ANTI-TUMOR MECHANISMS OF LUTEOLIN, A MAJOR FLAVONOID OF CHRYSANTHEMUM MORIFOLIUM

SHI RANXIN

(***M. Sc., Institute of Oceanology, Chinese Academy of Sciences***)

A THESIS SUBMITTED
FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF COMMUNITY, OCCUPATIONAL AND FAMILY MEDICINE,
YONG LOO LIN SCHOOL OF MEDICINE
NATIONAL UNIVERSITY OF SINGAPORE

2006

ACKNOWLEDGEMENTS

I would like to express my deepest respect and acknowledgements to my supervisors, Professor **Ong Choon Nam**, and co-supervisor, Dr. **Shen Han Ming**, for their consistent and invaluable guidance throughout my Ph.D. study. They are the persons who always encourage me, give me professional comments and lead me to the right way of doing scientific research. What I have learned from them will benefit my career and life.

I would also like to extend my sincere gratitude and appreciation to:

Prof. David Koh, Head of the department, for his general kind support during the course of this study.

Mr. Ong Her Yam, Mr. Ong Yeong Bing, Ms Su Jin and Ms Zhao Min for their kind help in the process of laboratory work.

Dr. Peter Colin Rose, Mr. Won Yen Kim, Dr Zhang Siyuan, Ms Huang Qing, Mr. Manav, Mr. Luo Guodong, Ms Zhou Jing and Ms Shi Jie for their critical discussions, invaluable comments and consistent help during whole course of my study.

Dr. Lai Jiaping, for his critical comments on the thesis.

All other staff in Department, for their general and unselfish help.

National University of Singapore, for the research scholarship

Especially, I would like express my deepest appreciation to my wife Ms Zhao Xiuli and my family members for their love, understanding and support.

TABLE OF CONTENTS

Acknowledgements ii

Table of contents iii

Summary xi

List of Publications xiii

List of Figures xiv

Abbreviations xix

CHAPTER ONE

INTRODUCTION

1.1 CHRYSANTHEM MORIFOLIUM 1

1.1.1 General introduction 1

1.1.2 Chemical components of chrysanthemum 4

1.1.2.1 Flavonoids in chrysanthemum 4

1.1.2.2 Terpenoids in chrysanthemum 7

1.1.3 Pharmacological properties of chrysanthemum 8

1.1.3.1 Anti-oxidant activities 8

1.1.3.2 Anti-hypertension 9

1.1.3.3 Anti-eye irritation 9

1.1.3.4 Anti-ulcerative colitis 9

1.1.3.5 Anti-inflammatory activity 10

1.1.3.6 Anti-tumor activities 10

1.2 PHARMACOLOGICAL MECHANISMS OF LUTEOLIN

1.2.1 Estrogenic and anti-estrogenic activity 12

1.2.2 Antioxidant activity 13

1.2.3 Anti-inflammatory activity 15

1.2.4 Anti-cancer property 19

1.2.4.1 Anti-carcinogenesis activity 19

1.2.4.2 Inhibition on proliferation 21

1.2.4.3 Induction of cell cycle arrest 24

1.2.4.4 Induction of apoptosis 26

1.2.4.5 Anti-angiogenesis 28

1.2.4.6 Inhibition on cancer metastasis 29

1.3 APOPTOSIS

1.3.1 General introduction 30

1.3.2 Caspases Apoptosis 31

1.3.3 Apoptosis pathways 34

1.3.3.1 Receptor-mediated apoptosis 34

1.3.3.2 Mitochondrial-mediated apoptosis 35

1.3.4 Apoptosis and cancer 36

1.3.5 TNFR signaling pathway 37

1.3.5.1 TNF-induced apoptosis 38

1.3.5.2 TNF-induced NF-κB activation 38

1.3.5.3 TNF-induced JNK activation 39

1.3.5.4 Regulation of TNF-induced apoptosis 40

1.3.6 TRAIL signaling pathway 40

1.3.6.1 TRAIL-induced apoptosis 41

1.3.6.2 NF-kappa B activation 41

1.3.6.3 Regulation of TRAIL-induced apoptosis 41

1.3.7 Cisplatin and its anti-cancer effects 42

1.3.7.1 Anti-cancer effect of cisplatin 42

1.3.7.2 Regulation of cisplatin-induced apoptosis 45

1.4 OBJECTIVES OF THE STUDY 47

CHAPTER TWO

INDENTIFICATION OF THE MAJOR ACTIVE COMPONENTS IN CHRYSANTHEM MORIFOLIUM

2.1 INTRODUCTION 50

2.2 MATERIALS AND METHODS 50

2.2.1 Materials 51

2.2.2 Cell lines and cell culture 51

2.2.3 Extraction and fractionation 51

2.2.4 Cytotoxicity assay 52

2.2.5 High-performance liquid chromatography-mass spectrum 52

2.3 RESULTS 53

2.3.1 Fractionation of Chrysanthemum water extract 53

2.3.2 Cytotoxicity of each fraction 53

2.3.3 Flavonoids are the major components in EtOAc fraction 56

2.4 DISCUSSION 72

CHAPTER THREE

CYTOTOXICITY OF FLAVONOIDS FROM CHRYSANTHEMUM

3.1 INTRODUCTION 74

3.2 METERIALS AND METHODS 75

3.2.1 Regents and chemicals 75

3.2.2 Cell lines and cell culture 75

3.2.3 Assessment of cell viability using MTT assay 76

3.2.4 Assessment of apoptosis using DAPI staining 76

3.2.5 Assessment of DNA content using flow cytometry 76

3.2.6 Caspase 3-like activity assay 76

3.2.7 Western blotting 77

3.3 RESULTS

3.3.1 Cytotoxicity of chrysanthemum flavonoids on human cancer cells 77

3.3.2 Chrysanthemum flavonoid extract induces apoptosis in cancer cells 77

3.3.3 Chrysanthemum flavonoid extract causes apoptosis by inducing caspase cascade 78

3.3.4 Cytotoxicity of luteolin and apigenin in human cancer cells 82

3.3.5 Luteolin induces apoptosis in COLO205 cells but not in HCT116 and HT29 cells 82

3.3.6 Luteolin induced apoptosis in COLO205 by activating caspase-3 83

3.4 DISCUSSION 91

CHAPTER FOUR

LUTEOLIN SENSITIZES TUMOR NECROSIS FACTOR (TNF)-INDUCED APOPTOSIS IN TUMOR CELLS

4.1 INTRODUCTION 94

4.2 MATERIALS AND METHODS 96

4.2.1 Cell culture and treatment 96

4.2.2 Measurement of cell death and apoptosis 96

4.2.3 Caspase 3-like and caspase 8 activity assay 97

4.2.4 Transient transfection 97

4.2.5 NF-κB luciferase reporter assay 98

4.2.6 Preparation of whole cell lysate, cell fractionation, co-immunoprecipitation and western blot 98

4.2.7 Electrophoretic mobility shift assay (EMSA) 98

4.2.8 RNA extraction and RT-PCR 99

4.2.9 Statistical analysis 99

4.3 RESULTS 100

4.3.1 Luteolin sensitizes TNFα-induced cell death in cancer cells 100

4.3.2 Luteolin sensitizes TNFα-induced cell death through apoptosis 103

4.3.3 Luteolin-induced sensitization is associated with enhanced caspase-8 activation 106

4.3.4 TNFα-induced NF-κB activation is inhibited by luteolin 111

4.3.5 Luteolin inhibits TNFα-activated NF-κB by interfering with CBP-p65 interaction 116

4.3.6 P65 expression protects the cell death induced by luteolin and TNFα 116

4.3.7 Luteolin suppresses the expression of NF-κB anti-apoptotic target genes A20 and c-IAP1 119

4.3.8 JNK activation contributes to the sensitization effect of luteolin on TNFα-induced apoptosis 119

4.3.9 Ectopic expression of A20, c-IAP1 and dominant negative forms of JNKK1 and JNKK2 prevents apoptosis induced by luteolin and TNFα 122

4.4 DISCUSSION 127

CHAPTER FIVE

LUTEOLIN SENSITIZES TRAIL-INDUCED APOPTOSIS IN CANCER CELLS

5.1 INTRODUCTION 133

5.2 MATERIALS AND METHODS 134

5.2.1 Reagents and Plasmids 134

5.2.2 Cell culture and treatments 135

5.2.3 Apoptosis assessment-DAPI staining 135

5.2.4 Transient transfection and luciferase assay 135

5.2.5 Western blot 136

5.2.6 Immunostaining for detection of death receptors 136

5.2.7 RNA extraction and RT-PCR 137

5.2.8 Statistical analysis 138

5.3 RESULTS 138

5.3.1 Luteolin sensitizes cancer cells to TRAIL-induced apoptosis 138

5.3.2 Luteolin facilitates TRAIL-initiated caspase-3 maturation 139

5.3.3 Luteolin does not alter surface expression of death receptors 144

5.3.4 NF-κB is not involved in the sensitization of luteolin 149

5.3.5 XIAP down-regulation contributes to the cell death 149

5.3.6 XIAP down-regulation is mediated by ubiquitination and proteasomal degradation 156

5.3.7 PI3K/AKT is not involved in cell death induced by luteolin and TRAIL 161

5.3.8 PKC activation blocks XIAP degradation and prevents the cell death induced by luteolin and TRAIL 164

5.3.9 PKC inhibition promotes XIAP down-regulation and apoptosis in TRAIL- treated cells 167

5.4 DISCUSSION 170

CHAPTER SIX

LUTEOIN SENSITIZES ANTI-CANCER DRUG INDUCED APOPTOSIS IN CANCER CELLS

6.1 INTRODUCTION 178

6.2 MATERIALS AND METHODS 180

6.2.1 Reagents and chemicals 180

6.2.2 Cell culture and treatments 180

6.2.3 Apoptosis assessment-4',6-diamidino-2phenylindole staining 180

6.2.4 RNA interference 181

6.2.5 Immunoprecipitation, cell fractionation and Western blot 181

6.2.6 RNA extraction and real time-PCR 182

6.2.7 *In vivo* xenograft experiments 182

6.2.8 Immunohistochemistry for p53 staining 183

6.3 RESULTS 184

6.3.1 Luteolin enhances cisplatin-induced caspase-dependent apoptosis in human cancer cells 184

6.3.2 Luteolin and cisplatin elevate p53 protein level 188

6.3.3 Luteolin does not enhance cisplatin-induced apoptosis in mutant p53 cells 188

6.3.4 p53 knockdown abolishes the apoptosis induced by luteolin and cisplatin 191

6.3.5 Luteolin elevates p53 by increasing its protein stability 194

6.3.6 Luteolin increases p53 protein stability by inhibiting MDM2 and disrupting their interaction 197

6.3.7 Luteolin and cisplatin induces p53 and Bax mitochondrial translocation 201

6.3.8 Luteolin enhances the anti-cancer effect of cisplatin *in vivo* 202

6.3.9 Luteolin enhanced the anti-cancer effect of cisplatin *in vivo* by elevating p53 202

6.4 DISCUSSION AND SUMMARY 211

CHAPTER SEVEN

DISCUSSION AND CONCLUSION

7.1 Flavonoids are the major anti-tumor components of chrysanthemum water extract 218

7.2 Luteolin sensitizes TNF induced apoptosis in human cancer cells 219

7.3 Luteolin sensitizes TRAIL induced apoptosis in human cancer cells 221

7.4 Luteolin enhances the anticancer effect of cisplatin *in vitro* and *in vivo* 223

7.5 Luteolin as a chemosensitizer in cancer therapy 224

7.6 Conclusions 225

CHAPTER EIGHT

REFERENCE

References 227

SUMMARY

The flower heads of *Chrysanthemum morifolium* have been used as traditional medicine as well as a beverage for centuries in many Asian countries. Recently, it was found that the water extract of chrysanthemum significantly inhibited tumor growth in mice, suggesting the anti-tumor potential of this herbal plant. To investigate the anti-tumor properties of chrysanthemum and its major active components, we conducted the following studies: 1) identification of the major active components of the water extract of chrysanthemum; 2) evaluation of the anti-tumor effects of the major active components; 3) investigation of the combined effects of luteolin, its main flavonoid, with cancer therapeutic agents *in vitro* and *in vivo*.

Initially, we applied a bioassay-driven fractionation strategy, and sequentially obtained four fractions from chrysanthemum. Flavonoids were then identified as the major components in the fraction showing the most potent cytotoxicity against human cancer cells. Further studies showed that the flavonoids extracted from chrysanthemum exerted significant cytotoxic effect on several human cancer cells via inducing caspase-dependent apoptosis.

Among a number flavonoids identified, luteolin is one of the most abundant found in chrysanthemum. In this study, we focused on the combined effect of luteolin with several cancer therapeutic agents, including tumor necrosis factor (TNF), TNF-related apoptosis-inducing ligand (TRAIL) and cisplatin.

First, we found that luteolin significantly sensitized TNFα-induced apoptosis in a number of cancer cell lines. The sensitization was due to the inhibitory effect by luteolin on TNFα-induced activation of nuclear transcription factor-kappaB (NF-κB). As a result, luteolin suppressed the expression of NF-κB targeted anti-apoptotic genes,

including *A20* and cellular inhibitor of apoptosis protein-1 (*c-IAP1*), and augmented and prolonged c-Jun N-terminal kinase (JNK) activation.

Next, we found that luteolin significantly sensitized the apoptosis induced by TRAIL in TRAIL-resistant cancer cells. Such sensitization was achieved through down-regulation of X-linked inhibitor of apoptosis protein (XIAP), which was due to enhanced XIAP ubiquitination and proteasomal degradation. Further, we demonstrated that inhibitory effect of luteolin on protein kinase C (PKC) contributed to the XIAP down-regulation. In addition, our data reveal a novel function of PKC in cell death: PKC activation may stabilize XIAP and thus suppress TRAIL-induced apoptosis.

Third, we examined the effect of luteolin on the anti-cancer activities of cisplatin, a potent DNA damaging agent that has been widely used as a cancer chemotherapeutic in clinic. Our data showed that luteolin was able to enhance the apoptosis-inducing effect of cisplatin. Interestingly, p53 played a critical role in the apoptosis induced by combination of luteolin and cisplatin. We found that the rapid elevation of p53 protein level was due to stabilization effect of luteolin by decreasing MDM2 protein. Furthermore, combined treatment of luteolin and cisplatin induced significant p53 and Bax mitochondrial translocation as well as Bax conformation change. Finally, the anti-cancer potential of a combination of luteolin and cisplatin was investigated in a xenograft nude mice model. We found that luteolin could significantly enhance the anti-cancer activity of low dose of cisplatin by elevating p53 protein.

In conclusion, the present study provides a new insight of the anti-tumor property of chrysanthemum and its major active component, luteolin. The evidence from both *in vitro* and *in vivo* experiments clearly demonstrates the anti-tumor potential of luteolin as a chemo-sensitizer in cancer therapy.

LIST OF PUBLICATIONS

I. Various parts of this study have been published in international peer-reviewed journals as below:

1. **Shi Ran-Xin**, Ong Choon-Nam, Shen Han-Ming. PKC inhibition and XIAP downregulation contribute to luteolin sensitized TRAIL-induced apoptosis in cancer cells. ***Cancer Research***. 2005, September 1, 65:7815-7823.
2. **Shi Ran-Xin**, Ong Choon-Nam, Shen Han-Ming. Luteolin sensitizes tumor necrosis factor alpha-induced apoptosis in human cancer cells. ***Oncogene***. 2004, October 7; 23(46):7712-7721.

II. Manuscripts submitted for publication or in preparation:

3. **Shi Ran-Xin**, Ong Yeong-Bing, Ong Choon-Nam, Shen Han-Ming. Luteolin enhances the anti-cancer effect of cisplatin by activating p53 in vitro and in vivo (Manuscript submitted to Cancer Research)
4. **Shi Ran-Xin**, Ong Choo-Nam, Shen Han-Ming. Identification of flavonoids as major anti-tumor components of Chrysanthemum water extract. (Manuscript in preparation)

III. Presentations at scientific conferences:

5. **Shi Ran-Xin**, Ong Choon-Nam, Shen Han-Ming. PKC inhibition and XIAP downregulation contribute to luteolin sensitized TRAIL-induced apoptosis in cancer cells. **96th Annual Meeting of American Association of Cancer Research,** April 16-20, Anaheim, California, 2005
6. **Shi Ran-Xin**, Ong Choon-Nam, Shen Han-Ming. Luteolin sensitized TNF-induced apoptosis in human cancer cells. **International Congress on Complementary and Alternative Medicines (ICCAM) 2005,** February 26-28, Singapore, 2005
7. **Shi Ran-Xin**, Ong Choon-Nam, Shen Han-Ming. Luteolin Sensitizes Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand (TRAIL)-Induced Apoptosis in Cancer Cells by Inhibiting Protein Kinase C and Down-regulating X-Linked Inhibitor of Apoptosis Protein (XIAP). **Combined Scientific Meeting-shaping a new era in healthcare,** November 4-6, Singapore, 2005

IV. Book chapters:

8. **Shi Ran-Xin,** Ong Choon-Nam, Shen Han-Ming. Pharmacological and Chemopreventive studies of Chrysanthemum. **Herbal and Traditional Medicine-Molecular Aspects of Health.** Marcel Dekker (New York) 2004, pp407-439

V. Awards

9. NUS-NMRC Young Scientist Award, Combined Scientific Meeting 2005 (CSM05), 4-6 November 2005

LIST OF FIGURES

Figure 1.1 *Chrysanthemum morifolium* Ramat (A) has been used as an herbal medicine as well as a beverage (B) 3

Figure 1.2 Structure of flavonoids 5

Figure 2.1 Bioassay-driven fractionation from Chrysanthemum 54

Figure 2.2 Cytotoxicity of fractions from Chrysanthemum on human colorectal cancer cells HCT116 55

Figure 2.3 Flavonoids in the EtOAc fraction 58

Figure 2.4 Structure elucidation of peak 1, RT 18.31 min 59

Figure 2.5 Structure elucidation of peak 2, RT 20.23 min 60

Figure 2.6 Structure elucidation of peak 3, RT 21.22 min 61

Figure 2.7 Mass spectrum of peak 4, RT 22.79 min 62

Figure 2.8 Structure elucidation of peak 5, RT 23.5 min 63

Figure 2.9 Structure elucidation of peak 6, RT 25.2 min 64

Figure 2.10 Structure elucidation of peak 7, RT 23.18 min 65

Figure 2.11 Structure elucidation of peak 8, RT 21.22 min 66

Figure 2.12 Structure elucidation of peak 9, RT 28.70 min 67

Figure 2.13 Structure elucidation of peak 10, RT 33.20 min 68

Figure 2.14 Structure elucidation of peak 11, RT 36.61 min 69

Figure 2.15 Structure elucidation of peak 12, RT 42.09min 70

Figure 2.16 Structure elucidation of peak 13, RT 43.08min 71

Figure 3.1 Cytotoxicity of EtOAc extract on human colorectal cancer cells 79

Figure 3.2 EtOAc extract induces apoptosis in cancer cells HCT116 80

Figure 3.3 EtOAc extract causes apoptosis by inducing caspase cascade in HCT116 81

Figure 3.4 Cytotoxicities of luteolin and apigenin in human cancer cells 84

Figure 3.5 Luteolin induces apoptotic cell death in COLO205 cells but not in HCT116 or HT29 cells 85

Figure 3.6 Morphological change of COLO205 after luteolin treatment 86

Figure 3.7 Luteolin induces PARP cleavage time- and dose-dependently in COLO205 cells 87

Figure 3.8 Luteolin induces caspase-3 cleavage in COLO205 cells 88

Figure 3.9 Luteolin activates caspase-3 like activity in COLO205 89

Figure 3.10 z-VAD-fmk inhibits cell death induced by luteolin in COLO205 cells 90

Figure 4.1 Luteolin pretreatment sensitizes TNFα-induced cell death in cancer cells 101

Figure 4.2 Effect of luteolin treatment sequence on sensitization 102

Figure 4.3 Luteolin and TNFα induce typical apoptosis in COLO205 cells 104

Figure 4.4 Effect of luteolin on c-myc protein level in COLO205 cells 105

Figure 4.5 Effect of luteolin and TNFα on caspase 107

Figure 4.6 Effect of luteolin and TNFα on caspase activity 108

Figure 4.7 Effect of caspase inhibitors on luteolin and TNFα-induced apoptosis 109

Figure 4.8 Effect of caspase inhibitors on luteolin and TNFα-induced apoptosis 110

Figure 4.9 Luteolin inhibits TNFα-induced NF-κB transcriptional activity 112

Figure 4.10 Effect of luteolin pretreatment on IκBα degradation and p65 nuclear translocation in COLO205 cells 114

Figure 4.11 Effect of luteolin pretreatment on NF-κB-DNA binding activity 115

Figure 4.12 Effect of luteolin on CBP-p65 interaction 117

Figure 4.13 Effect of p65 overexpression on cell death induced by luteolin and TNF 118

Figure 4.14 Luteolin pretreatment down-regulates expression of NF-κB anti-apoptotic target genes 120

Figure 4.15 Luteolin pretreatment leads to augmented and prolonged JNK activation induced by TNFα 121

Figure 4.16 SP600125 Inhibits caspase 8 and caspase 3 activation and PARP cleavage in cells treated with luteolin and TNFα 124

Figure 4.17 Ectopic expression of A20, c-IAP1 and JNKK1(DN)+JNKK2(DN) protects cell death induced by luteolin and TNFα 125

Figure 4.18 Ectopic expression of A20, c-IAP1 and JNKK1(DN)+JNKK2(DN) protects cell death induced by luteolin and TNFα (Quantification) 126

Figure 5.1 Sensitivity of human cancer cells to TRAIL-induced apoptosis 140

Figure 5.2 Luteolin sensitizes human cancer cells to TRAIL-induced apoptosis 141

Figure 5.3 Luteolin sensitizes human cancer cells to TRAIL-induced apoptosis 142

Figure 5.4 Luteolin and TRAIL induces caspase activation 143

Figure 5.5 Effect of caspase inhibitors on caspase activation induced by luteolin and TRAIL 145

Figure 5.6 Effect of caspase inhibitors on cell death induced by luteolin and TRAIL 146

Figure 5.7 Effect of luteolin on expression level of various TRAIL death receptors 147

Figure 5.8 Effect of luteolin and TRAIL on death receptor mRNA level 148

Figure 5.9 Effect of TRAIL and luteolin on NF-κB transcriptional activity 151

Figure 5.10 Effect of luteolin and TRAIL on expression of anti-apoptotic proteins 152

Figure 5.11 Down-regulation of XIAP in cells treated with luteolin and TRAIL 153

Figure 5.12 Ectopic expression of XIAP protects cell death induced by luteolin and TRAIL 154

Figure 5.13 Ectopic expression of XIAP protects cell death induced by luteolin and TRAIL (Quantification) 155

Figure 5.14 Effect of luteolin and TRAIL on XIAP mRNA level 158

Figure 5.15 XIAP down-regulation is through proteasomal degradation in cells treated with luteolin and TRAIL 159

Figure 5.16 A combination of luteolin and TRAIL promotes XIAP ubiquitination 160

Figure 5.17 Effect of PMA on the cell death induced by luteolin and TRAIL 162

Figure 5.18 Effect of luteolin and TRAIL on PI3K/AKT pathway 163

Figure 5.19 PKC activation protects cell death and XIAP down-regulation induced by luteolin and TRAIL 165

Figure 5.20 Effect of LY, Wort and BIM on PMA-induced PKC activation 166

Figure 5.21 Effect of luteolin on PKC activation 168

Figure 5.22 A combination of PKC inhibition and TRAIL enhances XIAP degradation and cell death 169

Figure 5.23 Illustration of the pathways involved in the sensitization activity of luteolin on TRAIL-induced apoptosis in cancer cells 175

Figure 6.1 Luteolin enhances cisplatin-induced apoptosis in cancer cells 184

Figure 6.2 Luteolin enhances cisplatin-induced apoptosis in HCT116 cells 185

Figure 6.3 A combination of luteolin and cisplatin causes caspase activation 186

Figure 6.4 A combination of luteolin and cisplatin elevates p53 protein level 188

Figure 6.5 A combination of luteolin and cisplatin does not cause apoptosis in mutant p53 cancer cells 189

Figure 6.6 p53 RNA interference 191

Figure 6.7 p53 RNA interference suppresses the apoptosis induced by luteolin and cisplatin in HCT116 cells 192

Figure 6.8 Luteolin does not affect p53 mRNA level in HCT116 cells 194

Figure 6.9 Luteolin elevates p53 stability in HCT116 cells 195

Figure 6.10 Luteolin disrupts the p53-MDM2 interaction in HCT116 cells 197

Figure 6.11 Luteolin decreases MDM2 protein level 198

Figure 6.12 Luteolin decreases MDM2 mRNA level 199

Figure 6.13 A combination of luteolin and cisplatin induces cytochrome c release to cytosal 203

Figure 6.14 Luteolin and cisplatin induced p53 and bax mitochondrial translocation 204

