism, but also provide valuable insight into mechanisms of many other active compounds with similar effects. Moreover, it will make it possible to design potent inhibitors of this target and yield better anticancer agents targeting NF-κB signaling.

### 6.3.3 Evaluation of possible synergistic effect of PA with other anticancer drugs

Activation of NF-κB signaling is one common feature of therapeutic treatment of cancer (Nakanishi and Toi 2005). Since NF-κB regulates cell survival, NF-κB activation contributes to resistance of cancer cells to chemotherapeutic agents, and thus compromises apoptotic potential of these agents (Nakanishi and Toi 2005). In our study, we have found that PA is an inhibitor of NF-κB signaling. Combination of PA with other chemotherapeutic agents that unnecessarily activate NF-κB may thus result in synergistic effects in killing cancer cells. Therefore, studying the combinatorial effect of PA with other chemotherapeutic agents may represent a feasible research direction. *In vivo* experiments can be carried out upon observation of such synergistic effects in *in vitro* studies.

### 6.3.4 Pharmacokinetic study on PA and PPAC

In this PhD project, we have shown that PA and PPAC inhibit lung cancer cell proliferation and induce apoptosis in lung cancer cells. However, it is not known whether the concentrations effective for these biological effects can be achieved *in vivo*. A
pharmacokinetic study will provide useful information on the pharmacokinetic profiles of these two compounds. It can also be used to facilitate dosage determination in tumor xenograft studies.

### 6.3.5 Evaluation of *in vivo* efficacy of PA and PPAC against lung tumor xenograft growth

PA and PPAC have been shown to induce apoptotic cell death of human lung cancer cells. They are also effective in inhibiting colony formation of lung cancer cells, suggesting that these compounds may inhibit cancer growth *in vivo*. In addition, PA has been demonstrated to modulate arachidonic acid metabolism, thereby possibly impeding cancer progression. Therefore, *in vivo* evaluation of these two compounds on xenograft tumor model is warranted. To determine whether PA or PPAC is able to repress or reduce the size of an established tumor, a dose-escalation study can be carried out in nude mice carrying lung tumor xenografts.

### 6.3.6 Determination of *in vivo* efficacy of PA against breast cancer metastasis

In this project, we have demonstrated the potential of PA against breast cancer cell invasion using *in vitro* experiments; we showed that PA inhibits *MMP-9* gene transcription, and thus reduces MMP-9 protein synthesis and the subsequent extracellular secretion of MMP-9. Our results therefore suggest that PA is an effective anti-invasive agent, and future research can be carried out to investigate the *in vivo* efficacy of PA against breast cancer metastasis.
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Appendix I \(^{13}\)C NMR spectrum of Compound 1 (pachymic acid)
Appendix II $^{13}$C NMR spectrum of Compound 2 (dehydropachymic acid)
Appendix III $^{13}$C NMR spectrum of Compound 3 (3-acetyloxy-16α-hydroxytrametenolic acid)
Appendix IV $^{13}$C NMR spectrum of Compound 4 (polyporenic acid C)
Appendix V $^{13}$C NMR spectrum of Compound 5 (3-epi-dehydropachymic acid)
Appendix VI $^{13}$C NMR spectrum of Compound 6 ($3$-epi-dehydrotumulosic acid)
Appendix VII $^{13}$C NMR spectrum of Compound 7 (tumulosic acid)
Appendix VIII $^{13}$C NMR spectrum of Compound 8 (29-hydroxypolyporenic acid)