Figure 6.15 Luteolin and cisplatin induced bax mitochondrial translocation 205

Figure 6.16 Luteolin enhances the anti-cancer effect of cisplatin *in vivo* 206

Figure 6.17 Luteolin enhances the anti-cancer effect of cisplatin *in vivo* (Quantification) 207

Figure 6.18 Luteolin and cisplatin elevate p53 protein level *in vivo* 208

Figure 6.19 Luteolin and cisplatin elevate p53 protein level *in vivo* (Quantification) 209

LIST OF ABBREVIATIONS

ActD	actinomycin D
AIF	apoptosis inducing factor
AKT	
AO	acridine orange
AP-1	activator protein-1
Apaf-1	apoptotic protease-activating factor 1
ATM	ataxia telangiectasia mutated kinase
ATR	ataxia telangiectasia and Rad3-related kinase
BIM	bisindolylmaleimide I
CARD	caspase recruitment domains
CBP	CRE binding protein
CDK	cyclin-dependent kinases
CHX	cycloheximide
c-IAP	cellular inhibitor of apoptosis protein
COX-2	cyclooxygenase-2
CrmA	cytokine response member A
DAPI	4',6-diamidino-2-phenylindole
DcR	death decoy receptor
DD	death domain
DED	death effector domain
DISC	death-inducing signaling complex
DMSO	dimethyl sulfoxide
DR	death receptor

EB	ethidium bromide
EBV-EA	epstein-Barr virus early antigen
EDTA	ethylenediaminetraacetic acid
EGCG	epigallocatechin gallate
EGF	epidermal growth factor
EGFP	enhanced green fluorescence protein
EGFR	epidermal growth factor receptor
EtOH	ethanol
EtOAc	ether acetate
EMSA	electrophoretic mobility shift assay
ER	estrogen receptor
FADD	Fas-associated death domain
FAK	focal adhesion kinase
FBS	fetal bovine serum
FLIP	FLICE inhibitory protein
FITC	fluorescein isothiothyanate
G3PDH	glyceraldehydes-3-phosphate dehydrogenase
GFP	green fluorescence protein
GSH	glutathione
GSSG	oxidized glutathione
H2O2	hydogen peroxide
HPLC	high performance liquid chromtagraphy
ICAM-1	intercellular adhesion molecule-1
IFN	interferon
IκB	inhibitor of κB

IKK	IκB kinase
IL	interleukin
iNOS	inducible NO synthase
JNK	c-Jun N-terminal kinase
MAPK	mitogen-activated protein kinases
MeOH	methanol
MMP	matrix metalloproteases
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium
MS	mass spectrometry
NADH	nicotinamide-adenine hydrogen
NF-κB	nuclear transcription factor-kappaB
NO	nitric oxide
LPS	lipopolysaccharide
LY	LY-294002
PARP	poly(ADP-ribose) polymerase
PBMC	peripheral blood mononuclear cells
PDGF	platelet-derived growth factor
PI	propidium iodide
PI3K	phosphatidylinositol 3'-kinase
PLC	phospholipase C
PKC	protein protein kinase C
PMA	phorbol 12 myristate 13 acetate
PMSF	phenylmethylsulfonyl fluoride
RIP	receptor-interacting protein
ROS	reactive oxygen species

RTK	receptor tyrosine kinase
TNFα	tumor necrosis factor-α
TNFR1	TNF receptor 1
TRADD	TNF receptor-associated death domain
TRAF2	TNF receptor-associated factor 2
TRAIL	TNF-related apoptosis-inducing ligand
TUNEL	TdT-mediated Dutp nick-end labelling
VEGF	vascular endothelial growth factor
VSMCs	vascular smooth muscle cells
Wort	wortmannin
XIAP	X-linked inhibitor of apoptosis
z-VAD-fmk	N-benzyloxycarbonyl-valyl-alanyl-aspartyl fluoromethylketone

CHAPTER ONE

INTRODUCTION

1.1 CHRYSANTHEM MORIFOLIUM

1.1.1 General introduction

Chrysanthemum morifolium Ramatuelle (also called *Dendranthema morifolium* or Hang Bai Ju in Chinese, referred as chrysanthemum hereafter in this thesis) is a member of the Compositae family. Its dried flower-heads have been used as a traditional herbal medicine in several Asian countries, such as China, Korea and Japan, for centuries. They have also been used as an herbal beverage in Chinese folklore and known as chrysanthemum tea (Figure 1.1).

The biological characters of chrysanthemum are "A perennial herb. 60-150 cm high. Stem erect, striate, hairy. Leaves alternate, petiolate, ovate or oblong, 3.5-5 cm long by 3-4 cm wide, variously lobed and divided. Inflorescence small head, 5 cm in diameter. Flowers yellowish-white. Calyxgreenish; ligulate unisexual, tubular bisexual. Stamens 5, syngenesious, epipetalous. Gynoecium bicarpellary, syncarpous, unicular, inferior; ovule one, basal placentation; style one with bifid curled stigmas whose receptive surfaces is on the inside. Fruit a one-seeded cypselia, crowned. Seed fills the fruit" (The Institute of Chinese Materia Medica, 1989).

Chrysanthemum is widely distributed in most habitats of China. In China, they are cultivated mainly in Zhejiang province along the Grand Canal. Tong Xiang City of this province, also referred to as the 'City of Chrysanthemum', produces about 4000-5000 tons of dried chrysanthemum flowers each year, which accounts for more than 90% of the total chrysanthemum production in China. The plants are usually grown in early spring and the flowers are harvested in autumn of each year. Although the components of chrysanthemum may vary slightly according to the different cultivation environments, the flowers are processed using similar methods. After

A

B

Figure 1.1 ***Chrysanthemum morifolium*** Ramat **(A) has been used as an herbal medicine as well as a beverage (B)**

steam treatment, the flowers were dried under the sun and then packed into an air-tight plastic bag to prevent absorption of moisture.

1.1.2 Chemical components of chrysanthemum

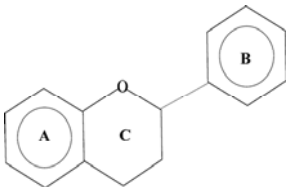
Volatile oil and flavonoids are believed to be the main active components in chrysanthemum. The most abundant and biologically active components flavonoids, in the form of glycoside derivatives, are more polar than volatile oil and hence are readily dissolved in water. Another group is terpenoids, which are present in the volatile oil.

1.1.2.1 Flavonoids in chrysanthemum

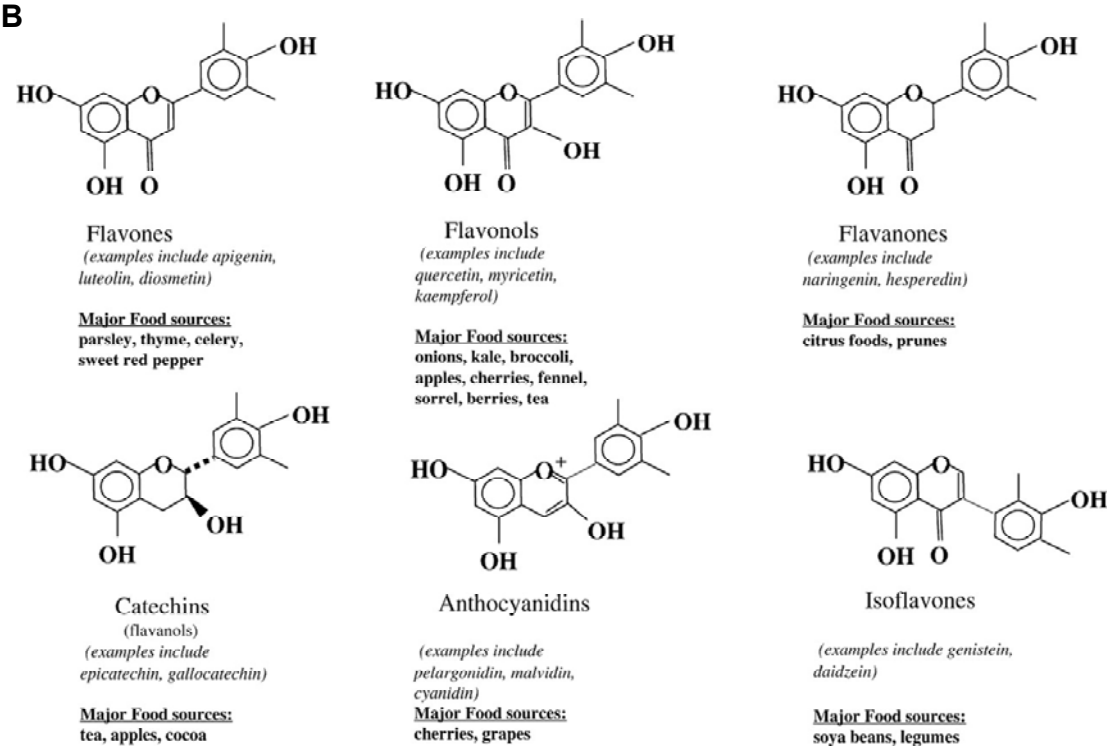
Flavonoids are ubiquitous plant components with a common C6-C3-C6 structure, consisting of two aromatic rings linked through three carbons (Figure 1.2A). The carbon skeleton can be regarded as being made up of a C6 fragment (A ring) and a C6-C3 fragment that contains a B ring. According to the variations in the heterocyclic C-ring, flavonoids can be further grouped into six major subclasses, including flavones, flavonols, flavanones, catechins, anthocyanidins, and isoflavones (Figure 1.2B) (Ross and Kasum, 2002).

Most flavonoids in plant cells are present as glycosides which are aglycons with sugar substitution. Sugar substitution on the flavonoid skeleton may occur through hydroxyl groups in the case of *O*-glycosides (Figure 1.2C), which is more common, or directly to carbon atoms in ring A as *C*-glycosides. The most important variations in their structure arise from the level of oxygenation (hydroxyl or methoxyl groups) and the position of attachment of ring B (flavonoids and isoflavonoids). The number of sugar rings substituted on the aglycone varies from one to four. All these render the great structure variation in flavonoids and so far more than 4000 types of flavonoids have been identified.

A



B



Adopted from Ross and Kasum 2002

C

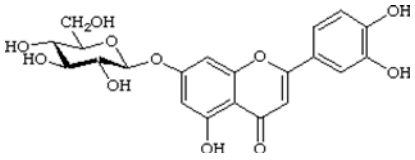


Figure 1.2 Structure of flavonoids

A, The skeleton of flavonoids; B, subgroups of flavonoids; C, example structure of a flavonoid glycoside

Both flavonoid aglycons and flavonoid glycosides can be extracted from plants by methyl alcohol (MeOH) or ethyl acetate (EtOAc). Since flavonoid glycosides are more polar than flavonoid aglycons, its solubility in water is higher than that of aglycons. The structure of an individual flavonoid in a mixture can be identified using liquid chromatography (LC) and mass spectrometry (MS) (Stobiecki, 2000).

Flavonoids play an important role in defense of plants against microorganisms and insects, and act as UV protectants in plant cells (Harborne and Williams, 2000). These phytochemicals also affect the human and animal health because of their significance in the diet, which is ascribed to their antioxidant properties, estrogenic action and a wide spectrum of antimicrobial and pharmacological activities (Birt *et al.*, 2001). The evidence comes from *in vivo* animal studies, *in vitro* cell culture experiments and human epidemiological studies (Hollman and Katan, 1999). For instance, epidemiological studies show a clear correlation between the flavonoid consumption and lower risk of cancer of the gastrointestinal tract (Hollman and Katan, 1999). A cohort study in Finland also supported that flavonoids intake in some circumstances may be involved in slowing cancer process and lowered cancer risks (Knekt *et al.*, 1997). Several studies also suggested an inverse correlation of flavonoids intake with stroke and cardiovascular disease (Hollman and Katan, 1999). Flavonoids may block several points in the process of tumor promotion, including inhibiting kinases, reducing transcription factors and regulating cell cycle (Birt *et al*., 2001).

In the last 3 decades, extensive studies have been conducted on isolation and identification of flavonoids in chrysanthemum. By extraction using 70 % MeOH and partition using hexane, $CHCl_3$, ethanol (EtOH), n-butanol (n-BuOH), and H_2O or similar methods, more than 12 flavonoids have been identified in chrysanthemum (Hu

et al., 1994; Liu *et al.*, 2001; Lee *et al.*, 2003; Hu *et al.*, 2004). Most of the flavonoids are glycosides, which are conjugated with sugars. Based on their aglycon form, these flavonoids can be grouped into the following six major types: (i) luteolin (luteolin, luteolin-7-O-beta-D-glucoside), (ii) apigenin (apigenin-7-O-beta-D-glucoside, apigenin 7-O-beta-D-(4'-caffeoyl)glucuronide), (iii) acacetin (acacetin-7-O-beta-D-glucoside, acacetin-7-O-beta-D-galactopyranoside, acacetin-7-O-(6”-rhamnosyl)-beta-D-glucopyranoside), (iv) hesperetin (hesperetin-7-O-beta-D-glucopyranosyl (6”-alpha-L-rhamnopyranoside, hesperetin-glucoside), (v) quercetin, and (vi) baicalin (Hu *et al*., 1994; Liu *et al*., 2001; Lee *et al*., 2003; Hu *et al*., 2004).

The flavonoid components of chrysanthemum have been proven to be responsible for the many pharmacological properties of this herbal plant, which will be discussed in more details in Section 1.1.3. However, it should be noted that the flavonoids present in chrysanthemum are not restricted in this plant. Most of them are also widely distributed in other plants, for example green tea, parsley, celery and berries. The studies on the bioactivities of flavonoids were extensively reviewed by Harborne and Williams (2000).

1.1.2.2 Terpenoids in chrysanthemum

Terpenes are a class of naturally occurring chemicals derived from five-carbon isoprene units assembled and modified in various ways. They consist of one isoprenoid skeleton or of a polymer made up of several such units. According to the number of isoprene units that they contain, terpenes can be subdivided into several subclasses, including monoterpenes ($C_{10}H_{16}$, 2 isoprene units), sesquiterpenes ($C_{15}H_{24}$, 3 isoprene units), diterpenes ($C_{20}H_{32}$, 4 isoprene units), triterpenes ($C_{30}H_{48}$, 6 isoprene units), tetraterpenes ($C_{40}H_{60}$, 8 isoprene units) and polyterpenes with a large number of isoprene units (Hanson, 2001).

Terpenoids are terpenes which have substitute groups. The substitute groups may have varying degrees of oxygenation, such as alcoholic and ketonic, at different positions. The large variety of this compound makes terpenoids the largest group of natural products. Among the more than 23,000 terpenoids described, various interesting substances are already known to be present. For example, plant hormones, flavour, flagrances and biopolymers (latex) are terpenoids. Because many terpenoids are biologically active, they are also used for medical purposes. For instance, the antimalarial drug artemisinin and the anticancer drug paclitaxel (Taxol) are terpenoids with an established medical application (Linden *et al.*, 2001)

Until now, more than 50 triterpenoids and several sesquiterpenoids have been isolated and identified in chrysanthemum (Akihisa *et al.*, 1996). Ukiya *et al.* identified 32 triterpenoids which are present as 3-*O*-fatty acid esters in the *n*-hexane soluble fraction and 24 triterpenoids as 3-*O*-palmitoyl esters in the nonsaponifiable lipid fraction (Ukiya *et al.*, 2001).

1.1.3 Pharmacological properties of chrysanthemum

Traditionally chrysanthemum is mainly used for common cold, fever, migraines, conjunctivitis, eye irritation, hypertension, ulcerative colitis, vertigo and ophthalmia with swelling and pain etc (Jiang, 2002). As a mixture with other herbs, it has been claimed to be able to relieve migraines and eye irritation, improve vision and cure keratitis. For instance, the effective rates against ulcerative colitis and hypertension are reported to be more than 90% and 80%, respectively (Liu, 1998; Jiang, 2002).

1.1.3.1 Antioxidant activities

The antioxidant properties of flavonoids extracted from chrysanthemum could have been responsible for its broad pharmacological effects. It was found that its

water extract showed significant antioxidant activities, suggesting that the extract may reduce lipid peroxidation and play a role in protecting against damages to the cell membrane (Chen *et al.*, 2003).

The water extract of chrysanthemum also possessed direct inhibitory effects on various free radicals (Duh, 1999). The significant correlation between phenolic compounds and antioxidant activity indicates that the flavonoids may contribute directly to the antioxidant activity of the extract. The flavonoids can also be absorbed into the cell membrane and hence protect the cells from the damages of free oxygen radicals (Duthie and Dobson, 1999).

1.1.3.2 Anti-hypertension

The flavonoids of chrysanthemum have been proven to increase blood circulation in experimental animals, suggesting a potential role in reducing hypertension (Zhou, 1987). Several fractions from the ethanol extract also showed significant anti-myocardial ischemia and anti-arrhythmias activities in rats (Jiang *et al.*, 2004).

1.1.3.3 Anti-eye irritation

Aldose reductase catalyzes the reduction of glucose to sorbitol, which is responsible for eye irritation (Terashima *et al*., 1991; Matsuda *et al*., 2002). Hot water extract of chrysanthemum has been reported to inhibit rat lens aldose reductase. Flavones and flavone glycosides were found to be the active components (Matsuda *et al.*, 2002).

1.1.3.4 Anti-ulcerative colitis

Chrysanthemum water extract was found to inhibit ulcerative colitis by decreasing the contents of adherent glycoproteins, which are responsible for the adherence and communication between cells (Liu *et al*., 2001).

1.1.3.5 Anti-inflammatory activity

Chrysanthemum has long history for treatment of inflammation (Yu and Xie, 1987). Eleven triterpene alcohols, isolated from chrysanthemum, were tested for their inhibitory effects on phorbal myristate acetate (PMA)-induced inflammation in the ears of mice. All eleven triterpene alcohols showed remarkable inhibitory effect with a 50% inhibitory dose at 0.1-0.8 mg per ear, which was roughly at the level comparable to that of indomethacin, an anti-inflammatory drug as positive control (Ukiya *et al*., 2001). Helianol, the most predominant component in the triterpene alcohol fraction, exhibited the strongest inhibitory effect among the 11 compounds tested. Since anti-inflammation activity of the inhibitors is highly related to their anti-cancer-promoting activities, helianol is also expected to be a potent anti-tumor agent (Akihisa *et al*., 1996).

Flavonoids of chrysanthemum also have been showed to exert anti-inflammatory effects (Cheng, 2005) and the mechanisms have been extensively studied. For example, luteolin is able to inhibit lipopolysaccharide (LPS)-induced release of TNF or interleukins (ILs) or directly inhibit the signaling transduction such as nuclear factor-kappa B (NF-κB) that mediates inflammatory responses (Xagorari *et al.*, 2001; Xagorari *et al.*, 2002; Kim *et al.*, 2005b). The functional role of those molecules will be discussed in more details in Section 1.2.3.

Recently, extracts of chrysanthemum were investigated on their anti-inflammatory effect in animal models. A butanol soluble fraction, which mainly contains flavonoids, caused a significant inhibition on the auricle edema induced by dimethylbenzene in mice (Cheng *et al.*, 2005b)

1.1.3.6 Anti-tumor activities

Recently the potential anti-tumor activity of chrysanthemum has interested many researchers. For instance, fifteen pentacylic triterpenes isolated from chrysanthemum have been screened for their anti-tumor-promoting activities. All of the compounds showed inhibitory effects against Epstein-Barr virus early antigen (EBV-EA) activation induced by the tumor promoter, PMA in Raji cell, which means that they can inhibit tumor promotion (Ukiya *et al.*, 2002). The terpenoids faradiol, heliantriol $B_{0,}$ heliantriol, arnidiol, faradiol α-epoxide and maniladiol also showed significant inhibitory activity against almost all 60 human tumor cell lines derived from seven cancer types (lung, colon, melanoma, renal, ovarian, brain and leukemia) (Ukiya *et al*., 2002).

The anti-tumor activities of flavonoids have also been well documented. For example, as one of the flavonoids from chrysanthemum, luteolin has been reported to inhibit proliferation or induce cycle arrest or induce apoptosis in some cancer cells (more details in Sections 1.2.4.2, 1.2.4.3, and 1.2.4.4). The anti-tumor properties of luteolin can also be through inhibiting angiogenesis and metastasis (more details in Sections 1.2.4.5 and 1.2.4.6).

1.2 PHARMACOLOGICAL MECHANISMS OF LUTEOLIN

Luteolin is one of the major flavonoids in chrysanthemum. As a ubiquitious flavonoid, luteolin has been extensively studied for its various biological effects, such as estrogenic and anti-estrogenic activity, anti-oxidant activity, anti-inflammation, anti-proliferation, anti-carcinogenesis, and anti-tumor effects. Many of these activities are functionally related to each other. For instance, its anti-inflammatory effects may also attribute to its anti-cancer effects. Anticancer property is closely related to the

effects on proliferation, cell cycle, apoptosis, topoisomerase and several protein kinases.

1.2.1 Estrogenic and anti-estrogenic activity

Estrogens are hormones involved in the proliferation and differentiation of target cells. In response to estrogens, estrogen receptor (ER) will be activated and it then stimulate DNA synthesis and cell proliferation (Colditz, 2005). Flavonoids are naturally occurring phytoestrogens because they can bind to ER and activate its signaling pathway (Collins-Burow, 2000). So, it is suggested that these groups of natural compounds may be used to replace conventional hormones in therapy of menopause disorder. Luteolin possesses potent estrogenic activity at very low concentration (Zand, 2000), suggesting that it may be useful in hormone replacement therapy.

However, there were also reports about the anti-estrogenic effects of luteolin, similar to genistein, a well studied soy isflavone with both estrogenic and anti-estrogenic properties (Wang, 1996; Han, 2002). The mechanisms behind this still remain controversial. A possible explanation is that flavonoids are estrogenic because they have a high affinity towards ER and thus activate ER if the estrogen is deficient. Nevertheless, their estrogenic activity is relatively weak, 10^3-10^5 fold less than 17β-estradiol (Murkies *et al.*, 1998; Zand, 2000). Thus, in the presence of estradiol, flavonoids could possibly inhibit estrogen by competing for its receptors.

Since ER is one of the major risk factors in breast cancer, the anti-estrogenic activity of flavonoids has been suggested to be closely related to their anti-proliferation activity and potential in breast cancer therapy and prevention. Luteolin, as well as other flavonoids such as daidzein, genistein and quercetin, is able to inhibit the proliferation-stimulating activity in MCF-7 cells caused by environmental

estrogens such as diethylstilbestrol, clopmiphene and bisphenol (Han, 2002). The suppressive effect of flavonoids suggests that these compounds have anti-estrogenic and anti-cancer activities. Wang and Kurzer (1998) also found that luteolin inhibits estradiol-induced DNA synthesis (Wang, 1998). In an *in vivo* test, Holland and Roy (1995) proved that luteolin reversed the estrogen-stimulated proliferation of mammary epithelial cells in female Noble rats, suggesting that it may play a preventive role in estrogen-induced mammary carcinogenesis (Holland and Roy, 1995).

It is however important to point out that the anti-estrogenicity of flavonoids does not always correlate with their ER binding capacity, suggesting that alternative signaling mechanisms could have been involved in their antagonistic effects (Collins-Burow, 2000). Mammalian cells contain two classes of estradiol binding sites, type I (Kd~1.0 nM) and type II (Kd ~20 nM), named according to their affinity (Markaverich, 1988). Luteolin was found to compete for estradiol binding to cytosol and nuclear type II sites but it did not interact with estrogen receptors (Markaverich, 1988). In an *in vivo* study, injection of luteolin blocked estradiol stimulation of nuclear type II sites in the immature rat uterus and this correlated with an inhibition of uterine growth (Markaverich, 1988). Further studies also showed that luteolin could bind to nuclear type II sites irreversibly due to covalent attachment (Markaverich, 1988).

1.2.2 Antioxidant activity

Flavonoids are well known antioxidants and there were also many reports about the antioxidant effects of luteolin. Robak *et al* (1998) found that luteolin inhibits lipoxygenase activity, cyclooxygenase activity and ascorbic acid-stimulated malonaldehyde formation in liver lipids (Robak *et al.*, 1988). In other reports, luteolin

also inhibits DNA damage induced by hydrogen peroxide or singlet molecular oxygen in human cells (Devasagayam *et al.*, 1995; Noroozi *et al.*, 1998). The glycosylated form of luteolin, luteolin-7-O-glucoside, demonstrates a dose-dependent reduction of LDL oxidation, although it is less effective than luteolin (Brown and Rice-Evans, 1998). Studies of the copper-chelating properties of luteolin-7-O-glucoside and luteolin suggest that both of them act as hydrogen donors and metal ion chelators (Brown and Rice-Evans, 1998). Since oxidative stresses is closely related to mutagenesis and carcinogenesis, luteolin, as an anti-oxidant, may act as a chemopreventive agent to protect cells from various forms of oxidant stresses and thus prevent mutagenesis and carcinogenesis.

Although the ability of flavonoids to protect cells from the oxidative stress has been demonstrated, there is also increasing evidence for their pro-oxidant property (Cao *et al.*, 1997; Lapidot *et al.*, 2002; Sakihama *et al.*, 2002; Galati and O'Brien, 2004). It is believe that flavonoids could behave as antioxidants or pro-oxidants, depending on the concentration and the source of the free radicals (Cao et al., 1997). The pro-oxidant activity of flavonoids may be related to the ability of flavonoids to undergo autoxidation catalyzed by transition metals to produce superoxide anions (Hanasaki *et al.*, 1994). In other reports, however, it was observed that the phenol rings of flavonoids are metabolized by peroxidase to form pro-oxidant phenoxyl radicals, which are sufficiently reactive to cooxidize glutathione (GSH) or nicotinamide-adenine hydrogen (NADH) accompanied by extensive oxygen uptake and reactive oxygen species formation (Galati *et al.*, 2002).

One important understanding is that the pro-oxidant properties of flavonoids could contribute to their ability in induction of tumor cell apoptosis and cancer chemoprevention (Ueda *et al.*, 2002). Exposure of mammalian cells to flavonoids is

accompanied by an increase in intracellular ROS levels and lipid peroxidation, which lead to apoptotic or necrotic cell death (Yoon *et al.*, 2000; Morin *et al.*, 2001; Mouria *et al.*, 2002; Salvi *et al.*, 2002; Shen *et al.*, 2004).

Structure-activity relationship study on pro-oxidant cytotoxicity of flavonoids showed that flavonoids containing a phenol ring are generally more bioactive than that containing a catechol ring (Galati *et al.*, 2002). Further studies showed that an increase in cytotoxicity is correlated with an increase in ease of electrochemical oxidation of flavonoids and their lipophilicity (Sergediene *et al.*, 1999). Although luteolin has been shown to induce apoptosis in several cancer cells (section 1.2.4.3), it remains to be determined whether the pro-oxidant activity of luteolin is part of the mechanisms causing apoptotic cell death.

1.2.3 Anti-inflammatory activity

Inflammation is a defense mechanism to guard against infection and help heal injury. During an inflammation, monocytes and macrophages become activated by various immune molecules, such as cytokines, or endotoxin, such as lipopolysaccharide (LPS), an outer membrane component of gram-negative bacteria. The activated macrophages will vigorously produce inflammatory molecules such as TNFα (Tracey and Cerami, 1994), ILs (Akira *et al.*, 1993), free radicals and nitric oxide (NO) etc (Nathan and Xie, 1994), which will lead to inflammation and turn on a deadly cascade of events.

LPS triggers the secretion of a variety of inflammatory products, such as TNF-α (Tracey and Cerami, 1994), interleukins (Akira et al., 1993), intercellular adhesion molecule-1 (ICAM-1), as well as inducible nitric oxide synthase (iNOS), which produces excessive amounts of nitric oxide (Nathan and Xie, 1994). Production and release of inflammatory cytokines by LPS depends on inducible gene expression

mediated by the activation of transcription factor NF-κB (Baeuerle and Henkel, 1994; Baeuerle and Baltimore, 1996; Beuvink *et al.*, 2005). The signals from LPS converge upon the IκB kinase (IKK) complex, which phosphorylates the inhibitor of NF-κB (IκB), causing its ubiquitination and degradation. Removal of IκB liberates NF-κB proteins such as p65 for nuclear translocation, binding to κB-promoter elements and induction of gene transcription.

Macrophages participate in host defense and are main targets for the action of LPS. Pretreatment of murine macrophages RAW 264.7 with luteolin or luteolin-7-glucoside inhibits both the LPS-stimulated TNFα and IL-6 release. Furthermore, luteolin abolishes the LPS-induced phosphorylation of Akt, which may link LPS activation to NF-κB activation (Zhou *et al.*, 2000; Xagorari *et al.*, 2001). However, overexpression of a dominant negative form of AKT does not alter LPS-induced TNF-α release, suggesting that inhibition of this kinase does not mediate the inhibitory action of luteolin (Xagorari *et al*., 2002). It is possible that luteolin interferes with LPS signaling by reducing the activation of MAPK family members ERK and p38, but not c-Jun N-terminal kinase (JNK) (Xagorari *et al*., 2002). The active anti-inflammatory components of *Glossogyne tenuifolia* were identified as oleanolic acid and luteolin-7-glucoside. Both of them inhibited LPS-stimulated inflammatory mediator production and NF-κB activation (Wu *et al.*, 2004b).

Similar effects and mechanisms of luteolin on innate immunity were found in intestinal epithelial cells and dendritic cells. Luteolin significantly blocks LPS-induced IκB phosphorylation and degradation, NF-κB transcriptional activity and intercellular adhesion molecule-1 (ICAM-1) gene expression in rat IEC-18 cells (Kim and Jobin, 2005). This effect is by directly inhibiting the LPS-induced IKK activity.

Interestingly, although luteolin shows potent inhibition on LPS-stimulated NF-κB transcriptional activity in Rat-1 fibroblasts, it does not inhibit either IκBα degradation, ,NF-κB nuclear translocation, or DNA binding induced by LPS (Kim *et al.*, 2003b). Rather, luteolin prevents LPS-stimulated interaction between the p65 subunit of NF-κB and the transcriptional coactivator CBP, suggesting that the effect of luteolin on NF-κB signaling varies depending on the cell types.

Luteolin not only inhibits LPS stimulated release of proinflammatory cytokines such as TNF and ILs, but also directly inhibits the signaling triggered by TNF or ILs. Intercellular adhesion molecule-1 (ICAM-1) is an immunoglobulin superfamily expressed on endothelial cells and important for adhesion of leukocytes and transendothelial migration (Hubbard and Rothlein, 2000). Luteolin inhibits TNF-α-stimulated ICAM-1 expression by inhibiting IKK activity, IκBα degradation, NF-κB DNA-protein binding, and NF-κB luciferase activity in respiratory epithelial cells (Hubbard and Rothlein, 2000)**.** The inhibitory effects of luteolin on ICAM-1 expression are also mediated by the sequential attenuation of the three MAPKs activities, the c-fos and c-jun mRNA expressions, and the activator protein-1 (AP-1) transcriptional activity (Chen *et al.*, 2004). Through a similar mechanism, luteolin can inhibit TNF-alpha-induced IL-8 production in human colonic epithelial cells *(Kim et al*., 2005b).

Another important inflammation mediator, NO is synthesized by inducible NO synthase (iNOS), which is activated by LPS. Luteolin and its glycoside, luteolin-7-O-glucoside, suppress the production of NO and prostaglandin E2 (PGE2) in LPS activated-mouse macrophage RAW264.7 cells (Kim *et al.*, 1999; Hu and Kitts, 2004). The inhibitory effect is attributed to the suppression of both iNOS and

cyclooxygenase-2 (COX-2) protein expression by luteolin, without affecting the enzymatic activity directly (Kim *et al*., 1999; Hu and Kitts, 2004).

It should be pointed out that it seems unlikely that the inhibitory action of luteolin on proinflammatory cytokine production is the result of antioxidant properties. This is based on observations that some flavonoids with strong antioxidant properties are completely ineffective in reducing LPS-stimulated TNF-production (Devasagayam *et al*., 1995). A structure-activity study shows that the presence of a double bond at position C2-C3 of the C ring with oxo function at position 4, along with the presence of the OH groups at positions 3' and 4' of the B ring are required for optimal inhibition of LPS-stimulated TNF- release (Xagorari *et al.*, 2001).

The anti-inflammatory ability of luteolin has been also evaluated *in vivo*. Mice receiving LPS exhibited high mortality after the LPS challenge. On the contrary, mice that had received luteolin (0.2 mg/kg, intraperitoneally) before LPS showed an increased survival (Kotanidou *et al.*, 2002). Luteolin pretreatment also reduces LPS-stimulated TNF-α release in serum and ICAM-1 expression in the liver (Kotanidou *et al*., 2002), which is in agreement with many *in vitro* observations. The effect of luteolin was also tested in an acute *Chlamydia pneumoniae* infection model in C57BL/6J mice. Luteolin was found to suppress inflammation in lung tissue that was caused by *Chlamydia pneumoniae,* however, luteolin treatment had no effect on iNOS but significantly decreased the expression of constitutive eNOS enzyme (Tormakangas *et al.*, 2005).

In summary, the anti-inflammation effect of luteolin has been well documented. It is via not only inhibiting LPS-stimulated release of cytokines such as TNF and ILs but also directly inhibiting the signal transductions triggered by these

cytokines. Both mechanisms may attribute to the strong inhibitory effects of luteolin on NF-κB.

1.2.4 Anti-cancer property

1.2.4.1 Anti-carcinogenesis activities

Carcinogenesis is a long-term and multi-stage process that results from accumulation of mutation and dysfunction of important molecules regulating cell proliferation and cell death. The process of chemical carcinogenesis may be divided into three stages: initiation, promotion and progression. During initiation, a potential carcinogen is transformed into a mutagen by phase I enzymes such as cytochrome P450. The mutagen may react with cellular molecules such as DNA and result in genetic mutation. During the promotion stage, the genetic alterations will lead to enhanced cell proliferation and/or reduced cell death. During the promotion stage, the mutations are enhanced and the cells are proliferating in an uncontrolled manner (Pitot, 1993).

The inhibitory effects of flavonoids, including luteolin, on carcignogenesis have been well documented. In an *in vivo* study, it was observed that luteolin significantly decreased the incidence of fibrosarcoma induced by 20-methylcholanthrene (20-MC), a strong carcinogen, in male Swiss albino mice (Elangovan *et al.*, 1994). Other studies showed that, to prevent tumor development, different stages of carcinogenesis can be targeted by luteolin. In the initiation stage, luteolin were found to inhibit the metabolism of carcinogens in isolated liver microsomes (Buening, 1981). In another study, Huang *et al* (1983) found that luteolin inhibits the mutagenic activity resulting from the metabolic activation of benzo-pyrene and trans-7,8-dihydroxy-7,8-dihydrobenzo-pyrene in rat liver microsomes (Huang, 1983). Later in 1998, Oguri *et al* proved that luteolin suppresses formation of

mutagenic and carcinogenic heterocyclic amines (Oguri, 1998). Recently, it was found that luteolin acts as a potent inhibitor of human cytochrome P450 (CYP) 1 family enzymes, such as CYP1A1, CYP1A2, and CYP1B1, and thus the transformation of potential carcinogen into a potent mutagen product is inhibited (Kim, 2005). During the promotion stage, luteolin could directly scavenge the ROS/RNS generated by various mutagens, or by inhibiting pro-oxidant enzymes, such as xanthine oxidase (Nagao, 1999), myeloperoxidase (Kostyuk *et al.*, 2003), and lipoxygenases (Sadik *et al.*, 2003). In addition, luteolin can inhibit the lipid peroxidation induced by CCl_4 (Cholbi, 1991) or by $FeSO_4^+$ cysteine in rat liver microsomes (Mora, 1990).

On the other hand, luteolin targets the enzymes involved in DNA synthesis, for example, DNA topoisomerases, to suppress tumor promotion. DNA topoisomerases are the essential enzymes that catalyze the interconversion of topological isomers of DNA molecules (Corbett and Berger, 2004). Acting by sequential breakage and reunion strands of DNA, two topoisomerases (topoisomerase I and topoisomerase II) are involved in many vital cellular processes such as DNA replication, transcription, recombination, integration and chromosomal segregation (Corbett and Berger, 2004). The dysfunction of these vital enzymes will result in DNA damage that may induce cell cycle arrest or apoptosis. Several flavonoids have been shown to exert their action by interacting with DNA topoisomerases and promoting site-specific DNA cleavage (Constantinou, 1995). Luteolin inhibits topoisomerase II activity of HL-60 cells by forming a luteolin- topoisomerase II-DNA ternary complex and then induces apoptosis in the cells (Yamashita and Kawanishi, 2000). By inhibiting DNA synthesis and promoting topoisomerase-II-mediated cleavage of kinetoplast DNA minicircles, luteolin inhibits the growth of *Leishmania*

donovani promastigotes and arrest its cell cycle progression, leading to apoptosis (Mittra *et al.*, 2000).

In addition, luteolin also strongly inhibits the catalytic activity of eukaryotic DNA topoisomerase I (Chowdhury *et al.*, 2002). Luteolin intercalates directly with the enzyme as well as the substrate DNA to stabilize the topoisomerase-DNA covalent complex and thus to block the subsequent rejoining of the DNA breaks.

1.2.4.2 Inhibition on cell proliferation

One character of cancerous cells is that they are undergoing rapid and unlimited proliferation. Proliferation requires the success of DNA synthesis and then cell division, which is controlled by signaling pathways triggered by growth factors, such as epidermal growth factor receptor (EGF), platelet-derived growth factor (PDGF), insulin-like growth factor (IGF), fibroblast growth factor (FGF), and vascular endothelial growth factor (VEGF).

Many flavonoids, including luteolin, were found to be able to inhibit the proliferation of cancer cells derived from nearly all tissues, such as human breast cancer cells MCF-7, human neuroblastoma cells SHEP and WAC2 (Fotsis, 1997; Han, 2002), Raji lymphoma cells (Ramanathan, 1994), pancreatic cancer cells MiaPaCa-2 (Lee *et al.*, 2002), human leukemia cells HL-60 (Ko *et al.*, 2002), hepatic stellate cells (Zhao, 2002), human thyroid carcinoma cell lines (Yin, 1999), human melanoma cells OCM-1 (Iwashita, 2000), human epidermoid carcinoma A431 (Huang *et al.*, 1999a) and human prostatic tumor cells (Knowles, 2000).

The anti-proliferation mechanisms of luteolin have been explored extensively in several aspects.

Firstly, it is controversial whether the anti-proliferation effect of luteolin is dependent on endoplasmic reticulum (ER) (Ross and Kasum, 2002). Luteolin, as well

as several other flavonoids, suppresses the proliferation of human prostatic tumor cells (PC-3), androgen-independent cells, indicating that flavonoids show their anti-proliferation activity in an androgen-independent manner (Knowles, 2000). On the other hand, it was found that luteolin inhibits estradiol-induced DNA synthesis in both estrogen-dependent MCF-7 and estrogen-independent MDA-MB-231 human breast cancer cells (Wang, 1997; Wang, 1998; Han, 2002). In a separate study, luteolin was shown to inhibit the proliferation of several human thyroid carcinoma cell lines, UCLA NPA-87-1 (with estrogen receptor), UCLA RO-82W-1 (with anti-estrogen binding site) and UCLA RO-81A-1 (lacking both estrogen receptor and anti-estrogen binding site), suggesting that the inhibitory activity of luteolin on cancer cell proliferation is not dependent on estrogen receptor or androgen receptor, but via other mechanisms (Yin, 1999).

Secondly, since luteolin is able to inhibit the activity of topoisomerases, which is critical for DNA synthesis (Constantinou, 1995; Mittra et al., 2000; Chowdhury et al., 2002), it has been suggested that luteolin inhibits cell proliferation by inhibiting topoisomerases and DNA synthesis (Makino, 2001).

Thirdly, the inhibitory effect of luteolin on cancer cell proliferation is related to its effects on various growth factors and their signaling pathways, including epidermal growth factor (EGF), platelet-derived growth factor (PDGF), and vascular endothelial growth factor (VEGF). For instance, EGF activates EGF receptor which is a typical receptor tyrosine kinase that stimulates cell growth as well as cell migration through receptor phosphorylation and the subsequent activation of downstream signaling pathways (Zhang, 1998). Luteolin was found to inhibit the proliferation of pancreatic cancer cell MiaPaCa-2 and its effect is closely related to the inhibition of the activity of EGF receptor, but not protein synthesis (Lee et al., 2002). The same

group found that luteolin inhibits proliferation of human epidermoid carcinoma A431 cells via a similar mechanism (Huang *et al.*, 1999b).

PDGF is one of the principal regulators of proliferation and migration of vascular smooth muscle cells (VSMCs). PDGF binding to its receptor leads to its phosphorylation on multiple tyrosine residues. The activated PDGF receptor is associated with a number of proteins including the phosphatidylinositol 3'-kinase (PI3K), which mediates Raf-MEK-ERK transduction (Claesson-Welsh, 1994). Luteolin inhibits PDGF-induced proliferation and DNA synthesis of rat aortic VSMCs by inhibiting PDGF receptor phosphorylation (Kim *et al.*, 2005b). As a consequence, luteolin significantly inhibits PDGF-induced ERK, Akt and phospholipase C (PLC)-γ1 activation as well as *c-fos* gene expression (Kim *et al*., 2005b). These results suggest that the inhibitory effect of luteolin on the PDGF-induced proliferation of rat aortic VSMCs may be mediated by blocking phosphorylation of PDGF receptor (Kim *et al.*, 2005c).

Another important growth factor is VEGF, which is one of the most important factors regulating key angiogenic responses of endothelial cells. They include proliferation, migration, and differentiation, as well as protection from apoptosis (Ferrara, 2001). In a murine xenograft model, luteolin was demonstrated to inhibit tumor growth and angiogenesis (Bagli *et al.*, 2004). Furthermore, it was found that luteolin inhibits proliferation of human umbilical vein endothelial cells by inhibiting VEGF-induced PI3K activity and activation of Akt, a downstream target of PI3K. However, luteolin does not affect VEGF-induced ERK activation, which is considered important for the mitotic effects of VEGF (Bagli *et al*., 2004).

Protein kinase C is a family of serine-threonine protein kinases that regulate growth factor response, cell proliferation, differentiation and apoptosis (Lucas and

Sanchez-Margalet, 1995; Weinstein *et al.*, 1997). It was found that PKC is inhibited in a concentration-dependent manner by many flavonoids including luteolin in both cell-free systems and intact cells (Ferriola *et al.*, 1989). Another study showed that luteolin is a potent inhibitor of human mast cell activation through the inhibition of Ca^{2}+ influx and PKC activation (Kimata *et al.*, 2000).

Thus, it appears that luteolin is able to inhibit activity of a range of kinases, such as RTKs, PI3K and PKC. The question to ask is whether this inhibitory effect on different kinases is via a common mechanism. Apigenin, with a structure similar to luteolin, is also able to inhibit a wide range of kinases and this inhibitory effect is by competing with adenosine triphosphate (ATP) (Huang, 1996; Conseil, 1998). It remains to be determined whether luteolin inhibits various kinase activity through the same mechanism.

Finally, the inhibitory effect of luteolin on cell proliferation is related to its effect on cell cycle progression or cell death. For example, luteolin inhibits proliferation of human melanoma cells OCM-1 by arresting the cells at phase G1 (Casagrande and Darbon, 2001). In human leukemia HL-60 cells, luteolin inhibits its proliferation at low dose and induces apoptosis at higher dose (Ko *et al*., 2002). Detailed discussion about cell cycle arrest- or apoptosis-inducing effects of luteolin will be covered in subsequent sections.

1.2.4.3 Induction of cell cycle arrest

In eukaryotic cells, cell proliferation proceeds through DNA replication followed by division of nucleus and separation of cytoplasm to yield two daughter cells. The sequential process, called cell cycle, contains 4 distinct phases biochemically. G1 phase is a period when cells decide whether to start proliferation or to stay quiescent. Once cells decide to proliferate, their DNA will be replicated during

a DNA synthesis phase (S phase). The phase after DNA synthesis is called G2 phase which allows for the repair of DNA damage and replication errors. When there is no DNA damage or replication errors, the nucleus and cytoplasm will be equally divided into two and yield two daughter cells, which is call mitosis phase (M phase) (Massague, 2004). Cell cycle progression is timely regulated by cyclin-dependent kinases (CDKs) and their cyclin subunits (Ekholm and Reed, 2000). G1 progression and G1/S transition are regulated by CDK4-cyclin D, CDK6-cyclin D and later CDK2-cyclin E. While CDK2 controls S-phase when associated with cyclin A and G2/M transition is regulated by CDK1 in combination with cyclins A and B (Donjerkovic and Scott, 2000). Activation of CDKs is regulated by cyclin as well as CDK inhibitors (CKIs). Two families of mammalian CKIs have been identified: the INK4 family, which specifically inhibits CDK4 and CDK6, and the CIP/KIP family, including p21cip1/waf1, p27kip1 and p57kip2, which have a broad range of inhibition (Ekholm and Reed, 2000).

Cell cycle checkpoints have been the targets for chemotherapeutic and chemopreventive agents. In several *in vitro* experiments, flavonoids have been found to inhibit the proliferation of many cancer cells by arresting cell cycle progression either at G1 or at G2/M phase (Zi *et al.*, 1998; Lindenmeyer *et al.*, 2001).

Although luteolin has been found to inhibit cell growth and proliferation of many cancer cells, its effect on cell cycle distribution was found only in several cell lines. Luteolin arrests the cell cycle at G1 phase in the following three cancer cell lines: human gastric cancer HGC-27 cells (Matsukawa *et al.*, 1993), human melanoma cells OCM-1 (Casagrande and Darbon, 2001) and human prostate cancer cells LNCaP (Kobayashi *et al.*, 2002). The G1 cell cycle arrest induced by luteolin on OCM-1 is mediated by inhibiting the activity of CDK2, which is attributed to the up-regulation

of its inhibitors p27/kip1 and p21/waf1 (Casagrande and Darbon, 2001). On the other hand, luteolin was found to arrest mouse cancer cell tsFT210 at G2/M phase (Li *et al.*, 2005). Another study suggests that the G2/M arresting effect might be related to the activation of the tumor suppressor protein p53. Luteolin was also found to induce G2/M arrest in a non-tumor cell line C3H10T1/2CL8 due to the rapid activation of p53 (Plaumann *et al.*, 1996).

1.2.4.4 Induction of apoptosis

During the development, apoptosis is critical in eliminating unwanted cells in a specific site to form organs. Apoptosis is also critical in eliminating any damaged cells that may be caused by carcinogens and keep the whole organism healthy. Insufficient apoptosis of the damaged cells is believed to be a reason of cancer formation. Therefore, many cancer therapeutics work through induction of cancer cell apoptosis (Ghobrial *et al.*, 2005).

In addition to the inhibitory effect on cancer growth, luteolin is able to kill cancer cells by inducing apoptotic cell death. It has been reported that luteolin can induce apoptosis in several cancer cell lines, including human epidermoid carcinoma A431(Huang et al., 1999b), human leukemia HL-60 (Ko *et al*., 2002; Cheng *et al.*, 2005a) and U937 (Monasterio *et al.*, 2004), pancreatic tumor cell MiaPaCa-2 (Lee et al., 2002), and human hepatoma cell HepG2 (Lee *et al.*, 2005).

One possible mechanism of apoptosis-inducing effect of luteolin is that this flavonoid is a RTK inhibitor and it may mimic deprivation of growth factors by blocking the growth factor-triggered signaling pathway. The possibility was partially supported by the finding that apoptosis induced by luteolin in pancreatic cancer cell MiaPaCa-2 is concomitant with dampened EGF receptor triggered-signals (Lee et al., 2002).

Topoisomerases are essential in catalyzing sequential breakage and reunion strands of DNA, which is involved in many vital cellular processes such as DNA replication and transcription. Inhibition of topoisomerases not only suppresses DNA replication and cell proliferation but also caused DNA damage, which might lead to apoptosis. Luteolin has reported to inhibit topoisomerases II (Yamashita and Kawanishi, 2000) and induced apoptosis in HL-60 cells. The inhibition is through forming a luteolin-topoII-DNA ternary complex. Luteolin has been also proven to inhibit the catalytic activity of DNA topoisomerase I (Chowdhury *et al.*, 2002). Luteolin intercalates directly not only with the enzyme but also with substrate DNA. Further study showed that it can cause DNA damage by stabilizing the topoisomerase-DNA covalent complex and block the subsequent rejoining of the DNA break (Chowdhury *et al*., 2002).

In response to DNA damage, a number of signals are activated sequentially. Ataxia telangiectasia mutated kinase (ATM) and ataxia telangiectasia and Rad3-related kinase (ATR) are activated very rapidly and then activate p53, which eventurely leads to cell cycle arrest or apoptosis. The apoptosis mediated by p53 may be through either a transcription-dependent pathway, which involves the activation of proapoptsotic genes, or transcription–independent pathway, which involves p53 and Bax mitochondrial translocation (Chipuk *et al.*, 2003; Erster *et al.*, 2004). Plusseman (1996) found that luteolin, as well as apigenin and quercetin, induces apoptosis, which is accompanied by p53 protein accumulation and p53 transactivation (Plaumann *et al.*, 1996). Recently, two studies proved that change of mitochondria and Bcl-2 family member proteins are involved in the apoptosis induced by luteolin in cancer cells. Luteolin induces cleavage of the proapoptotic Bcl-2 proteins, Bax and Bak translocation to mitochondria and the release of cytochrome c to cytosol, whereas Fas

ligand remains unchanged, suggests that luteolin induced apoptosis via mechanisms involving mitochondria translocation of Bax/Bak (Cheng *et al*., 2005a; Lee *et al*., 2005).

1.2.4.5 Anti-angiogenesis

Angiogenesis, the generation of new blood vessels, occurs during many physiological processes, including development, wound healing, formation of the corpus luteum, endometrium and placenta (Folkman, 1995). Angiogenesis also occurs in some pathological processes, such as solid tumor growth and metastasis, which require nutrition and oxygen due to the fact that avascular tumors do not grow beyond a diameter of 1-2 mm (Folkman, 1995). Rapid growth of tumor and consumption of oxygen result in hypoxia, which activates transcription of genes such as vascular endothelial growth factor (VEGF) and matrix metalloproteases (MMP). In response to secretion of angiogenic stimuli, such as VEGF and MMP which degrade the extracellular matrix, the endothelial cell basal membrane is degraded by the action of protease and then the endothelial cells migrate and proliferate and finally organize into capillary tubes. Several important factors are involved in this progress. (1) VEGF receptor. This enzyme is one of the most potent and specific known angiogenic factors *in vivo*. It increases microvascular permeability in response to hypoxia, multiple growth factors, cytokines and estradiol (Benassayag *et al.*, 2002). (2) Hyaluronidase. Hyaluronic acid is one of the most abundant constituents of the extracellular matrix and acts as a barrier to neovascularization (Trochon *et al.*, 1997). Fragments of hyaluronic acid, as a result of catalytic activity of hyaluronidase, bind to CD44 receptor exposed on the membrane of endothelial cells and then are responsible for endothelial cell proliferation, migration and finally angiogenesis. (3) MMP. Metalloproteases also can promote angiogenesis by degrading the extracellular matrix.

Transcription of the VEGF gene is enhanced under hypoxic conditions (Semenza, 2001). Many flavonoids have been reported to be able to inhibit hypoxia and hypoxia-activated downstream events (Zhou *et al.*, 2004; Fang *et al.*, 2005; Liu *et al.*, 2005). Luteolin is also effective in inhibiting the transactivation of the hypoxia-response element (HRE) under hypoxic conditions (Hasebe, 2003). On the other hand, luteolin, as a RTK inhibitor (section 1.2.4), was also found to be able to inhibit the *in vitro* angiogenesis by inhibiting VEGF activity (Fotsis, 1997; Bagli *et al*., 2004).

In addition, luteolin has been found to be a potent competitive inhibitor of hyaluronidase (Kuppusamy, 1990), which catalyzes hyaluronic acid and activates receptors for endothial cell proliferation, migration and angiogenesis. As an inhibitor of several growth factors, luteolin inhibits EGF triggered MMP secretion in A431 cells (Huang et al., 1999b). Moreover, it was shown luteolin can also directly inhibit MMP activity. Interestingly, kinetic analysis revealed that the inhibition of MMP by luteolin is non-competitive, while luteolin inhibits hyaluronidase in a competitive manner (Ende, 2004).

The anti-angiogenesis effect of luteolin was confirmed in two *in vivo* studies. Luteolin can significantly inhibit corneal angiogenesis *in vivo* and corneal neovascularization induced by fibroblast growth factor (FGF) (Joussen *et al.*, 2000). Recently, in a murine xenograft model, luteolin is able to inhibit tumor growth and angiogenesis (Bagli et al., 2004). Furthermore, it has been shown that luteolin inhibits angiogenesis by inhibited VEGF-induced PI3K activity and activation of AKT (Bagli *et al.,* 2004).

1.2.4.6 Inhibition on cancer metastasis

In addition to rapid and continuous division and proliferation, another important feature of cancer cells is their ability to spread from the primary site to

other more distant sites. This process, called metastasis, contributes to over 90% of human cancer mortality. The deadly process involves several sequential steps, migration, invasion and adhesion, which are driven by growth factors such as EGF, and MMPs (Brinckerhoff and Matrisian, 2002).

Transactivation of the epidermal growth factor receptor (EGFR) tyrosine kinase activity is proposed to stimulate cell migration by regulating MMP expression. By blocking of the EGFR-signaling pathway, luteolin is able to reduce the level of phosphorylated FAK as well as the secreted MMP, which may lead to the suppression of cell invasion and metastasis (Huang et al., 1999b; Lee *et al.*, 2004).

In addition to many growth factors, cytokines also control MMP expression. For example, interleukin 6 (IL6) is known as a cytokine that induces MMP-1 expression. Luteolin is potent in inhibiting the production of IL-6 and suppressing the expression of MMP-1 (Kim, 2004). Since ILs production is regulated by NF-κB, it is possible that the inhibitory effect of luteolin on NF-κB may play a role in suppressing IL production and MMP expression. Interestingly, luteolin as well its glycoside, can directly inhibit the activity of MMP-2 and MMP-9, in a non-competitive manner (Ende, 2004).

On the other hand, luteolin is a potent inhibitor of *in vitro* invasion of human PC-3 prostate cancer cells (Lansky, 2005). Since elevation of Focal adhesion kinase (FAK) activity in human carcinoma cells is associated with increased invasive potential, the inhibitory effect of luteolin on FAK phosphorylation may contribute to suppression of cell invasion ability (Huang, 2005).

Intercellular adhesion molecule-1 (ICAM-1) has been implicated in the processes of adhesion and metastasis. Luteolin was found to suppress TNF-stimulated ICAM-1 expression in respiratory epithelial cells. The inhibitory effect of luteolin on

ICAM-1 expression is mediated by inhibiting NF-κB pathway, including IKK activity, IκBα degradation, NF-κB DNA-protein binding, and NF-κB luciferase activity, as well as AP-1 transcriptional activity (Chen *et al*., 2004).

Taken together, Section 1.2.4 summarizes different aspects of the anticancer properties of luteolin and the main mechanisms involved. Luteolin has been proved to be a potent anticancer agent in a variety of cancer cells *in vitro* as well as in a number of *in vivo* animal models. Firstly, it can inhibit carcinogenesis at different stages: initiation, promotion and progress. Secondly, it can inhibit cancer cell proliferation, or modulate cancer cell cycle progression, or induce apoptotic cell death or suppress angiogenesis. Lastly, luteolin possesses strong anti-metastasis effect, an important property in cancer therapy to restrict the mortality of cancer. It is possible that a number of common mechanisms are involved in the diverse activity of luteolin on cancer. For example, inhibition on receptor tyrosine kinases and topoisomerases contributes to its inhibitory effects on carcinogenesis, proliferation, cell cycle and apoptosis. The inhibition on NF-κB could contribute to its anti-inflammatory, anti-carcinogenesis, anti-angiogenesis and anti-metastasis activities.

1.3 APOPTOSIS

1.3.1 General introduction

Apoptosis is a tightly regulated cell death process characterized by unique morphological and biochemical changes including cell shrinkage, mitochondrial depolarization, nuclear fragmentation, chromatin condensation, membrane blebbing and formation of apoptotic bodies (Hengartner, 2000; Kaufmann and Hengartner, 2001). In receiving cell death signals, which might be either intrinsic or extrinsic, cells will undergo a chain of biochemical changes, which are characterized by

dramatic changes of mitochondria, the main apoptosis mediator, and activation of caspase cascade, the main apoptosis executors. Activated caspase will cleave a number of substrates, including DNase II, caspase-activated deoxyribonuclease and cytoskeletal proteins which responsible for the distinct morphological changes such as nuclear fragmentation, chromatin condensation and cell shrinkage. The cleaved substances of the cells will be packed into small apoptotic bodies, which, with phosphatidylserine flipped to outer cell membrane, will be recognized and removed by phagocytes by engulfment and degradation (Savill and Fadok, 2000). So, different from necrosis, apoptosis does not trigger inflammation responses and exert damages to surrounding cells, and thus are believed to be a physiological way to eliminate a cell.

1.3.2 Caspases

Caspases are a group of proteases which cleave its substrate proteins specifically on the carboxyl side of an aspartate residue (Strasser *et al.*, 2000). Up to now, there are about 14 caspases have been identified in mammalian cells and more than half of them are directly involved in apoptosis regulation (Nicholson and Thornberry, 1997). Caspases are produced initially in cells in an inactive form with an extended N-terminal prodomain that must be cleaved during activation. According to their structures and functions in activation, caspases can be subdivided into two groups, initiator caspases and effector caspases. Initiator caspases, including caspase-8, -10, -9 and -2, have caspase recruitment domains (CARD) or death effector domains (DED) which function in its activation. For example, upon death signals, such as Fas-FasL interaction, caspase 8 can be recruited through its DED domains to death receptors via an adaptor protein Fas-associating death domain protein (FADD) (Chinnaiyan *et al.*, 1995). The recruitment will bring pro-caspase-8 together and

trigger its auto-activation through proximity-induced dimerization. Another important initiator caspase, caspase-9, is activated in an apoptosome comprising of pro-caspase-9, cytochrome C, apoptotic protease-activating factor 1 (Apaf-1) and dATP (Kroemer, 2000). Activation of effector caspase, including caspase-3, -6 and -7, requires activated initiator caspase which cleave effector caspase at specific internal Asp residues and produce its active form. The active effector caspases will act as executors that cleave diverse cellular substrates which lead to apoptotic cell death (Fischer *et al.*, 2003). For instance, cleavage of ICAD by caspase-3 liberates the active CAD nuclease that mediates apoptotic DNA fragmentation.

Interestingly, initiator caspases also serve as the substrates of active effector caspases. For example, active caspase-3 can cleave and activate pro-caspase-8. The apoptosis process can be accelerated by this positive feedback loop (Shi, 2002).

On the other hand, the activation of caspase is negatively regulated by anti-apoptotic proteins (IAPs) that directly interact with caspases and inhibit their activation as well as activity. One of the most important anti-apoptotic proteins is X-linked inhibitor of apoptosis (XIAP), a member of IAPs family. XIAP can inhibit the activation of caspase-3 and -9 as well their active forms. XIAP has a baculovirus IAP repeat domain (BIR) which mediates its interaction with caspase-3 or -9 (Deveraux *et al.*, 1997). So, a success activation of caspase-9 requires neutralization of the inhibitory effect of XIAP, which is performed by SMAC/DIBLO released simultaneously with cytochrome C from mitochondria. Another potent anti-apoptotic protein is FLICE-inhibitory protein (FLIP), which specifically inhibits caspase-8 activation. FLIP, has similar structure with pro-caspase-8 but without protease activity, can compete with pro-caspase-8 to be recruited to activated death receptors (Hu *et al.*, 1997; Irmler *et al.*, 1997).

Although it has been well established that caspases are vital mediators of apoptosis, there are apoptosis independent of caspases but mediated by apoptosis inducing factor (AIF) (Cande *et al.*, 2002) or other proteases such as granzyme B and calpain (Kroemer and Martin, 2005).

1.3.3 Apoptosis pathways

Apoptosis is mediated through two signaling pathways, death receptor pathway (the extrinsic pathway) and mitochondrial pathway (intrinsic pathway). Notably, there is significant cross-talk between these two pathways.

1.3.3.1 Death receptor-mediated apoptosis pathway

In the death receptor pathway, the death receptors are triggered by their ligands or antagonist antibodies and lead to the recruitment of adaptor molecules that activate the caspase cascade. Death receptors are receptors on the cell surface which can be specifically activated by death ligands. The death receptors are intermembrane proteins characterized by its extracellular domains that can be recognized by death ligands, and its intercellular death domains (DD), which recruit a series of proteins and trigger caspase activation (Itoh and Nagata, 1993). Several groups of death receptors have been identified such as Fas/CD95, TNFR1, DR4 and DR5. Accordingly, the main death ligands which can bind to death receptors include FasL (for Fas), TNF (for TNFR), and TRAIL (for DR4 and DR5).

The signal transduction of receptor-mediated apoptosis has been well understood using Fas/FasL as a model (Chinnaiyan *et al*., 1995). The binding of FasL to Fas will trigger trimerization of Fas, which recruits Fas associated death domain (FADD) through DD domain. FADD is acting as an adaptor protein with its death domains binding to death receptors, and its death effector domain recruiting pro-caspase-8 and form the death-inducing signaling complex (DISC). Within the DISC,

pro-caspase-8 is processed by its auto-activation through proximity-induced dimerization. The long prodomain is cleaved and pro-caspase-8 forms intermediate but inactive forms, which will be cleaved further and maturate into active forms. The active caspase-8 will cleave its effector caspase, caspase-3, which activation is considered as "no-return" event during apoptosis.

Receptor-mediated apoptosis, however, can be triggered in the absence of death ligands. Under certain circumstances, cells overexpress death receptors, which may trigger receptor trimerization and caspase activation (Wu, 1997).

1.3.3.2 Mitochondria-mediated apoptosis pathway

Mitochondria, apart from its crucial role in supplying energy, are the pivotal mediator of apoptosis. One of the main evidence supporting the critical role of mitochondria in apoptosis is that mitochondria contain an array of apoptosis regulatory proteins including cytochrome c, Smac, apoptosis-inducing factors (AIFs), endonuclease G and HtrA2 (also known as Omi) (Kroemer, 2000). Whereas AIF and endonuclease G seem to be able to directly cause nuclear and DNA damage, cytochrome c works together with Apaf-1 and pro-caspase-9 to form a complex, called apoptosome, within which caspase-9 is activated and then activates the effector caspases that results in morphological and biochemical changes in apoptosis. On the other hand, Smac and HtrA2 can reverse the suppressive effects of the inhibitor-of-apoptosis proteins (IAPs), such as XIAP, on either caspase-9 or caspase-3/7, thus further enhancing the activation of the effector caspases.

The involvement of mitochondria in apoptosis is tightly regulated by bcl-2 family proteins, which can be either pro-apoptotic such as Bid, Bad and Bax, or anti-apoptotic such as Bcl-2, Bcl-xL, Mcl-1 (Adams and Cory, 1998). For example, Bcl-2 is an anti-apoptotic protein over-expressing in many cancer cells and is responsible

for their resistance to cell death. Down-regulation of Bcl-2 or inactivation of Bcl-2 has been suggested to be effective in restoring the sensitivity of cancer cells to anticancer treatment (Bartholomeusz *et al.*, 2005).

Mitochondria are also closely involved in death receptor-mediated apoptosis in certain types of cells. In type I cells, the presence of activated caspase-8 is sufficient to induce activation of effector caspases, which then act on death substrates in apoptosis (Wallach *et al.*, 1999). However, in type II cells, a small amount of activated caspase-8, although not enough to activate the effector caspases directly, is sufficient to trigger a mitochondria-dependent apoptotic amplification loop (Scaffidi *et al.*, 1998; Scaffidi *et al.*, 1999). Active caspase-8 will cleave its substrate Bid and form a truncated Bid (tBid). tBid, together with other pro-apoptotic bcl-2 members, then translocates to outer membrane of mitochondria and cause cytochrome C release and apoptotic cell death (Li, 1998; Luo *et al.*, 1998).

1.3.4 Apoptosis and cancer

Apoptosis, as a way to eliminate unwanted cells, is crucial for development, organ morphogenesis, and tissue homeostasis. There is accumulating evidence showing that the accumulation of damaged cells in the tissue resulted from lacking of proper apoptosis is closely associated with tumorigenesis (Hanahan and Weinberg, 2000). The resistance to apoptosis of cancer cells is acquired through a variety of biochemical changes, including over-expression or low-expression of certain functional proteins relevant to apoptosis. Moreover, these changes also attribute to the responsiveness of cancer cells to anticancer therapy. Therefore, apoptosis regulatory molecules are legitimate targets for anticancer treatment.

Apoptosis is executed by activated intracellular proteases, known as caspases, which are responsible for the specific apoptotic biochemical and morphological

changes. The activation of caspases is regulated by a fine balance with/between two opposite sides, i.e. proapoptotic signals that facilitate its activation and antiapoptotic signals that inhibit its activation. Changes at either side may perturb the balance and confer the cancer to be resistant or sensitive to apoptosis stimuli. For example, low expression of cell death receptors contributes to the resistance of some cancers (Wang and El Deiry, 2003). Apaf-1, a cell-death effector that acts with cytochrome c and caspase-9, is frequently inactivated in cancers such as malignant melanoma (Soengas *et al.*, 2001). Another well known example is p53, a tumor suppressor protein, which activation in response to DNA damage induced by anticancer drugs will lead to cell cycle arrest or apoptosis. However, many cancers have mutant p53, which confers the cancer cell to be resistant to anticancer therapy (Koechli, 1994). Thus activation of p53, for example by inhibiting its interaction with MDM2 (Vassilev *et al.*, 2004), a p53 antagonist, or restoration of wide type p53, for example by introducing wide type p53 using gene therapy (Quist, 2004), have been proved to be effective in anti-cancer therapy. The cancer resistance can also be acquired by over-expression of one or several anti-apoptotic proteins, such as Bcl-2, survivin, FLIP and IAPs (Deveraux *et al*., 1997; Deveraux and Reed, 1999). Understanding of the molecular basics of cancer resistance also helps to locate proper targets for activating apoptosis in cancer therapy.

1.3.5 TNFR signaling pathway

TNF, a cytokine produced mainly in macrophages, was initially regarded as an important player in inflammation and immuno responses. Upon the activation of LPS, macrophages produce a number of cytokines including TNF and ILs, which can mediate inflammation responses through activating NF-κB (Tracey and Cerami, 1993). It is now well understood that TNF, through binding to it receptors, can trigger caspase cascade and thus function as a death ligand. The bioactivities of TNF are

mainly elicited by TNF receptor 1 (TNFR1), through which the following three distinct signaling pathways are initiated: a caspase cascade, NF-κB, and mitogen-activated protein kinase JNK (Chen and Goeddel, 2002).

1.3.5.1 TNFR1-mediated apoptosis

Comparing to that of Fas, TNFR1-induced apoptosis pathway is more complicated, which involves more players, formation of two complexes, and feedback regulations. Upon TNF binding, the trimerized TNFR1 does not directly recruit FADD, like in Fas signaling pathway, but first binds to an adaptor protein TNF receptor-associated death domain (TRADD), which then binds to cytosolic proteins FADD as well as receptor-interacting protein (RIP) and TNF receptor-associated factor 2 (TRAF2). The above molecules form a big complex (complex I), which is bound to membrane via TNFR1. Pro-caspase-8 is not bound to TNFR directly and not found in complex I (Harper *et al.*, 2003a). However, the complex I is disassociated, together with FADD and pro-caspase-8, form a second complex (complex II), within which pro-caspase-8 is processed and activated (Chinnaiyan *et al.*, 1995; Yeh *et al.*, 1998; Micheau and Tschopp, 2003). The active caspase-8 will activate caspase-3 directly or through crosstalking with mitochondria via tBid.

1.3.5.2 TNFR1-induced NF-κB activation

NF-κB is a ubiquitous transcription factor playing an important role in inflammation responses and cell survival regulation. It consists of heterogenous dimeric proteins, such as p65 and p50, which all containing a Rel homology domain (Karin and Delhase, 2000). In TNFR1 signaling pathway, NF-κB is activated and provides a negative feedback for apoptotic cell death.

In resting cells, NF-κB binds to inhibitor of κB (IκB) proteins and localizes in cytoplasm. In response to TNFα-TNFR1 ligation, the activated IκB kinase (IKK)

phosphorylates IκB, which results in the proteasomal degradation of IκB through ubiquitination. The released NF-κB then translocates from cytoplasm to nuclei and binds to the promoter regions of its target genes to regulate the gene expression.

A number of genes such as A20, IAPs, c-FLIP, TRAF1 and TRAF2 have been identified as NF-κB-regulated anti-apoptotic genes (Krikos *et al.*, 1992; Wang *et al.*, 1998; Micheau *et al.*, 2001). Expression of these anti-apoptotic proteins can promote cell survival and inhibit cell death. For example, XIAP, a member of IAPs, can directly bind to and inhibit the activation of caspase-9 and -3 (Deveraux *et al.*, 1997). It is thus why some cancer cells are resistant to the TNF-induced apoptosis and inhibition of NF-κB by a transcription inhibitor (actinomycin D) or translation inhibitor (cycloheximide, CHX), can great facilitate TNF-induced apoptosis.

1.3.5.3 TNFR1-induced JNK activation

Another branch of signaling pathway triggered by TNF-TNFR1 is JNK. Upon TNF-TNFR1 interaction, JNK is readily activated in a transient manner. Persistent JNK activation was observed in TNFα-treated cells when the NF-κB signaling pathway was blocked. It is known now that the crosstalk between NF-κB and JNK plays a role in diminishing the activated JNK. For instance, upregulation of the Gadd45 and XIAP by NF-κB can block the JNK activation (Tang *et al.*, 2001; Papa *et al.*, 2004). Another important mediator between JNK and NF-κB is believed to be ROS as many of the NF-kB target proteins function as antioxidants to remove ROS and to suppress JNK activation (Bubici *et al.*, 2006).

In contrast to the well-established anti-apoptotic role of NF-κB, the exact function of JNK in TNF-mediated apoptosis remains largely controversial (Liu *et al.*, 1996; Natoli *et al.*, 1997). Recently it has been demonstrated that prolonged JNK activation by the suppression of NF-κB activity promotes TNFα-induced apoptosis

(De Smaele *et al.*, 2001; Tang et al., 2001). It appears that the exact function of JNK in TNF-induced apoptosis depends on a number of factors such as cell type and/or the presence of other signaling pathways such as NF-κB activation (Karin and Lin, 2002).

1.3.5.4 Regulation of TNF-induced apoptosis

Upon TNF-TNFR interaction, in addition to the recruitment of FADD that activate caspase cascade, the recruitment of RIP and TRAF2 results in the activation of NF-κB which mainly functions as a cell survival mechanism to protect cells against TNFα-induced apoptotic cell death (Ting *et al.*, 1996; Reinhard *et al.*, 1997; Kelliher *et al.*, 1998). As TNFα activates both cell death and cell survival pathways simultaneously, most cancer cells are resistant to TNFα-induced apoptosis and thus inhibition of NF-κB activation becomes a popular strategy to enhance the sensitivity of cancer cells to apoptosis mediated by TNF family proteins (Baldwin, 2001; Yamamoto and Gaynor, 2001). Various approaches have been developed, including the genetic disruption of NF-κB signaling pathway by overexpression of a mutant IκBα (Leverkus *et al.*, 2003) and chemical inhibitors of NF-κB. Some of these approaches have been in clinical trial with promising results in cancer therapy.

1.3.6 TRAIL signaling pathway

Tumor necrosis factor-related apoptosis inducing ligand (TRAIL) is newly identified member of the TNF superfamily. TRAIL elicits its function by binding to its receptors on the cell surface. To date, four types of receptors have been identified. Death receptor 4 (DR4) and death receptor 5 (DR5) have both an extracellular domain that binds to TRAIL, and intercellular domains with DDs that trigger caspase cascade. However, other two receptors, death decoy receptor 1 (DcR1) and death decoy receptors (DcR2), lack the functional intercellular domains. Therefore, TRAIL can

bind to DcR1 and DcR2 but does not trigger caspase cascade and cell death (Wang and El Deiry, 2003).

1.3.6.1 TRAIL-induced apoptosis

The apoptosis induced by TRAIL is quite similar with that induced by FasL. Ligation of TRAIL to its receptors (DR4 and DR5) results in trimerization of receptors and clustering of intracellular death domains (DDs), which then recruit Fas-associated death domain protein (FADD) and pro-caspase-8 to form the death-inducing signaling complex (DISC). Caspase 8 activation within DISC subsequently leads to activation of effector caspases or cleavage of Bid, which then crosstalks with mitochondria to activate caspase-9 and -3 (Green, 2000a; Ashkenazi, 2002).

1.3.6.2 TRAIL-induced NF-κB activation

The apparent difference from TNF-induced signaling is that TRAIL-DRs interaction does not recruit TRADD or RIP or TRAF2 that can activate the cell survival signal, NF-κB. However, there were several reports that, in some cell lines, TRAIL can trigger NF-κB activation as evidenced from the detection of NF-κB-DNA binding by electrophoretic mobility shift assay (EMSA), although it is much weaker comparing with that induced by TNF. Up to date, little is known about how TRAIL activates NF-κB. It suggests that PI3K/AKT might be involved in the process (Zauli *et al.*, 2004). On the other hand, a recent report provided a possible explanation why TRAIL fails to activate NF-κB in some cells: TRAIL triggered-caspase activation causes the cleavage of NF-κB protein and in the presence of caspase inhibitor, TRAIL can significantly activate NF-κB (Kim *et al.*, 2005a).

1.3.6.3 Regulation of TRAIL-induced cell death

TRAIL is a potent apoptosis inducer and might be potential in cancer therapy. However, its application in clinical is limited because many cancer cells are found to be resistant to the apoptosis induced by TRAIL.

The resistance of cancer cells to TRAIL has been studies extensively. It may attribute to the biochemical changes in the cells in a variety of ways. First, either low expression of DR4/DR5, or high expression of decoy receptors DcR1/DcR2 will render the cancer cell to be resistant to TRAIL (Sheridan *et al.*, 1997). A number of anti-cancer drugs can activate DR4 or DR5 via DNA damage-activated p53 because DR4/DR5 is under control of transcription factor p53 (Liu *et al.*, 2004). Thus, a combination of anti-cancer drug with TRAIL might be an effective anti-cancer therapy. Second, although TRAIL may not activate NF-κB in some cancer cells, the relatively high basal level of NF-κB activation may directly or indirectly inhibit caspase activation via an array of its anti-apoptotic genes. Many small molecules which can downregulate or antagonize these anti-apoptotic proteins have been shown to be potent in sensitizing TRAIL-induced apoptosis in both in vitro and in vivo experiments (McManus, 2004; Hyer *et al.*, 2005).

1.3.7 Cisplatin and its anti-cancer effects

Cisplatin (cis-diamminediachloroplatium) is a widely used anti-cancer drug. Since its approval in 1970's for the treatment of genitourinary tumors, cisplatin has become one of the most widely used and successful drugs for the treatment of a variety of cancers, including ovarian, head, neck, bladder, cervical, and small cell lung cancers (Loehrer, 1984).

1.3.7.1 Anti-cancer effects of cisplatin and mechanisms of action of p53

. Once cisplatin enters a cell, its chloride ligand will be replaced by water molecules and form positively charged species that can react with nucleophilic sites on intracellular macromolecules such as protein, RNA and DNA (Dijt, 1988). It is generally accepted that the anti-cancer effect of cisplatin is mainly mediated by its interaction with DNA to form DNA adducts, which induce DNA damage and activate several signaling transduction pathways including ATR, ATM, p53, and MAPK (Siddik, 2003).

The interaction between cisplatin and DNA not only inhibits DNA replication and cell division, but also leads to apoptosis (Gonzalez *et al.*, 2001). Tumor suppressor p53 activation is one of major factors responsible for apoptosis induced by cisplatin. It has been observed that cisplatin treatment can cause apoptosis in wide type p53 cancer cell but not in p53 deficient or mutant cancer cells (Song *et al.*, 1998; Kanata *et al.*, 2000; Tang and Grimm, 2004; Beuvink et al., 2005), suggesting that p53 is the key regulator for cisplatin-mediated apoptosis in cancer cells.

p53 is a tumor suppressor protein (Ko, 1996; Levine, 1997), which is readily activated by DNA damage as well as other stimuli. Activation of p53 contributes to the tumor suppression either by inducing cell cycle arrest, possibly providing opportunity for the cells to repair damaged DNA, or by inducing apoptosis in the injured cells. So, p53 is playing a critical role in avoiding genetic instability and acts as tumor suppressor protein. It is believed that the loss of p53 activity promotes malignant transformation, leading to the high incidence of p53 mutations in a wide spectrum of human cancer (Hollstein, 1991; Levine *et al.*, 1991).

At present, the molecular mechanisms controlling p53 activation have been studied extensively. Generally, there are two regulatory mechanisms working together

to control the function of p53: p53 transcriptional activation and p53 stability (Kubbutat *et al.*, 1997; Ashcroft and Vousden, 1999). The stability of p53 protein, a short-lived protein, is mainly regulated by its interaction with its transcriptional target mouse double minute 2 (MDM2), which act as an ubiquitin E3 ligase and promotes p53 ubiquitination and proteasomal degradation (Kubbutat, 1997). Meanwhile, MDM2 is a transcriptional target of p53 and expression of MDM2 will promote p53 degradation to maintain the negative feedback loop. Upon DNA damage or other stimuli, p53 is up-regulated by transcriptional activation as well as via a number of mechanisms that disrupt the interaction between MDM2 and p53 and thus to increase p53 stability. Modifications on either p53 or MDM2 may affect their interaction. For instance, phosphorylation of p53 affects its interaction with MDM2 or its binding to DNA or its transcriptional activity (Steegenga *et al.*, 1996). It is known that DNA damage-activated ATM, ATR and DNA-PK can phorphorylate p53 on Ser 15 and Ser 37 (Shieh, 1997), Chk2 is among the kinases that contribute to phosphorylation of p53 on serine 20 (Shieh, 1999), whereas JNK phosphorylates p53 on tyrosine 81 (Buschmann *et al.*, 2001). Any of above phosphorylations on p53 may affect its interaction with MDM2 and finally affect its stability. On the other hand, post-translational modifications on MDM2 or inhibition of MDM2 activity or decrease of MDM2 protein can disrupt the interaction between p53 and MDM2 and promote the rapid accumulation of p53 (Ryan *et al.*, 2001). MDM2 protein is also controlled by ubiquitination and proteasomal degradation (Chang, 1998).

One important role of p53 as a tumor suppressor is its involvement in apoptosis. p53 activates the caspase cascade and apoptosis mainly via an intrinsic pathway that involves mitochondria, a central regulator of apoptosis. The integrity of outer mitochondrial membrane is tightly regulated by Bcl-2 family proteins. Pro-

apoptotic members of the Bcl-2 family, such as Bax, Bak and Bid, form channels in membranes and to regulate preexisting channels. Anti-apoptotic members of the family, such as Bcl-2 and Bcl-X_L, tend to have the opposing effects on membrane channel formation. (Kelekar and Thompson, 1998). The pro-apoptotic functions of p53 can be mediated through a transcription-dependent pathway, which involves the activation of pro-apoptotic genes. It was shown that p53 can regulate the transcription of a group of pro-apoptotic proteins such as Bax (Miyashita and Reed, 1995), Noxa (Oda *et al.*, 2000a), PUMA (Nakano and Vousden, 2001; Chipuk *et al.*, 2005), DR5 (Wu, 1997), BID (Sax *et al.*, 2002), and CD95 (Muller *et al.*, 1998), which elicit caspase cascade and apoptosis.

In addition to transactivation of target genes, evidence has also implicated that p53 can induce apoptosis via a transcription–independent way. For example, the apoptosis induced by p53 may be through increasing surface Fas by transporting from the Golgi complex (Bennett *et al.*, 1998), or require FADD-independent activation of caspase-8 (Ding *et al.*, 2000). Further, it was found that p53 protein can translocate to mitochondria, form complexes with Bcl-XL and Bcl-2 proteins and directly induce cytochrome c release (Mihara *et al.*, 2003). Recently, it has been demonstrated that the apoptosis induced by p53 is mediated by Bax mitochondrial translocation and activation (Chipuk *et al.*, 2003; Erster *et al*., 2004). In non-stimulated cells, Bax exists as a monomer either in the cytosol or loosely attached to the outer mitochondrial membrane. Upon stimulation, the cytosolic Bax translocates to mitochondria and inserts into the membrane. Bax oligomerizes into large complexes which are believed to be crucial to mitochondrial membrane permeabilization (Goping *et al.*, 1998). The activated Bax on mitochondria can be distinguished by a conformational change in the

N-terminus that exposes the formerly buried 6A7 epitope (Desagher *et al.*, 1999; Nechushtan *et al.*, 1999)

1.3.7.2 Regulation of cisplatin-induced apoptosis

Despite the success against testicular cancer, the use of cisplatin against other cancers is limited due to acquired or intrinsic resistance. For example, cisplatin has minimal activity against some common cancer types, such as colorectal cancer (Natoli *et al.*, 2000). Thus, resistance is the major constraint that undermines the curative potential of cisplatin.

Efforts have been made to define the cellular and molecular mechanisms responsible for cisplatin resistance (Kartalou and Essigmann, 2001). The resistance may be through either limiting the extent of cisplatin-induced damage, for example, alterations in cellular pharmacology, including decreased drug accumulation, increased cellular thiol levels and increased repair of platinum–DNA damage. In addition, alterations in the cellular response to the damage also contribute to the resistance. Since p53 is a major mediator of the apoptosis induced by cisplatin, mutation of p53 or alteration of expression level of Bcl-2, Bcl-XL, Bax or MDM2 may affect the responses to cisplatin. For example, MDM2 overexpression confers the cancer cells to be resistant to cisplatin (Kondo *et al.*, 1995).

Therefore, cisplatin is usually not used alone for cancer therapy. Clinically, it is used in combination with other anti-cancer drugs, such as etoposide (Kovnar *et al.*, 1990; Ardizzoni *et al.*, 1999), bleomycin (Behnia *et al.*, 2000) and irinotecan (Sandler, 2002). Recently, new strategies to enhance the cytotoxicity of cisplatin has been investigated (Duan *et al.*, 2001; Iwase *et al.*, 2003; Kim *et al.*, 2003a; Fulda and Debatin, 2005; Mohanty *et al.*, 2005). For instance, downregulation of MDM2 using

MDM2 antisense oligonucleotides or RNA interference can enhance the sensitivity to cisplatin (Yu *et al.*, 2006).

1.4 OBJECTIVES OF THE STUDY

At present, many aspects of pharmacological activities of chrysanthemum have been well studied, including the anti-inflammatory and anti-oxidant function.. Preliminary results from our laboratory has shown that the water extract of chrysanthemum exerted significant anti-tumor effects in *in vivo* experiments (unpublished data), suggesting the anti-tumor potential of this herbal plant. However, the major anti-tumor components in the water extract of chrysanthemum are yet to be determined. It has been reported that chrysanthemum contains a wide range of flavonoids including luteolin, apigenin etc. However, it is currently not known how these flavonoids contribute the potential anti-tumor effect in chrysanthemum. Therefore, the goals of this study are to identify the major anti-tumor components in the water extract of chrysanthemum and to investigate the molecular mechanisms involved.

To achieve these goals, the following studies will be conducted:

(1) To identify the major active component(s) in the water extract of *Chrysanthemum morifolium* Ramat.

(2) To evaluate the anti-tumor property and mechanism of the water extract of chrysanthemum.

(3) To study the anti-tumor effect and mechanisms of luteolin, a major component found in Chrysanthemum, on human cancer cells.

(4) To investigate the potential synergistic effects of luteolin with known cancer therapeutics agents (cisplatin) or cell death ligands (TNF and TRAIL) on apoptosis in cancer cells and the molecular mechanisms involved.

(5) To evaluate the synergistic anti-tumor effects of luteolin and cancer therapeutic agents using *in vivo* animal models.

This study will help in understanding the anti-tumor properties of chrysanthemum and, at a molecular level, provide evidence of the potential effects of this herb on cancer prevention or therapy. Furthermore, the study of combined effects of luteolin with other cancer therapeutic agents could provide useful insight on the synergistic mechanisms, and the potential application of luteolin as a chemosensitizer in cancer therapy.

CHAPTER TWO

IDENTIFICATION OF THE MAJOR ACTIVE COMPONENTS IN CHRYSANTHEMUM

2.1 INTRODUCTION

The dried flower heads of *Chrysanthemum morifolium* Ramatuelle have been used as a traditional herbal medicine in Asian countries for centuries. Its traditional usage includes treatment of common cold, fever, migraines, conjunctivitis, eye irritation, hypertension, ulcerative colitis, vertigo and ophthalmia with swelling and pain etc (Liu, 1998; Jiang, 2002). There were also reports about its other activities, such as anti-tumor activities (Ukiya *et al*., 2002).

Due to its multiple pharmacological properties as well as the mild fragrance, the flower is used popularly as herbal beverage, chrysanthemum tea. Chrysanthemum tea is prepared in the same way as for traditional tea. The dried flowers are infused with hot water for a few minutes before it is served. For clinical usage, the chrysanthemum is boiled with water. Therefore, the water-soluble components in the flower are more likely related to its pharmacological properties.

Recently, preliminary data from our laboratory found that the water extract of chrysanthemum significantly inhibited growth of transplanted tumor in nude mice (Shen et al, unpublished data), suggesting that the aqueous components of chrysanthemum may have potent anti-tumor effects. More than 50 terpenoids, identified as the main components of volatile oil from chrysanthemum flower, were tested on their anti-tumor properties (Ukiya *et al.,* 2002). However, the anti-tumor components in the water extract are yet to be determined.

In this chapter, we attempted to identify the major water soluble anti-tumor components of chrysanthemum based on bioactivity-driven HPLC-MS analysis.

2.2 MATERIALS AND METHODS

2.2.1 Materials

Commercially available air-dried flower head of Chrysanthemum morifolium (commercial name: Hang Bai Jv) was obtained from Tong xiang city, Zhejiang Province, China. 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) and dimethyl formamide (DMF) were from Sigma (St. Louis, MO). Formic acid, ammonia solution and hydrochloric (HCl) acid were obtained from Merck (Darmstadt, Germany). All organic solvents and other chemical reagents used were analytical grade.

2.2.2 Cell lines and cell culture

Human colorectal cancer cells HCT116 were purchased from the American Type Culture Collection (ATCC, Rockville, MD) and maintained in completed RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), penicillin (100U/mL) and streptomycin (100 U/mL) at 37°C in 5% CO_2.

2.2.3 Extraction and fractionation

The flower head of chrysanthemum was pulverized by an electronic blender and kept airtight at 4°C for further use. 200g of the dried powder was boiled in 3000 ml distilled water for 20 min and filtered through paper filter (Whatman). The same process was applied to the residue twice. The combined filtrate was concentrated to 1400 ml using a rotary vacuum evaporator. 700 ml condensed filtrate was freeze-dried and resulted in crude water extract (Fraction A). Another 700 ml condensed filtrate was partitioned with 700 ml ethyl acetate (EtOAc) four times and freeze-dried (Fraction B), while the aqueous fraction was freeze-dried and then extracted three time with 250 ml 80% ethanol (EtOH). After concentrated by a rotary vacuum

evaporator and then freeze-dried, two fractions were obtained, the EtOH fraction (Fraction C), and water fraction (Fraction D) (Figure 2.1).

2.2.4 Cytotoxicity assay

MTT assays were used to assess the cytotoxicity of various extracts on cancer cells as described previously (Yang *et al.*, 1999). Briefly, HCT116 cells were plated on 96-well microplates (1×10^4 cells/well in 100 μL of medium) for 24 h. After discarding the medium, cells were then treated with various concentrations of extracts dissolved in RPMI 140 medium for 24 h. At the end of treatment, 25 μL of MTT (5 mg/mL) was added to each well and incubated for a further 2 h. Finally, 100 μL of lysing buffer (50% DMF and 20% SDS, pH 4.6) was added to each well and incubated for another 2 h. The plate was finally read using a microplate reader (BIO-RAD Model 3550) at a wavelength of 595 nm. Each assay was repeated 3 times.

2.2.5 High-performance liquid chromatography-mass spectrum

The major components of ethyl acetate fraction were identified by reverse phase high performance liquid chromatography coupled with mass spectrometer (RP-HPLC-MS). The ethyl acetate fraction was dissolved in methanol:acetic acid (95:5) and filtered through 0.5 μm filter before applying to HPLC-MS. Separation was performed on a Zorbax SB-C18 (5 μm) column (150 × 4.6 mm i.d.) from Agilent Technologies. The detectors are Finnigan MAT LCQ ion trap mass spectrometer equipped with an atmospheric pressure chemical ionization interface (APCI) and a diode array thermo separation product UV6000LP detector. Mobile phase: step gradient of 0.1% formic acid in water (A) and 0.1 % formic acid in acetonitrile (B) was used according to the following profile: 0–15 min, 100–77% A, 0–23 % B; 15–35 min, 77 % A, 23 % B; 35-45 min, 77-66% A, 23-34 % B; 45-50 min, 25-0 % A, 75-100 % B. The flow rate was 1.0 ml/min. Column temperature, controlled with a

column heater–cooler HP Series 1100 from Hewlett-Packard, was set at 35 °C. The mass scanning range of APCI detector was set in 50-1500.

2.3. RESULTS

2.3.1 Fractionation of chrysanthemum water extract

To determine the anti-tumor water-soluble components of chrysanthemum, we used a strategy applying a combination of bioactivity-directed fractionation and HPLC-MS analysis. First, we extracted the chrysanthemum flower powder using boiling water, which is to mimic the procedure of chrysanthemum tea preparation. The 200 g chrysanthemum flower power was boiled with water and half of them was freeze-dried and resulted in the crude water extract (Fraction A), which was about 38 g (yielding rate 38 %) (Figure 2.1). Another half was sequentially partitioned into three fractions according to their polarity and resulted in EtOAc (Fraction B), EtOH (Fraction C) and final water extract (Fraction D). The EtOAc fraction was about 2 g (yielding rate 2 %) and was the minimal fraction. EtOH fraction and final water extract were about 5 g and 30 g, respectively.

2.3.2 Cytotoxicity of each fraction

The cytotoxicity of each fraction was assessed using the MTT assay. As shown in Figure 2.2, the crude water extract showed a moderate cytotoxicity (IC50 = 3.6 mg/ml) on HCT116 cancer cells. The EtOAc fraction showed a higher cytotoxicity (IC50 = 0.2 mg/ml), which is about ten times higher than using crude water extract. However, two other fractions, sequentially extracted from the leftover of EtOAc extraction, showed much lower cytotoxicities (IC50 > 5 mg/ml for EtOH fraction and IC50 > 8 mg/ml for final water fraction). These results suggest that the EtOAc fraction (Fraction B) contains the major anti-tumor components in the water extract of chrysanthemum.

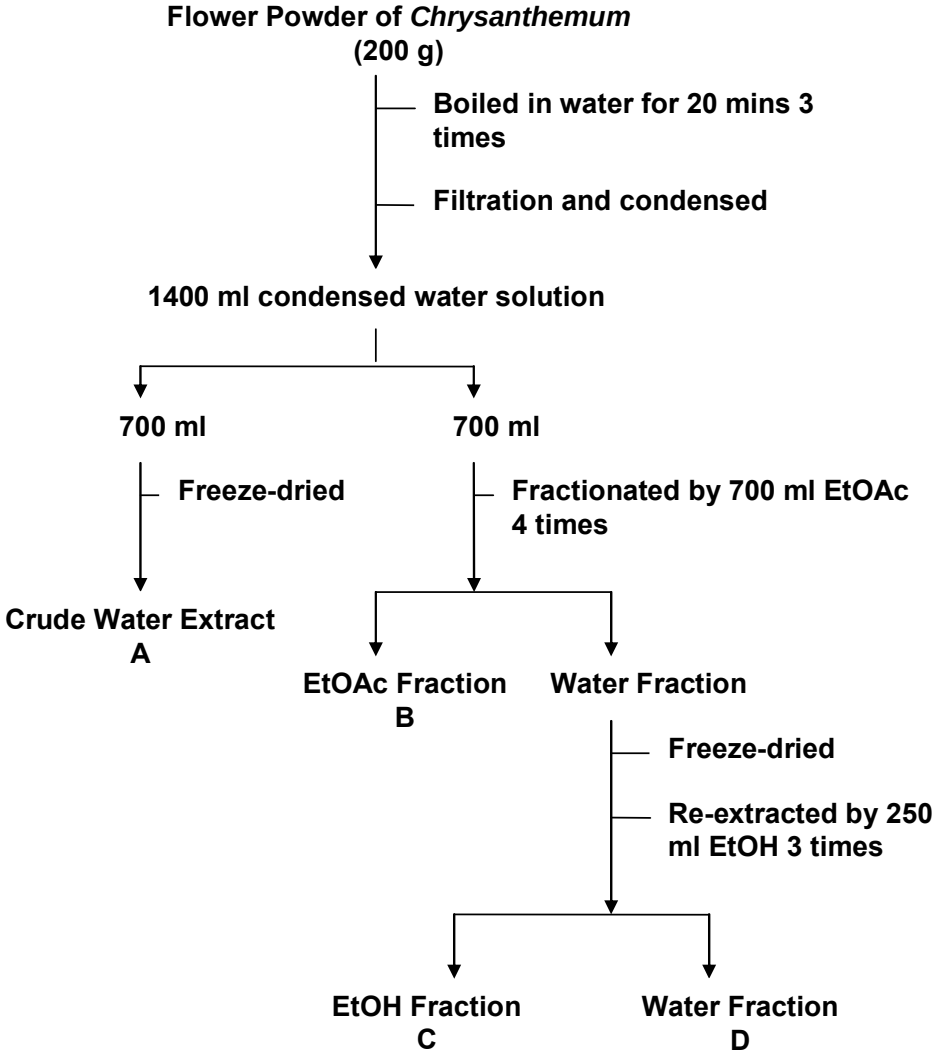


Figure 2.1 Bioassay-directed fractionation from Chrysanthemum

The water extract of chrysanthemum was separated into four fractions, A, B, C and D. Details were described in Section 2.2.3

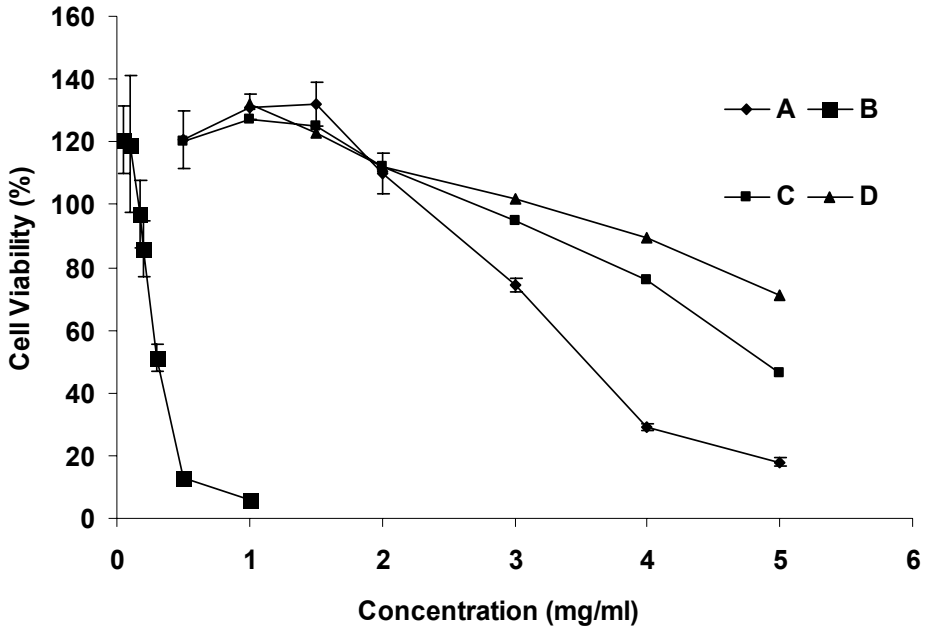


Figure 2.2 Cytotoxicity of fractions from Chrysanthemum on human colorectal cancer cells HCT116

HCT116 cells were treated with various fractions from chrysanthemum for 24 h. At the end of treatment, cell viabilities were determined by MTT assay. Cell viabilities were expressed as means of three experiments. A, crude water extract ; B, EtOAc fraction; C, EtOH fraction; D, final water fraction.

2.3.3 Flavonoids are the major components in EtOAc fraction

Flavonoids are ubiquitious in many edible plants. They consist of two aromatic rings linked through three carbons. Most flavonoids in plant are present as flavonoid glycosides, aglycone with sugar substitution (Figure 1.2) (Ross and Kasum, 2002).

A common method of flavonoid extraction is using ethyl acetate. In this study, EtOAc fraction was separated in a C18-HPLC column with a UV detector and a MS detector. HPLC is able to effectively separate various flavonoids under the specific conditions and mass spectrum and UV spectrum provides some structure information of each peak such as molecular weight, major stable ions and existence of phenol ring. A combination of HPLC separation and MS are useful in identification of flavonoids from a mixture (Stobiecki, 2000). Since the EtOAc fraction was originally from a water extract, it was separated in a reverse phase column using a gradient solution, a combination of water and acetonitrile, after optimization. Figure 2.3 shows that there are 13 major peaks.

The structure of each of the 13 peaks was identified according to the information provided by MS and UV and made reference to available literature (Hu *et al*., 1994; Liu *et al*., 2001; Lee *et al*., 2003; Hu *et al*., 2004). Mass spectrum provides important information about the peak, in particular the molecular weight. Increasing the voltage of APCI will result in more fragments and provide additional information about its possible structure. Take peak 2 (retention time 20.23 min) as an example, the mass spectrum using low collision energy of APCI showed two major peaks, m/z 449 and m/z 287.5 (Figure 2.5A). Thus, m/z 449 is the molecular weight ion peak $[M+H]^+$ and its molecular weight (MW) is 448. Higher collision energy of APCI caused the peak m/z 449 disappeared and only peak m/z 287.5 remained (Figure 2.5B). The loss

of 162 (from 449 to 287.5) is evidently due to the loss of a sugar residue ($C_6H_{12}O_6$ - H_2O = 162). Loss of 162 resulted in a stable structure m/z 287.5. According to literature (Hu et al., 2004), it is putatively considered as luteolin. UV spectrum confirmed the existence of a phenol ring (data not shown). According to the literature, the sugar is established as a glucose side chain (Hu *et al*., 2004; Hu and Kitts, 2004). Thus, peak 2 is luteolin glucoside. Figure 2.5C shows the conversion from peak m/z 449 to m/z 287 under APCI.

Similarly, the putative structures of other peaks were identified based on their mass spectrum and earlier reports (Hu et al., 1994; Liu et al., 2001; Lee et al., 2003; Hu et al., 2004) (Figures 2.4-2.16). Peak 8 appears to be a mixture of two flavonoids, one is a baicalein glucuronide and another is a hesperetin glycoside. However, the latter contains an unknown group attached to glucose. The structure of a small peak (peak 4) was not identified. In this investigation, a total of 13 flavonoids from 12 peaks were identified.

The 13 flavonoids of EtOAc fraction can be classified into five groups according to their aglycones (Figure 2.3). Four peaks are related to luteolin, including luteolin-rhamonosyl-glucoside (peak 1), luteolin-glucoside (peak 2), luteolin-glucuronide (peak 3) and luteolin-methoxyl-glucoside (peak 7). Two peaks are apigenin glycosides; including apigenin-glucoside (peak 6) and apigenin-methoxyl-glucoside (peak 10). There are three hesperetin glycosides, including hesperetin-rhamonosyl-glucoside (peak 5), heperetin-glucuronide (peak 9) and heperetin-methoxyl-glucoside (peak 8). Two peaks are baicalein glycosides, including baicalein-glucuronide (peak 8) and baicalein-methoxyl-glucoside (peak 12). The last group consists of two acacetin-glycosides, acacetin-rhmnosyl-glucoside (peak 11) and acacetin-glucoside (peak 13).

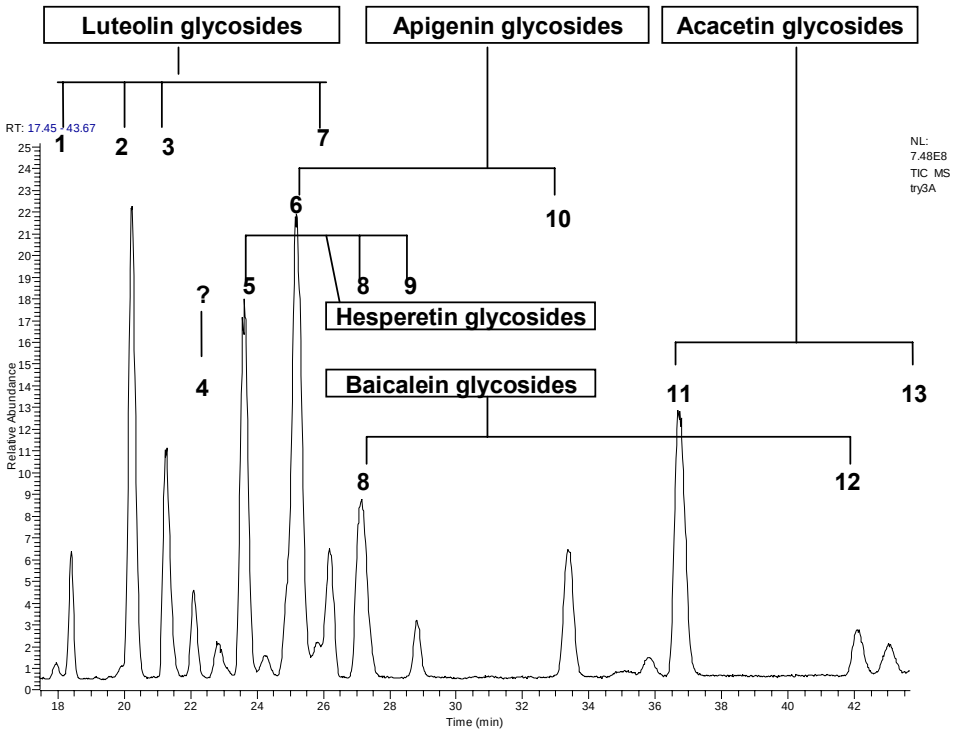


Figure 2.3 Flavonoids in the EtOAc fraction

The flavonoids in the EtOAc fraction are grouped into five groups, luteolin glycosides, apigenin glycosides, hesperetin glycosides, baicalein glycosides and acacetin glycosides.

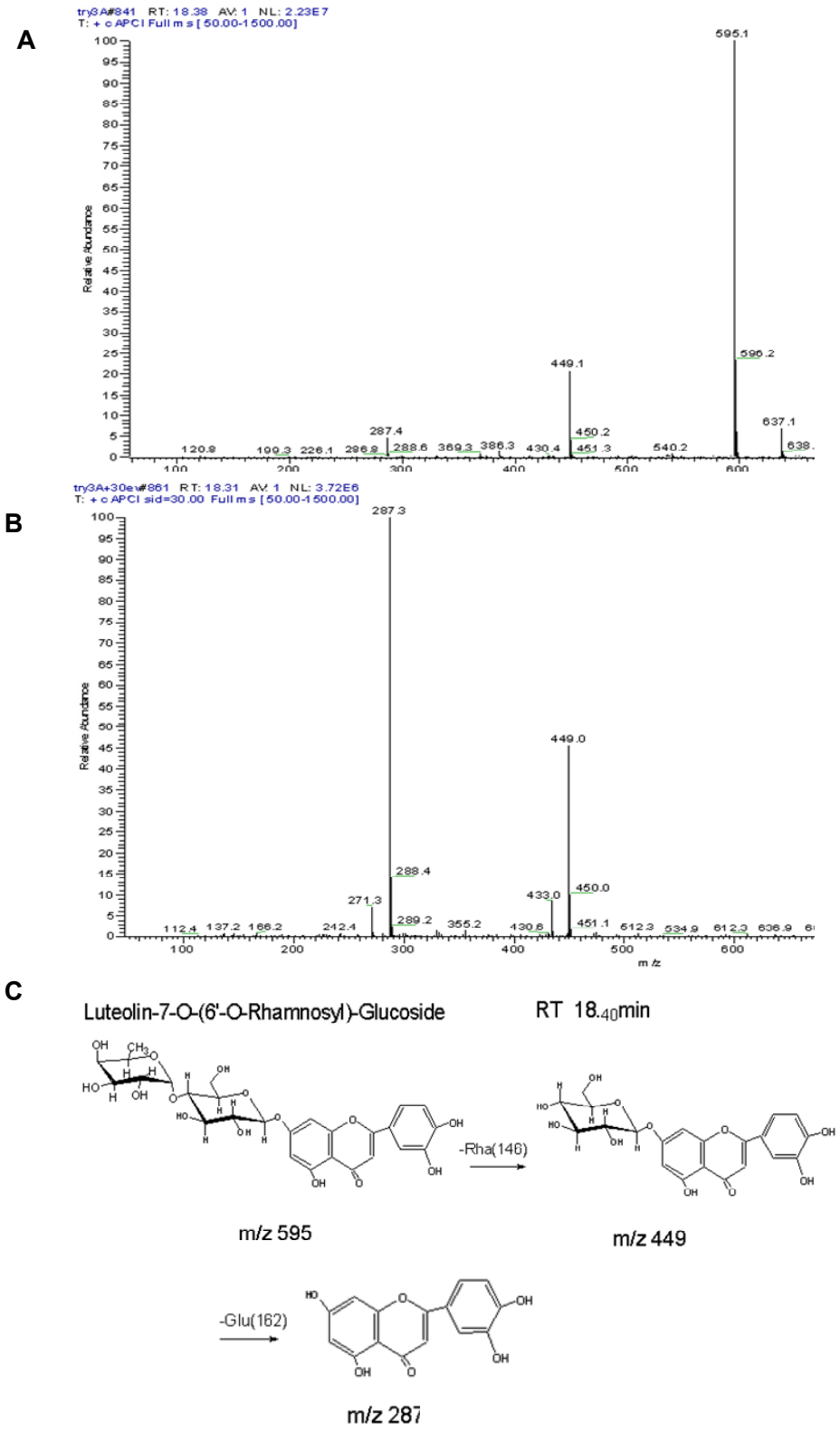


Figure 2.4 Structure elucidation of peak 1, RT 18.31 min

EtOAc fraction was separated on reverse phase-HPLC and detected by APCI. The mass spectrum of peak RT18.31 were shown. A, under low energy APCI; B, under high energy of APCI. Structure of peak 1 and its conversion under APCI.

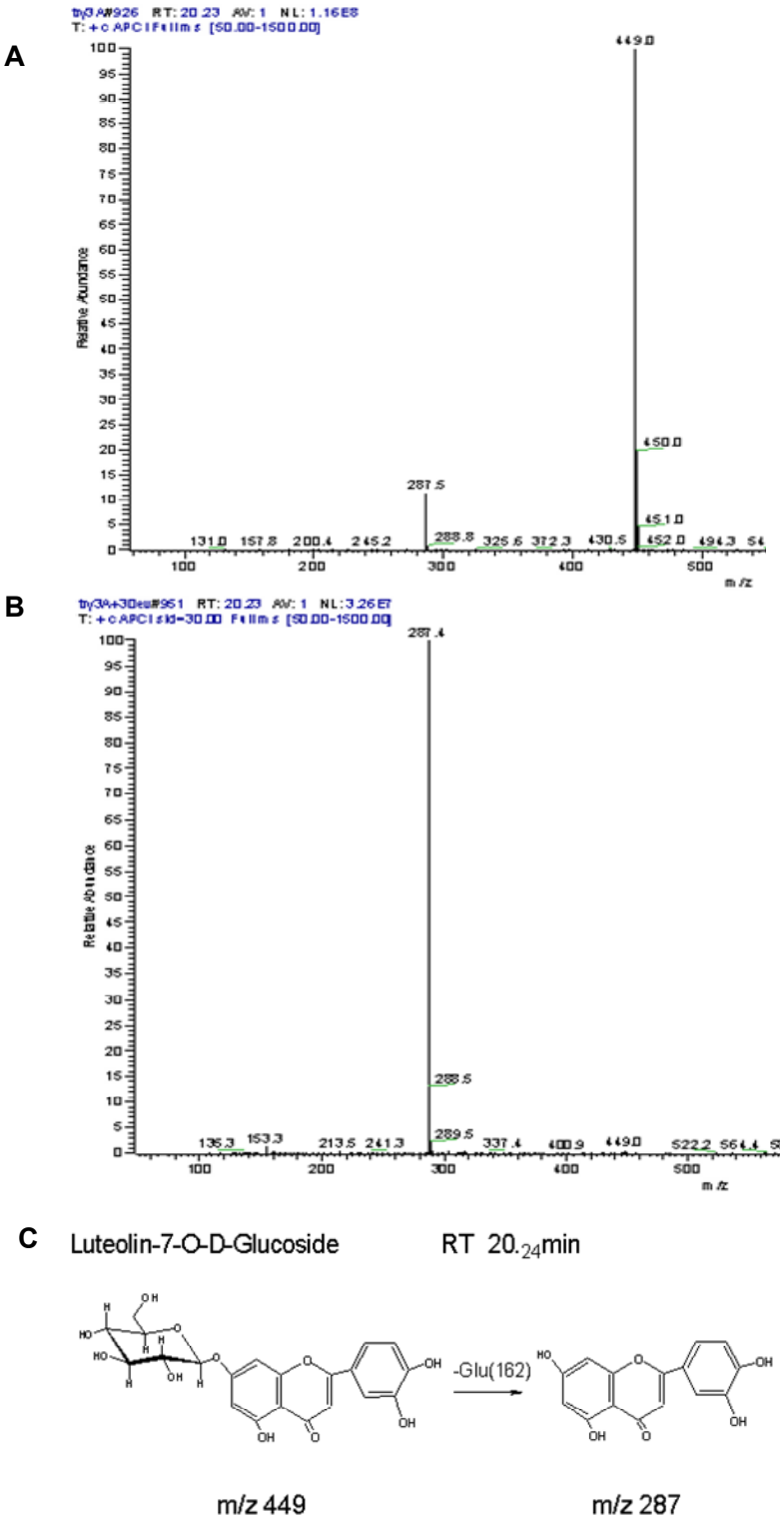
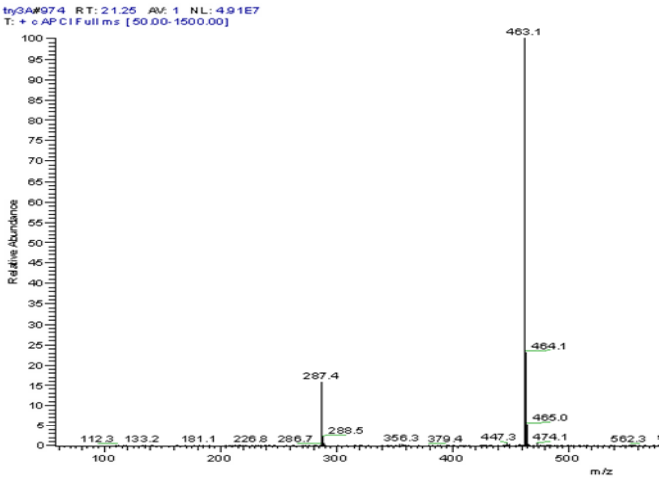


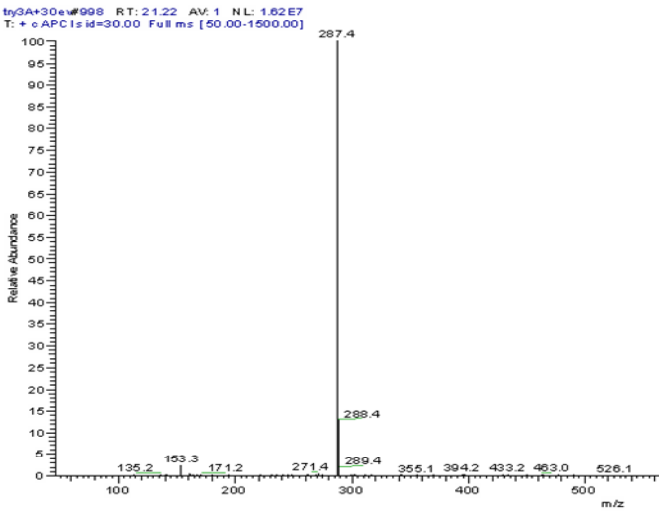
Figure 2.5 Structure elucidation of peak 2, RT 20.23 min

EtOAc fraction was separated on reverse phase-HPLC and detected by APCI. The Mass spectrums of the peak (RT20.23min) were shown in A and B. A, under low energy APCI; B, under high energy of APCI. C, Structure of peak 2 and its conversion under APCI

A

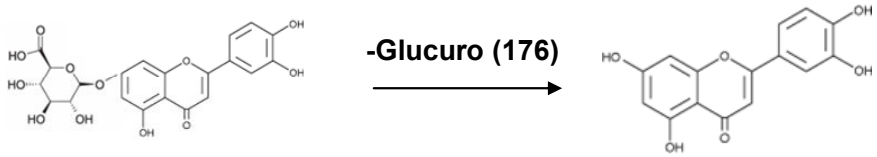


B



C

Luteolin-Glucuronide RT21.22 min



m/z 463 **m/z 287**

Figure 2.6 Structure elucidation of peak 3, RT 21.22 min

EtOAc fraction was separated on reverse phase-HPLC and detected by APCI. The mass spectrum of peak RT21.22 were shown. A, under low energy APCI; B, under high energy of APCI. Structure of peak 3 and its conversion under APCI.

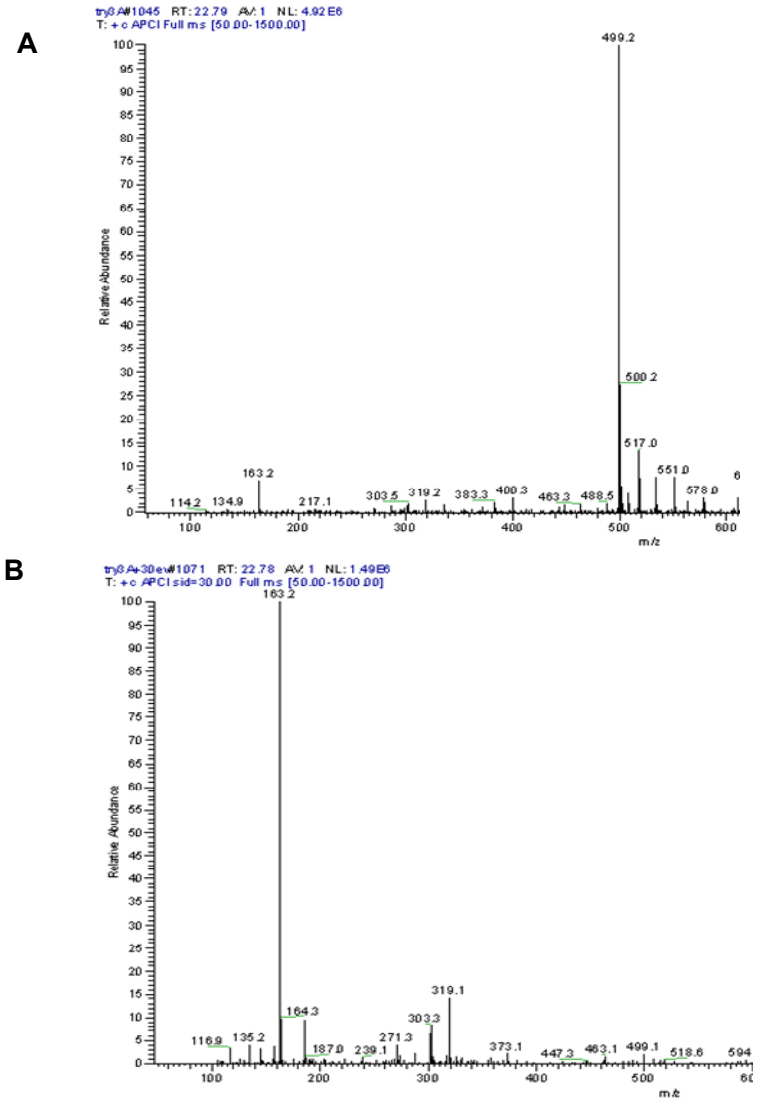
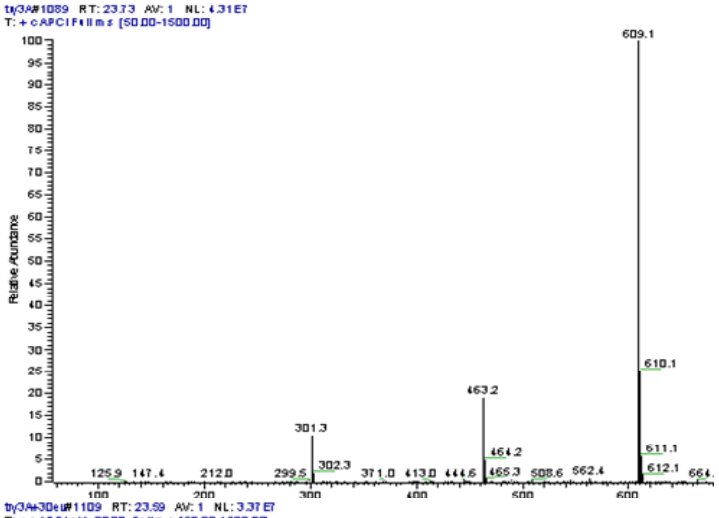


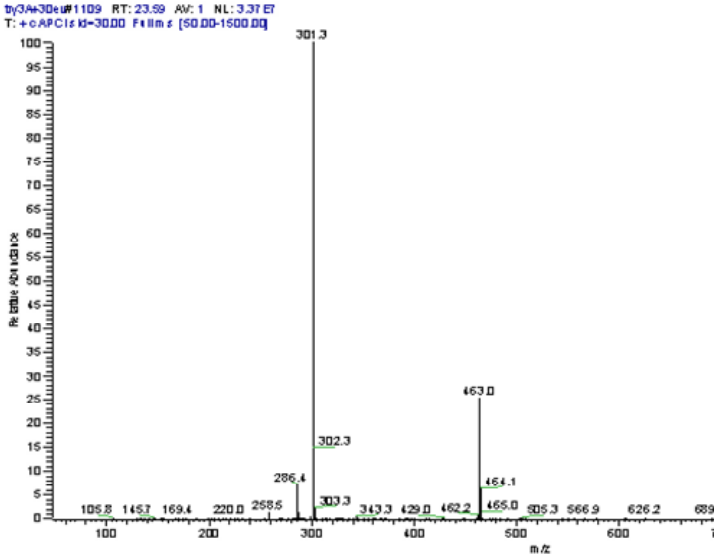
Figure 2.7 Mass spectrum of peak 4, RT 22.79 min

EtOAc fraction was separated on reverse phase-HPLC and detected by APCI. The mass spectrum of peak RT22.79 were shown. A, under low energy APCI; B, under high energy of APCI.

A



B



C **Hesperdin (Hesperetin-Glucoside-Rhamnoside) RT 23.50 min**

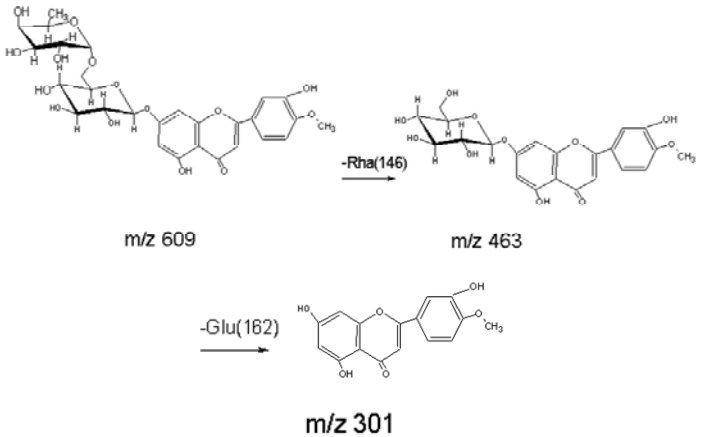


Figure 2.8 Structure elucidation of peak 5, RT 23.5 min

EtOAc fraction was separated on reverse phase-HPLC and detected by APCI. The mass spectrum of peak RT23.5 were shown. A, under low energy APCI; B, under high energy of APCI. Structure of peak 5 and its conversion under APCI.

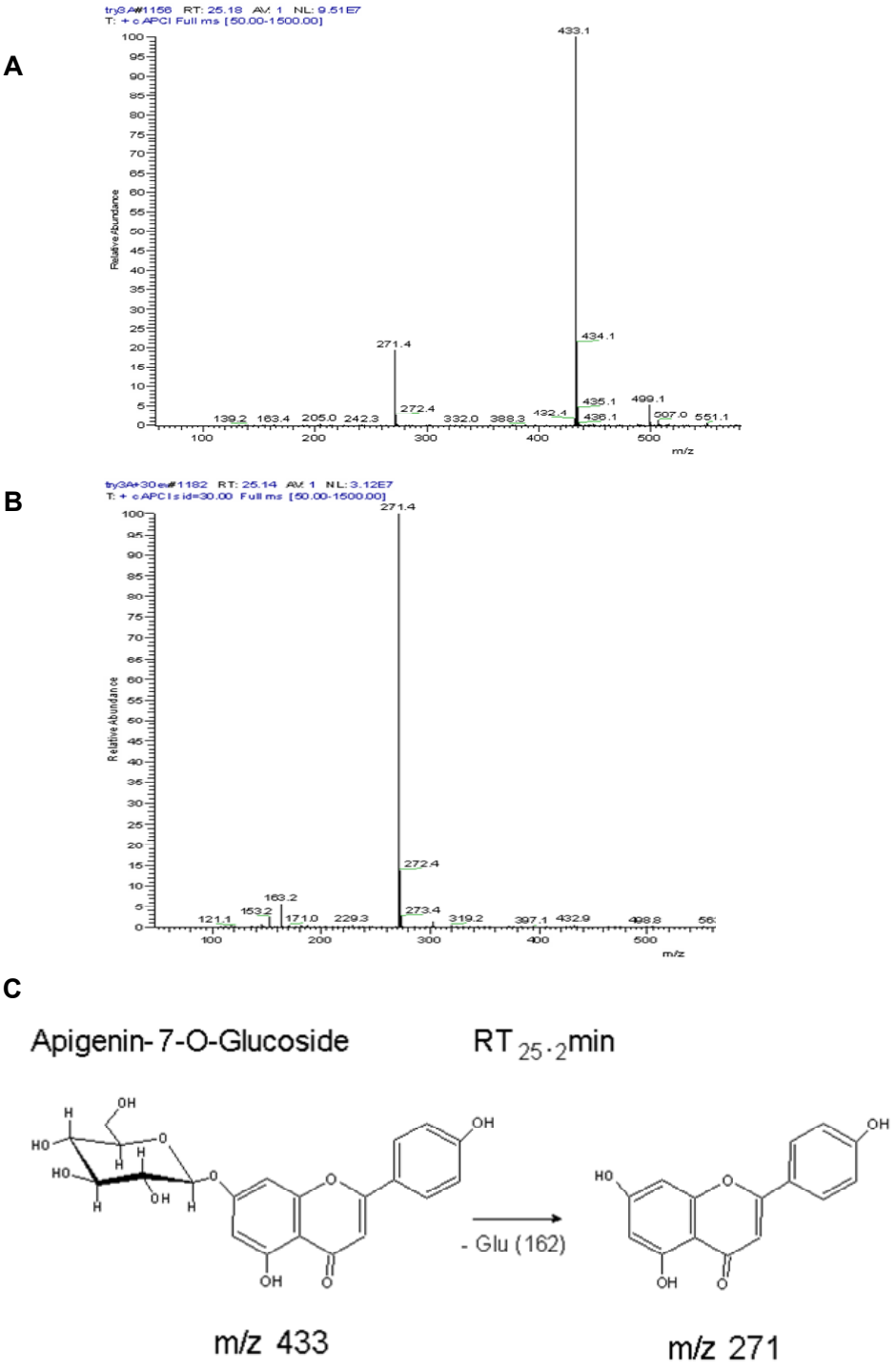


Figure 2.9 Structure elucidation of peak 6, RT 25.2 min

EtOAc fraction was separated on reverse phase-HPLC and detected by APCI. The mass spectrum of peak RT25.2 were shown. A, under low energy APCI; B, under high energy of APCI. Structure of peak 2 and its conversion under APCI

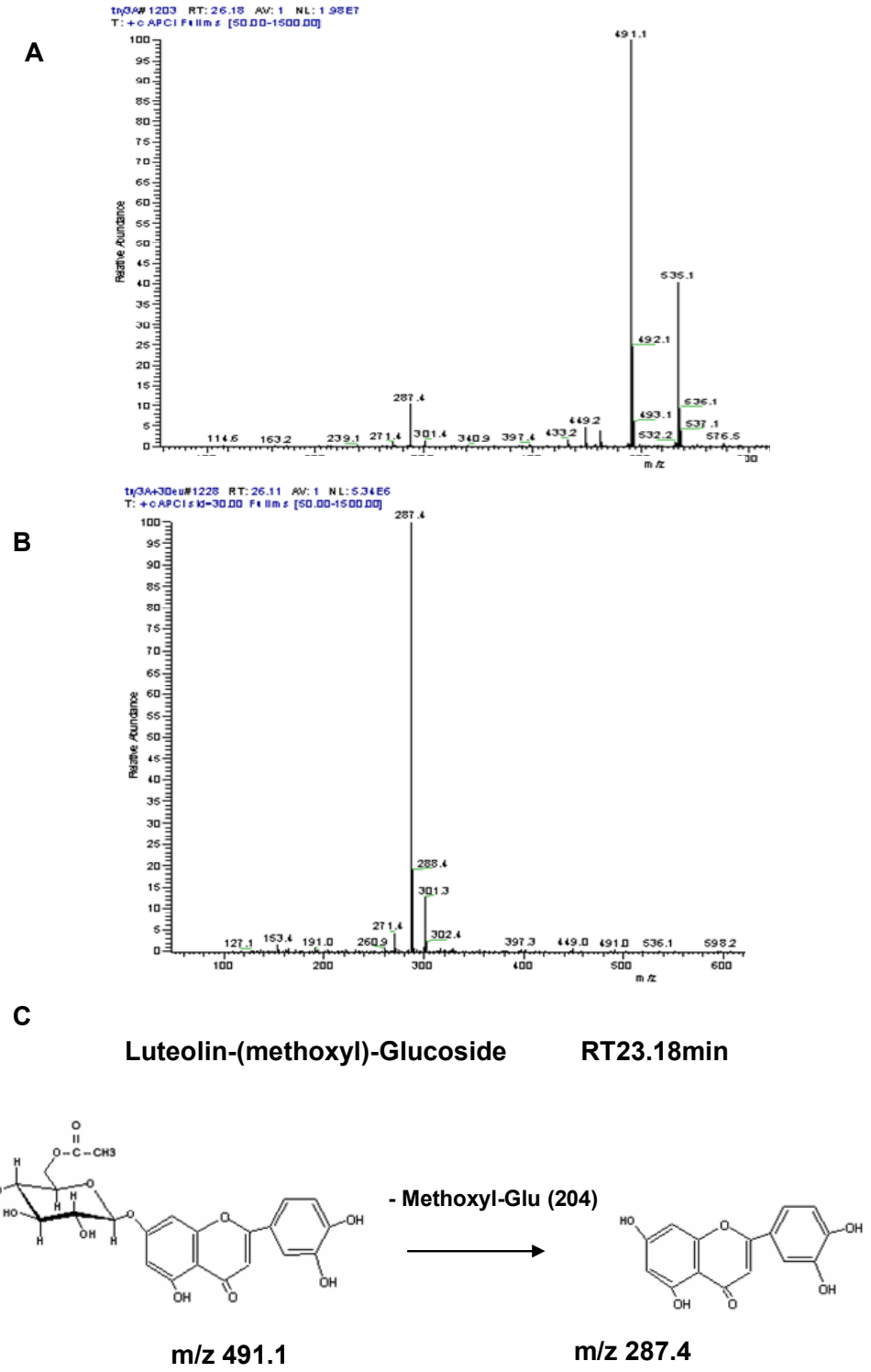


Figure 2.10 Structure elucidation of peak 7, RT 23.18 min

EtOAc fraction was separated on reverse phase-HPLC and detected by APCI. The mass spectrum of peak RT23.18 min were shown. A, under low energy APCI; B, under high energy of APCI. Structure of peak 3 and its conversion under APCI.

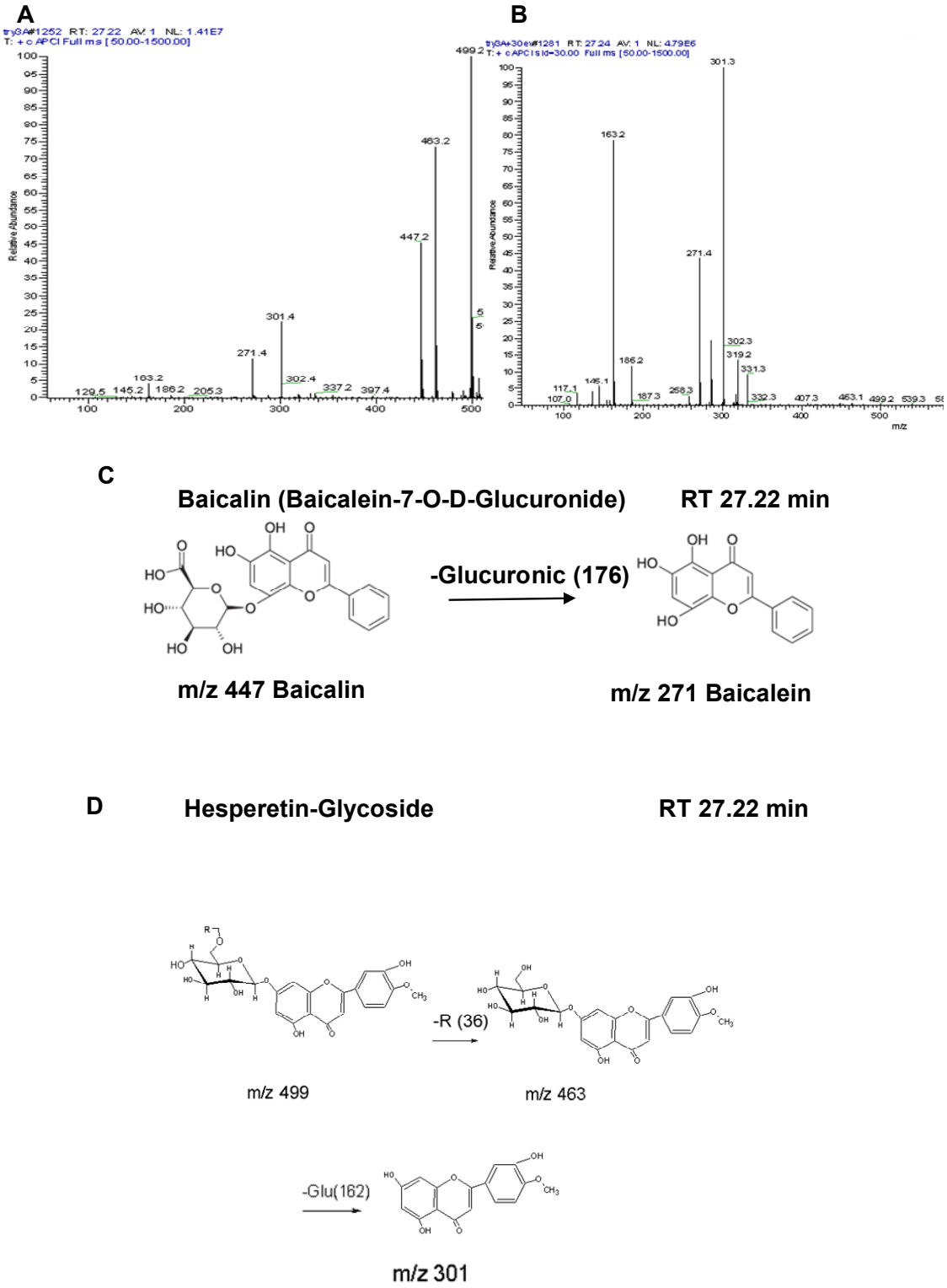


Figure 2.11 Structure elucidation of peak 8, RT 21.22 min

EtOAc fraction was separated on reverse phase-HPLC and detected by APCI. The mass spectrum of peak RT21.22 were shown. A, under low energy APCI; B, under high energy of APCI. Structure of peak 3 and its conversion under APCI.

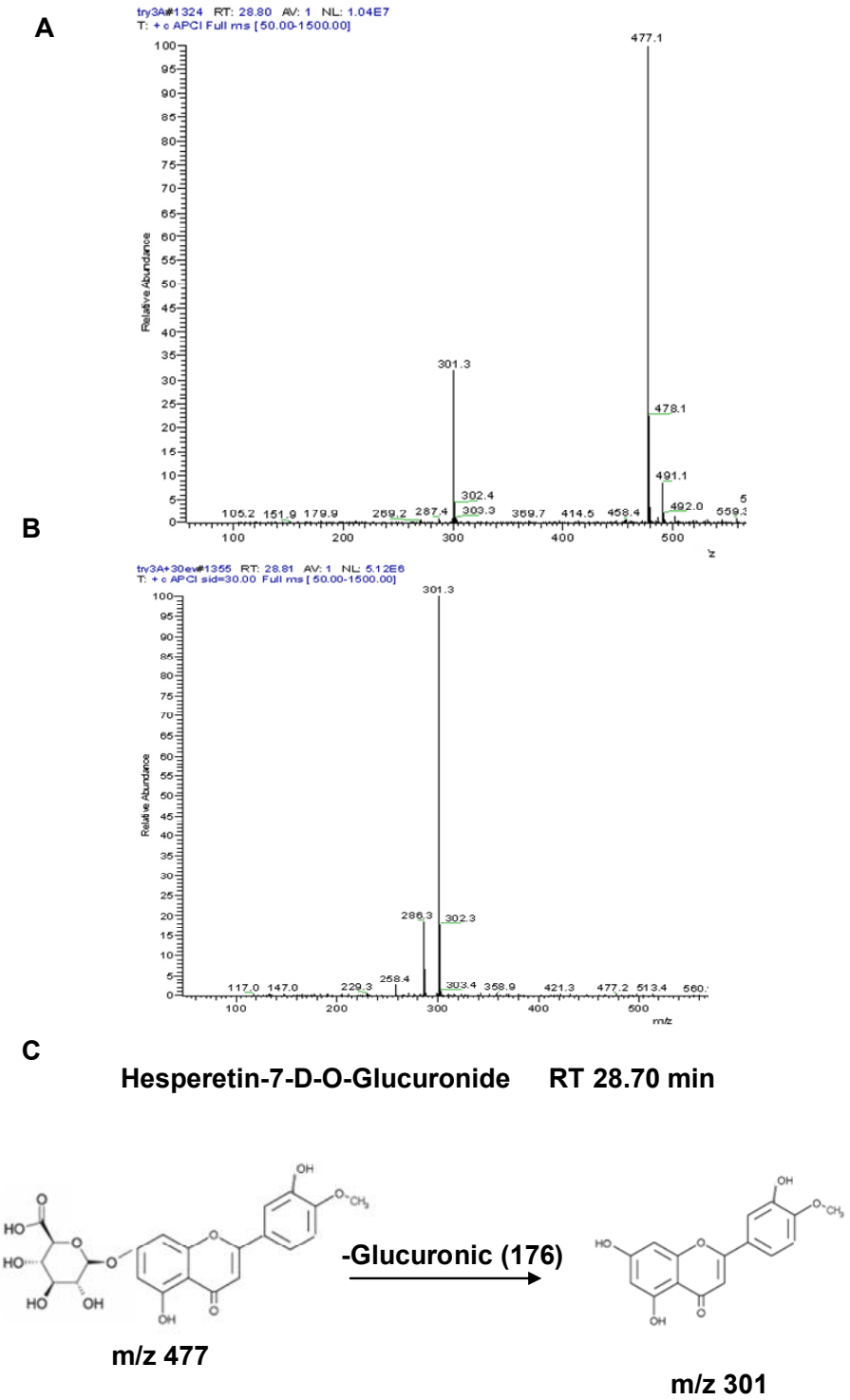


Figure 2.12 Structure elucidation of peak 9, RT 28.70 min

EtOAc fraction was separated on reverse phase-HPLC and detected by APCI. The mass spectrum of peak RT28.70 were shown. A, under low energy APCI; B, under high energy of APCI. Structure of peak 9 and its conversion under APCI.

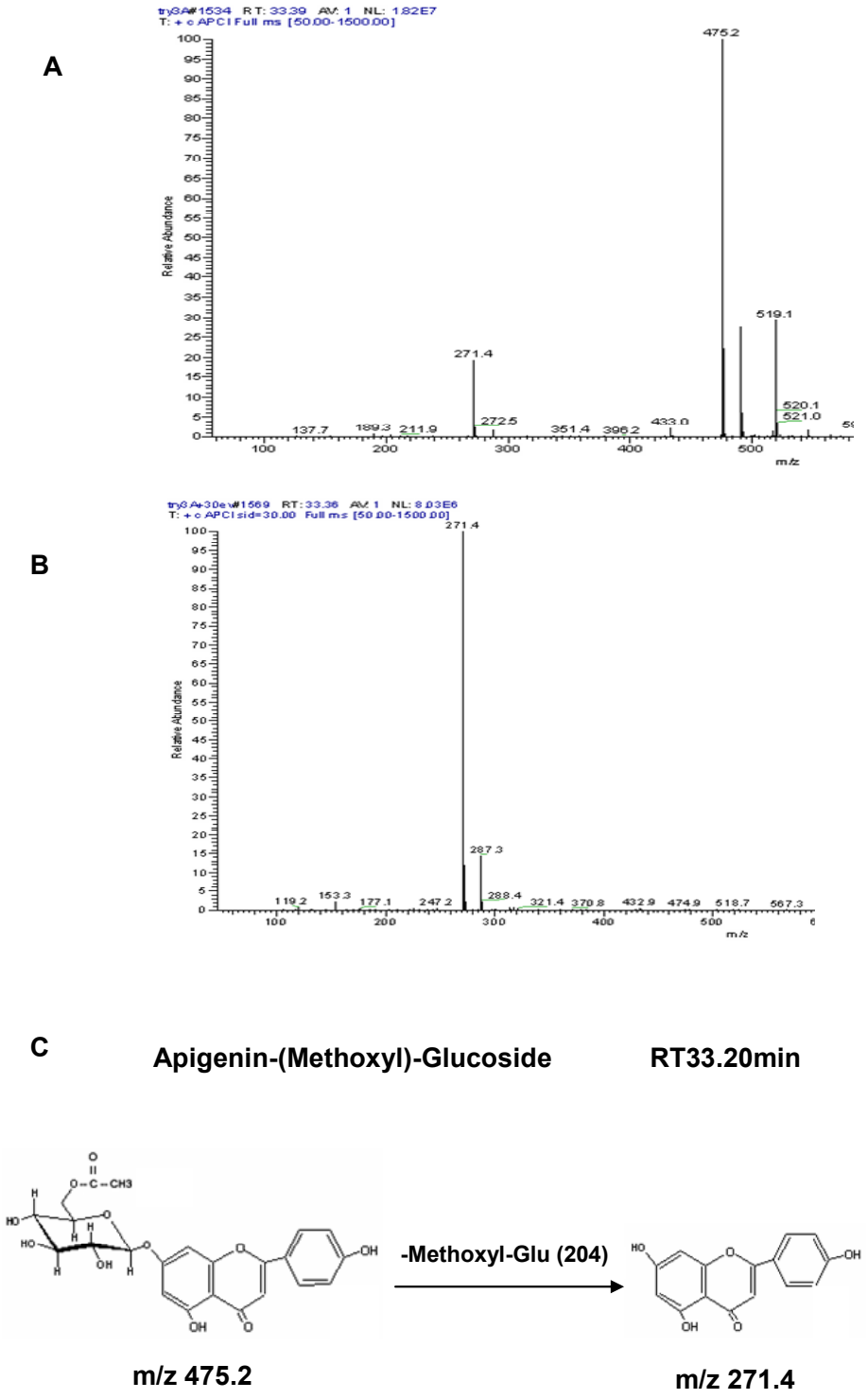


Figure 2.13 Structure elucidation of peak 10, RT 33.20 min

EtOAc fraction was separated on reverse phase-HPLC and detected by APCI. The mass spectrum of peak RT33.20 were shown. A, under low energy APCI; B, under high energy of APCI. Structure of peak 10 and its conversion under APCI.

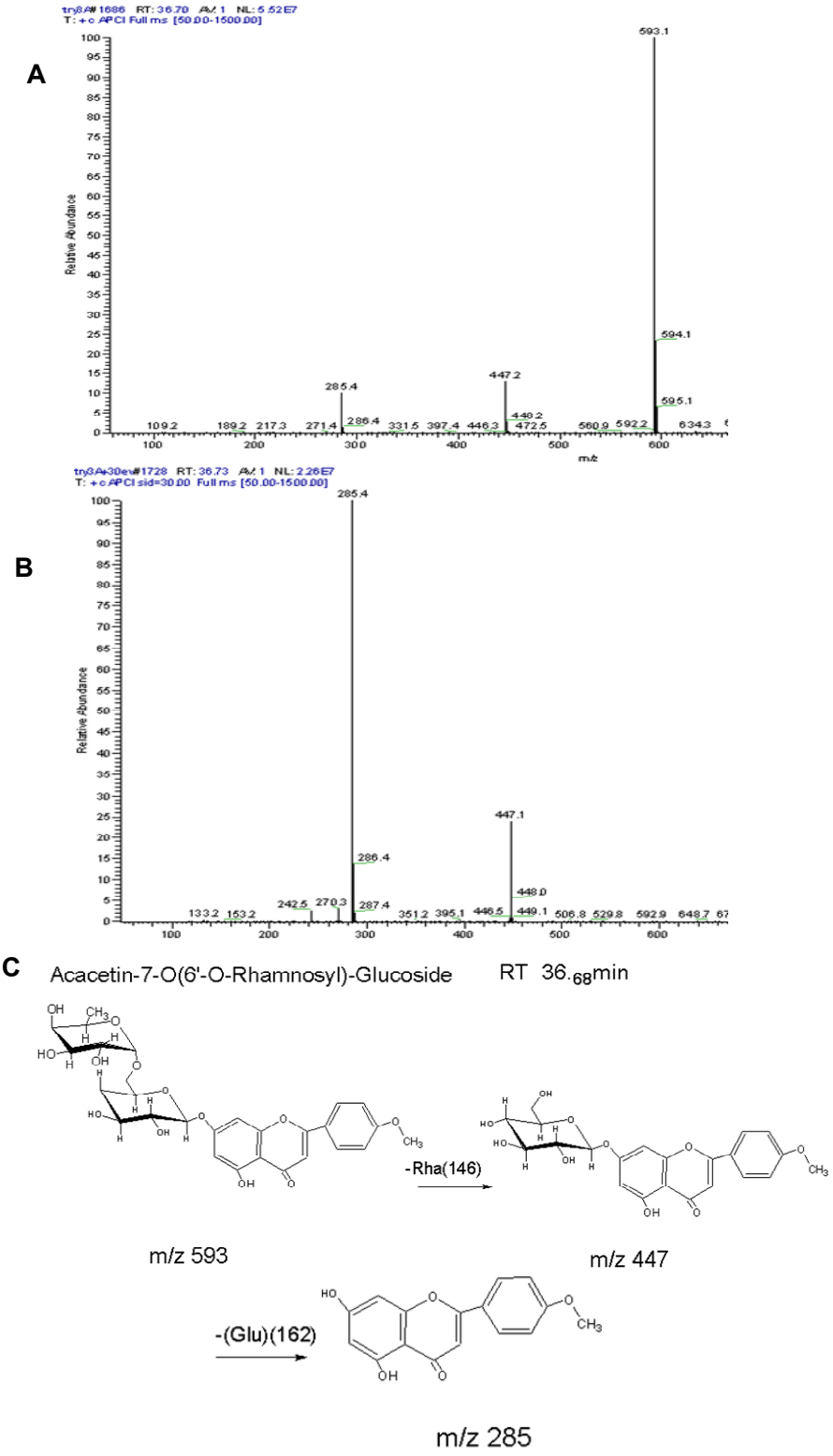


Figure 2.14 Structure elucidation of peak 11, RT 36.61 min

EtOAc fraction was separated on reverse phase-HPLC and detected by APCI. The mass spectrum of peak RT36.61 were shown. A, under low energy APCI; B, under high energy of APCI. Structure of peak 11 and its conversion under APCI.

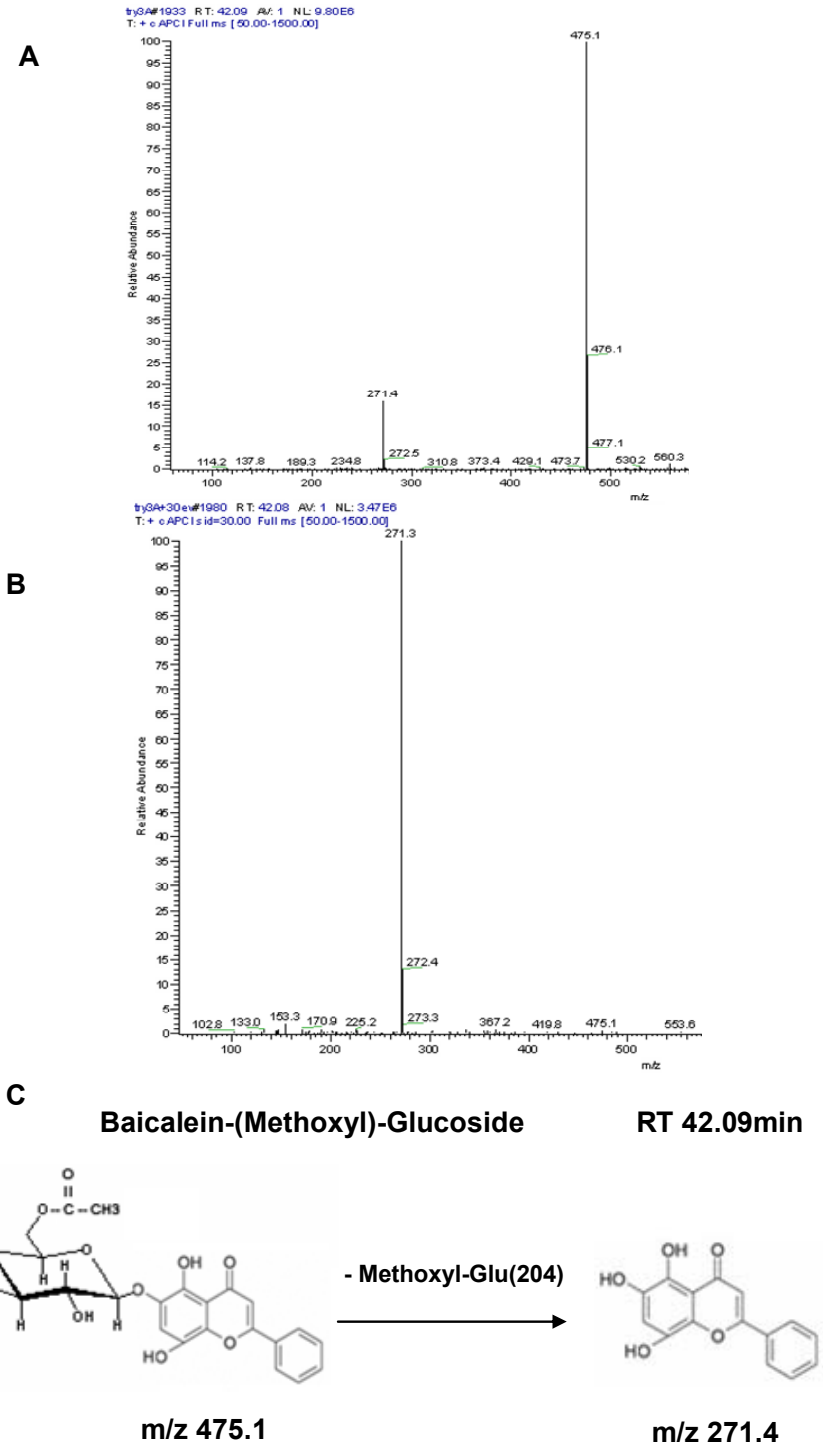


Figure 2.15 Structure elucidation of peak 12, RT 42.09min

EtOAc fraction was separated on reverse phase-HPLC and detected by APCI. The mass spectrum of peak RT42.09 were shown. A, under low energy APCI; B, under high energy of APCI. Structure of peak 12 and its conversion under APCI.

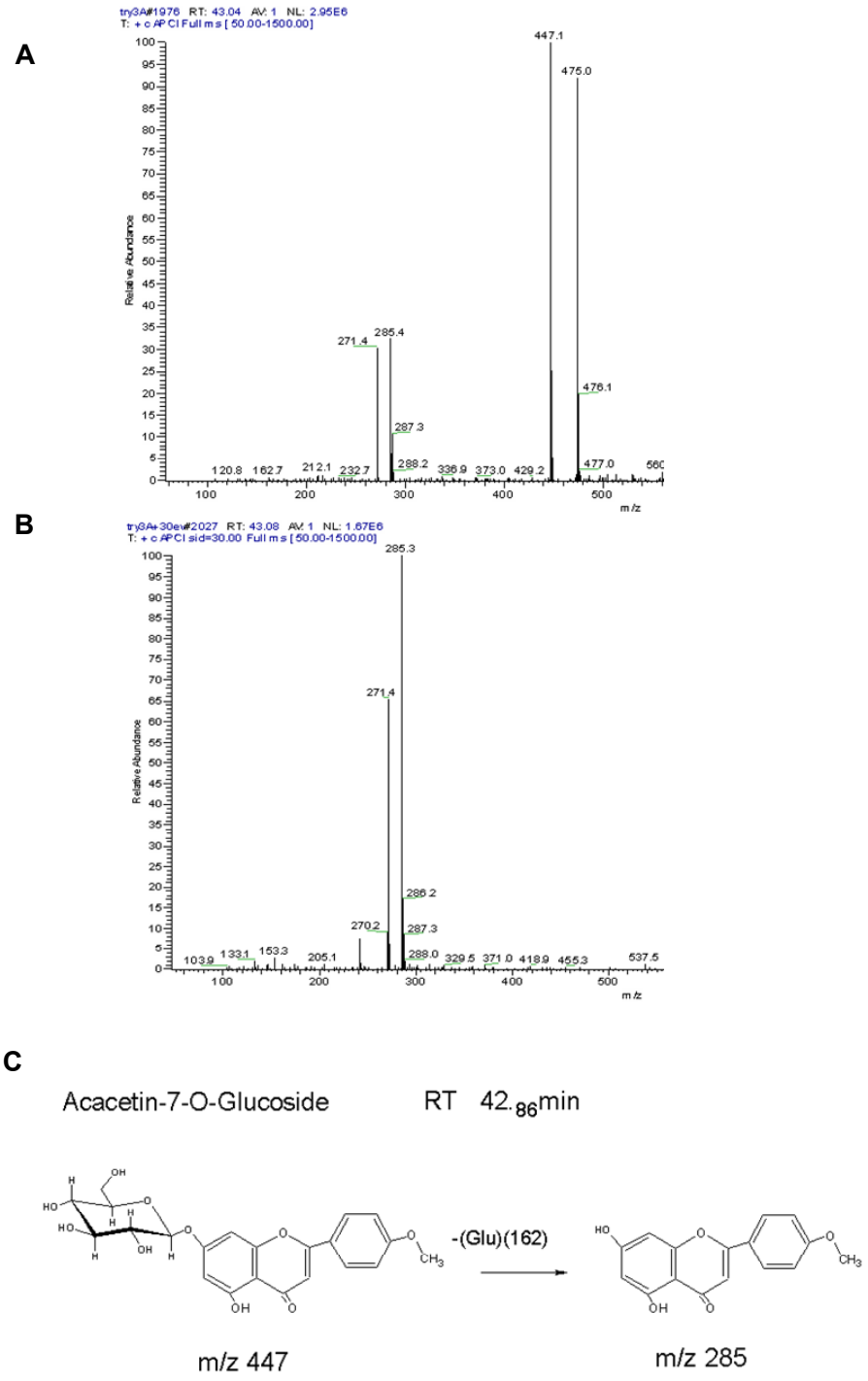


Figure 2.16 Structure elucidation of peak 13, RT 43.08min

EtOAc fraction was separated on reverse phase-HPLC and detected by APCI. The mass spectrum of peak RT43.08 were shown. A, under low energy APCI; B, under high energy of APCI. Structure of peak 13 and its conversion under APCI.

2.4 DISCUSSION

Flavonoids are a group of phytochemicals ubiquitiously present in plants. It has been well documented that flavonoids possess certain anti-tumor effects, including flavonoids from tea (Brown, 1999; Hibasami, 2000; Kinjo *et al.*, 2002), ginkgo biloba (Kim *et al.*, 2005d), grape (Ye *et al.*, 1999) and soybean (Kim *et al.*, 2004b) etc. In this study, the EtOAc extract of chrysanthemum was shown to have strong cytotoxicity on human cancer cells (Figure 2.2).

The components of this fraction were further identified using HPLC-MS and also made reference to various earlier reports (Hu *et al.*, 1994; Liu *et al.,* 2001; Lee *et al.*, 2003; Hu *et al.*, 2004). Twelve of the major peaks of this extract were identified as flavonoids (Figures 2.4-2.16). All are conjugated with either one or two sugar side chains.

Several flavonoids, which have not been reported previously in chrysanthemum, are also detected in the present study. We found four luteolin glycosides (only 1 was reported previously), three hesperetin glycosides (2 were reported previously) and two baicalein glycosides (1 was reported previously). However, apigenin-(4'-caffeoyl)-glucuronide, which was reported earlier, was not found in the present study. Instead, apigenin-methoxyl-glucoside was detected.

In summary, the EtOAc extract was found to be the most potent in its cytotoxic effect on human cancer cells. The flavonoids in the EtOAc fraction can be divided into 5 groups according to their aglycones. They are glycosides of luteolin, apigenin, hesperetin, baicalein and acacetin. Among them, luteolin glycosides and apigenin glycosides are the two major components in chrysanthemum EtOAc extract.

CHAPTER THREE

CYTOTOXICITIES OF FLAVONOIDS FROM CHRYSANTHEMUM

3.1 INTRODUCTION

The flower heads of chrysanthemum have been used as an herbal medicine as well as a popular beverage for centuries. Recently, it has been shown that the water extract of chrysanthemum exerted significant anti-tumor properties (Shen *et al.,* unpublished data). In order to find out the active components of chrysanthemum water extract, we obtained several fractions and then tested their cytotoxicity on human cancer cells (Figures 2.1 and 2.2). In the most potent EtOAc fraction, 13 flavonoids were identified as the major components (Figure 2.17). Thus, it is believed that chrysanthemum flavonoids are the major anti-cancer components of chrysanthemum water extract.

The anti-tumor effects of flavonoids have been well studied and extensively reviewed (Harborne and Williams, 2000). Many of them are capable of inhibiting tumor growth and/or inducing cancer cell apoptosis, which is characterized by characteristic morphological and biochemical changes including cell shrinkage, nuclear fragmentation, chromatin condensation, membrane blebbing and formation of apoptotic bodies (Hengartner, 2000; Kaufmann and Hengartner, 2001). In the previous Chapter, chrysanthemum flavonoids was shown to possess strong cytotoxicity in cancer cells, however, it remains to be determined whether they are capable of inducing apoptotic cell death in cancer cells.

Another important issue closely related to the biological effect of flavonoids is absorption. Most flavonoids in plants are conjugated with sugar substitute, named flavonoid glycosides. Absorption of flavonoid glycosides has been thought to occur in intestine after hydrolysis into their algycone forms (Griffiths and Barrow, 1972). The hydrolysis can be processed by microorganisms in intestine or oral cavity (Walle *et al.*, 2005). Once this hydrolysis occurs, the aglycones are absorbed more efficiently.

Recently, it was reported that flavonoid glycosides can also be absorbed directly (Hollman *et al.*, 1995). In contrary, intact flavonoid glycosides was not found in plasma (Sesink *et al.*, 2001). As shown in Chapter 2, most of the chrysanthemum flavonoids are glycosides of the five main aglycones. Luteolin glycosides and apigenin glycosides are the two major groups of chrysanthemum flavonoids, therefore luteolin and apigenin were the main focus of the subsequent studies.

The main aim of this chapter is to examine the anti-cancer property of chrysanthemum flavonoid extract and its major flavonoids, luteolin and apigenin. We found that chrysanthemum flavonoid extract (EtOAc fraction) and luteolin are strong inducers of apoptosis in colorectal cancer cell COLO205 via caspase activation.

3.2 MATERIALS AND METHODS

3.2.1 Regents and chemicals

Luteolin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), 4',6-diamidino-2-phenylindole (DAPI), Propidium iodide (PI), RPMI 1640 were obtained from Sigma (St. Louis, MO). Chrysanthemum flavonoid extract was prepared as described in Figure 2.1. Characterized Fetal Bovine Serum (FBS) was obtained from Hyclone (Logan,Utah). Apo-one Homogeneous Caspase-3/7 Assay Kit was obtained from Promega (Madison, WI). General caspase inhibitor z-VAD-fmk were purchased from Biomol (Plymouth Meeting, PA). Anti-caspase-3 antibody and CHAPS lysis buffer were purchased from Cell Signaling (Beverly, MA). Anti-PARP antibody was purchased from Pharmingen (San Diego, CA).

3.2.2 Cell lines and cell culture

Human colorectal cancer cells COLO205, HCT116 and HT29 were obtained from American Type Culture Collection (ATCC). COLO205 cells were routinely

maintained at 37° C in RPMI 1640 medium containing 10% filter-inactivated fetal bovine serum (FBS) in an atmosphere containing 5% CO_2. HCT116 and HT29 were maintained in McCoy 5A medium.

3.2.3 Assessment of cell viability using MTT assay

Cell growth was assessed by a MTT assay as described previously in Chapter 2 (Section 2.2.4)

3.2.4 Assessment of apoptosis using DAPI staining

The cells undergoing apoptosis were evaluated by chromatin condensation and nuclear shrinkage using 4',6-diamidino-2-phenylindole (*DAPI*) staining (Fuentes *et al.*, 2003). After various designated treatments, medium was removed and cells were fixed with 70% ethanol at room temperature for 10 min. Cells were then stained with 0.3 μg/mL DAPI (in PBS) at room temperature for 10 min and visualized under an inverted fluorescence microscope and photographed. For quantification, 200 cells were counted and the percentage of apoptotic cells was calculated.

3.2.5 Assessment of DNA content using flow cytometry.

After treatment, all cells were collected by trypsinization and fixed with ice-cold 70% ethanol. Before subject to flow cytometry, ethanol was removed from the fixed cells and then the cells were stained with 0.5 ml propidium iodide solution for 30 min at room temperature. 10,000 cells were counted by flow cytometer (Coulter Epics Elite ESP, Miami, FL, USA) using a filter with 488 nm excitation and 610 nm emission.

3.2.6 Caspase 3-like activity assay

Apo-one homogeneous caspase-3/7 assay kit (Promega) was used to measure the caspase 3-like activity according to manufacturer's protocol. Briefly, cells were plated on 96-well microplates. At the end of designated treatments, z-DEVD-

Rhodamine 110 was added into the cells as substrate and continued the incubation at 37°C for 1 h. The fluorescence intensity was then recorded using a spectrofluorimeter (Tecan) at excitation 485 nm and emission 535 nm. Cells treated by TNF (10ng/ml) with ActD (1 μg/ml) pretreatment were used as a positive control.

3.2.7 Western blotting

PARP cleavage and procaspase 3 cleavage were detected by western blotting. Equal amount of proteins were fractionated on SDS-polyacrylamide gel in the Mini-PROTEAN II system (Bio-Rad) and blotted onto PVDF membrane (Millipore). After blocked with 5% nonfat milk in TBST (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.1% Tween 20), the membrane was probed with various antibodies and developed with enhanced chemiluminescence (Pierce) using a Kodak Image Station (Kodak).

3.3 RESULTS

3.3.1 Cytotoxicity of chrysanthemum flavonoids on human cancer cells.

In Chapter 2, an EtOAc fraction was obtained from chrysanthemum water extract (Figure 2.1) and flavonoids have been identified as its major components (Figure 2.17). Further, our data showed the flavonoid fraction (Fraction B) exerted higher cytotoxicity than other fractions of chrysanthemum water extract (Figure 2.2), suggesting that flavonoids are the cytotoxic components of the chrysanthemum water extract. Here we aimed to further examine the cytotoxic effect of chrysanthemum flavonoids on various human cancer cell lines. As shown in Figure 3.1, the EtOAc fraction rich in chrysanthemum flavonoids exerted significant cytotoxicity on three human colorectal cancer cells HCT116, COLO205 and HT29.

3.3.2 Chrysanthemum flavonoid extract induces apoptosis in cancer cells

To examine whether the cytotoxicity of chrysanthemum flavonoids are due to induction of apoptosis, we then checked the morphological changes of HCT116 after 24 h treatment with 0.25 mg/ml chrysanthemum flavonoids. Under a normal light microscope, we observed that most cancer cells rounded up after treatments (upper panel of Figure 3.2A). Using DAPI staining, which specifically stains DNA, we could see that a large portion of cells showed a chromatin condensation (lower panel of Figure 3.2A). The above data were quantified and presented in Figure 3.2B, chrysanthemum flavonoids induced around 60% and 90% apoptosis at 0.25 mg/ml and 0.5 mg/ml, respectively, which is consistent with the dose response pattern of the cytotoxicity detemined by MTT assay (Figure 3.1). Significant apoptosis was also observed in COLO205 and HT29 cells after the Fraction B treatment (data not shown).

3.3.3 Chrysanthemum flavonoid causes apoptosis by inducing a caspase cascade

Caspases are the major executors of apoptosis (Strasser *et al*., 2000). To test whether the apoptosis induced by chrysanthemum flavonoids was through caspase activation, we then examined the caspase-3 activation as well as the cleavage of its substrate protein PARP using Western blot in HCT116 cells. The caspase-3 as well as PARP was cleaved significantly in cells treated with chrysanthemum flavonoids at 0.25 mg/ml and 0.5 mg/ml (Figure 3.3).

More importantly, the cleavage of caspase-3 and PARP induced by chrysanthemum flavonoids were completely prohibited by a general caspase inhibitor, z-VAD-fmk (Figure 3.3), suggesting that the apoptosis induced by chrysanthemum flavonoids was mediated via caspase activation.

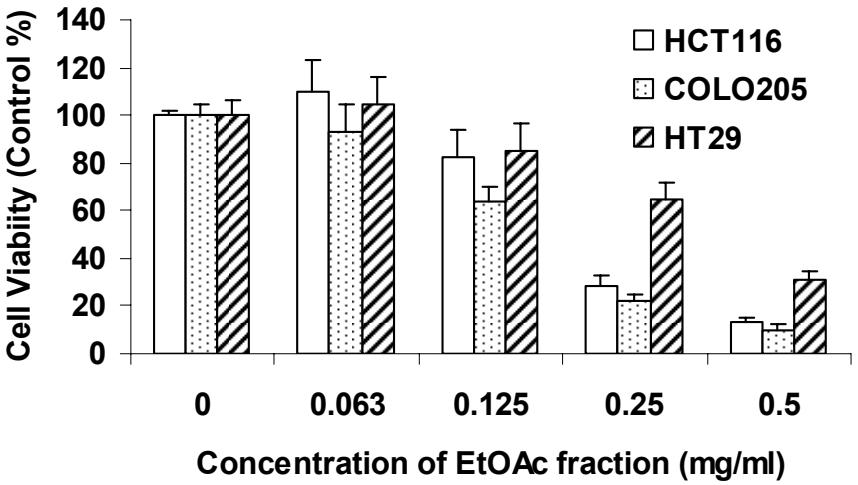


Figure 3.1 Cytotoxicity of EtOAc extract on human colorectal cancer cells

Human cancer cell HCT116, COLO205 and HT29 were plated on 96-well plates. After 24 h, the cells were treated with indicated concentration of EtOAc extract for 24 h. At the end of treatment, cell viabilities were determined by MTT assay. Cell viability was expressed as means of three experiments ± SE

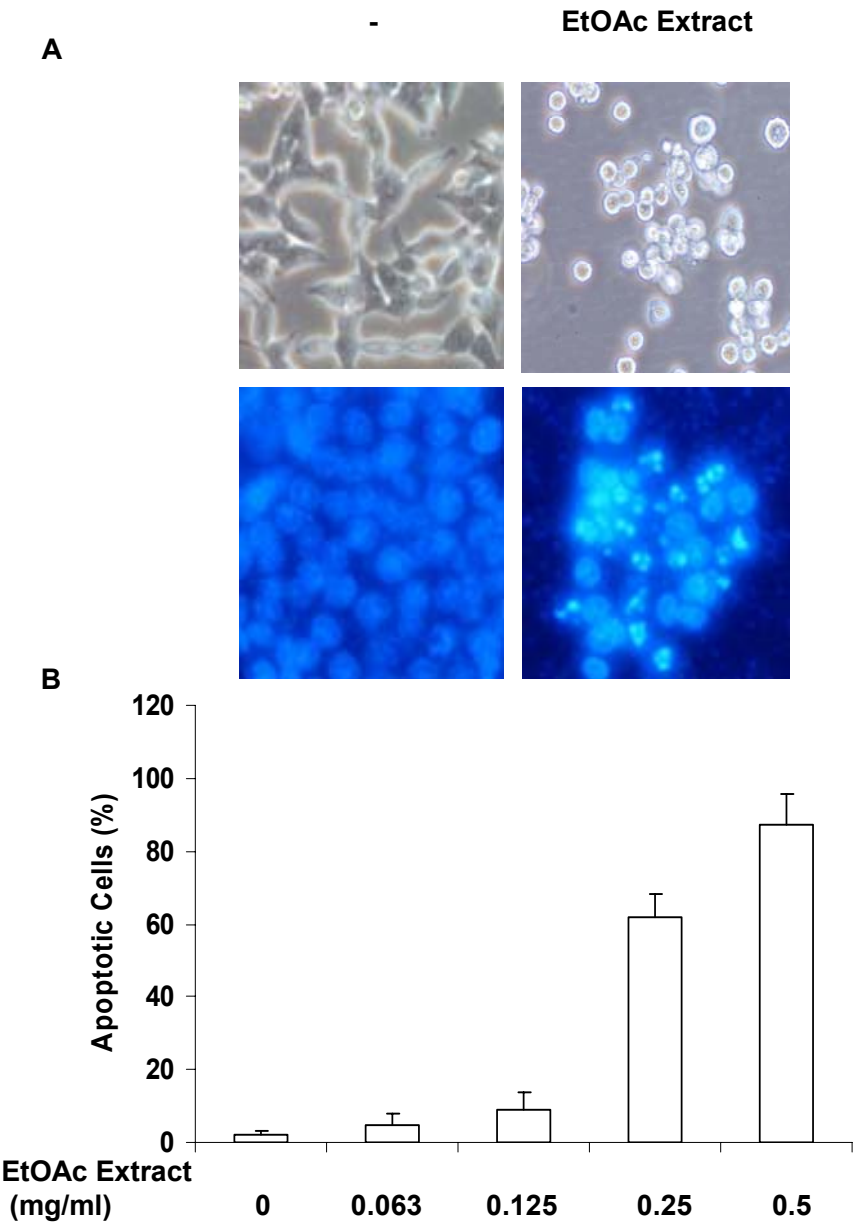


Figure 3.2 EtOAc extract induces apoptosis in cancer cells HCT116

A, HCT116 cells were treated with 0.5 mg/ml EtOAc fraction for 24 h, cells were observed under a light microscope directly (upper panel) or under UV after DAPI staining (lower panel); B, HCT116 cells were treated with indicated concentration of EtOAc fraction for 24 h, apoptotic cells were counted after DAPI staining.

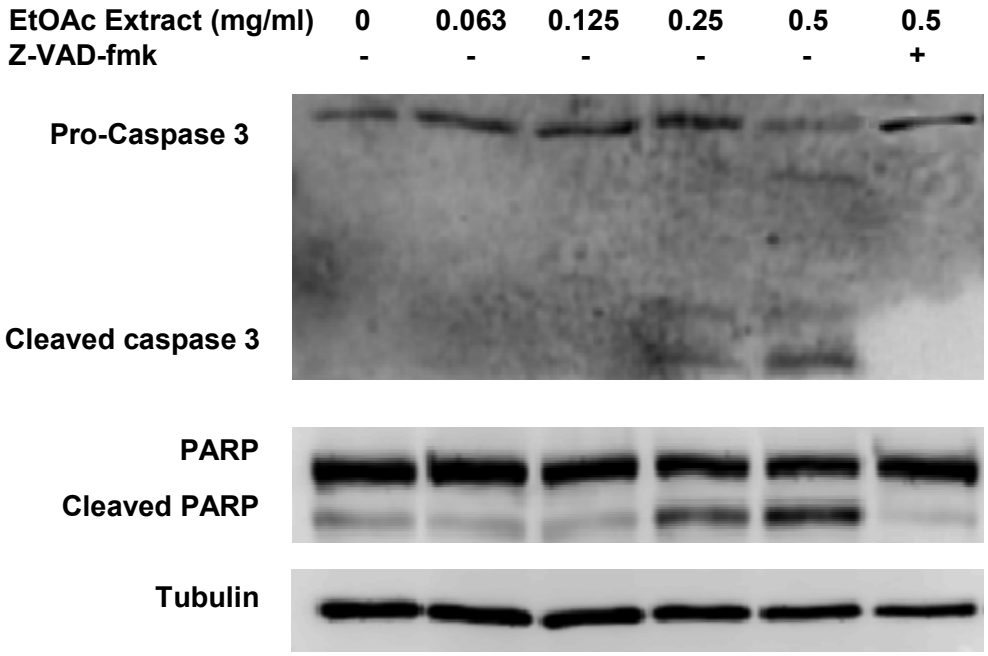


Figure 3.3 EtOAc extract causes apoptosis by inducing caspase cascade in HCT116

HCT116 cells were treated with indicated concentration of EtOAc fraction for 24 h, cells were collected for Western blotting using anti-caspase-3 and anti-PARP antibody. Tubulin was used as loading control

3.3.4 Cytotoxicity of luteolin and apigenin in human cancer cells.

Our chemical assay shows only flavonoid glycosides are detected in the EtOAc fraction, with apigenin and luteolin as the major components. Physiologically, when ingested the sugar moiety of their glycosides are also likely to be removed by colonic bacteria and left with the aglycones. We then tested the cytotoxicity of luteolin and apigenin, the two major flavonoid aglycones in chrysanthemum, on several human colorectal cancer cell lines using the MTT assay. Both of them showed significant cytotoxicity in all three cancer cell lines in a concentration-dependent pattern (Figure 3.4). Interestingly, both of them showed stronger cytotoxicity in COLO205 and HCT116 cells than in HT29 cells, similar to the cytotoxicity effect of the chrysanthemum flavonoids extract (Figure 3.1). To be noted, luteolin showed stronger cytotoxicity than apigenin in all three cell lines, especially on COLO-205 cells. Therefore in the subsequent studies we decided to focus on the anti-tumor properties of luteolin.

3.3.5 Luteolin induces apoptosis in COLO205 but not in HCT116 and HT29 cells.

We next tested whether luteolin exerts its cytotoxicity through induction of apoptosis in all three cancer cell lines. Since the COLO205 cells are semi-adherent, we had to use flow cytometry after propidium iodide staining, instead of DAPI staining, to quantify the extent of apoptosis. Cells undergoing apoptosis will lose DNA fragments after fixation and washing. So, the hypodiploid proportion (sub-G1) is generally regarded as apoptotic cells (Yang *et al.*, 2000a). As shown in Figure 3.5A, luteolin induced apoptosis in COLO205 cells in a dose-dependent pattern. Luteolin at 40 μM induced more than 70% apoptotic cell death after 24 h. Under a normal light microscope, COLO205 cells were undergoing typical apoptosis, as judged by the cell shrinkage and formation of apoptotic bodies (Figure 3.6).

To our surprise, luteolin treatment, even at higher doses, was unable to induce evident apoptosis in both HCT116 and HT29 cells (Figure 3.5B), suggesting that other mechanism may be involved in the cytotoxicity in these two cells. The molecular mechanisms involved in the cytotoxicity of luteolin on these two cell lines remain to further be elucidated.

To confirm the form of cell death induced by luteolin in COLO205 cells, we then tested the changes of a typical apoptosis marker, PARP cleavage. Figure 3.7A showed that luteolin caused significant PARP cleavage in a dose-dependent manner. The time course data showed that the cells started to undergo apoptosis after 18 h. (Figure 3.7B)

3.3.6 Luteolin induced apoptosis in COLO205 by activating caspase-3

To examine whether caspase activation is involved luteolin-induced apoptosis, we used western blot to detect the activation of caspase-3. As shown in Figure 3.8, two cleaved bands (21 kDa and 17 kDa), the active forms of caspase-3, were detected after 18 h and 24 h treatment with luteolin. In addition, Apo-ONE caspase 3/7 homogenous assay kit was used to detect the caspase-3-like activity. Significant caspase-3-like activity was detected after 18 h treatment by luteolin at 40 μM (Figure 3.9), which was consistent with the temporal pattern of caspase-3 cleavage (Figure 3.8) and apoptotic cell death (Figure 3.5A).

Next, we pretreated the cells with a general caspase inhibitor, z-VAD-fmk and found that the percentage of sub-G1 cells measured by flow cytometry was almost completely blocked by z-VAD (Figure 3.10). In addition, z-VAD-fmk could also inhibit cell shrinkage and formation of apoptotic bodies induced by luteolin (data not shown). Therefore, it is believed that luteolin induces apoptosis in COLO205 cells through activating caspases.

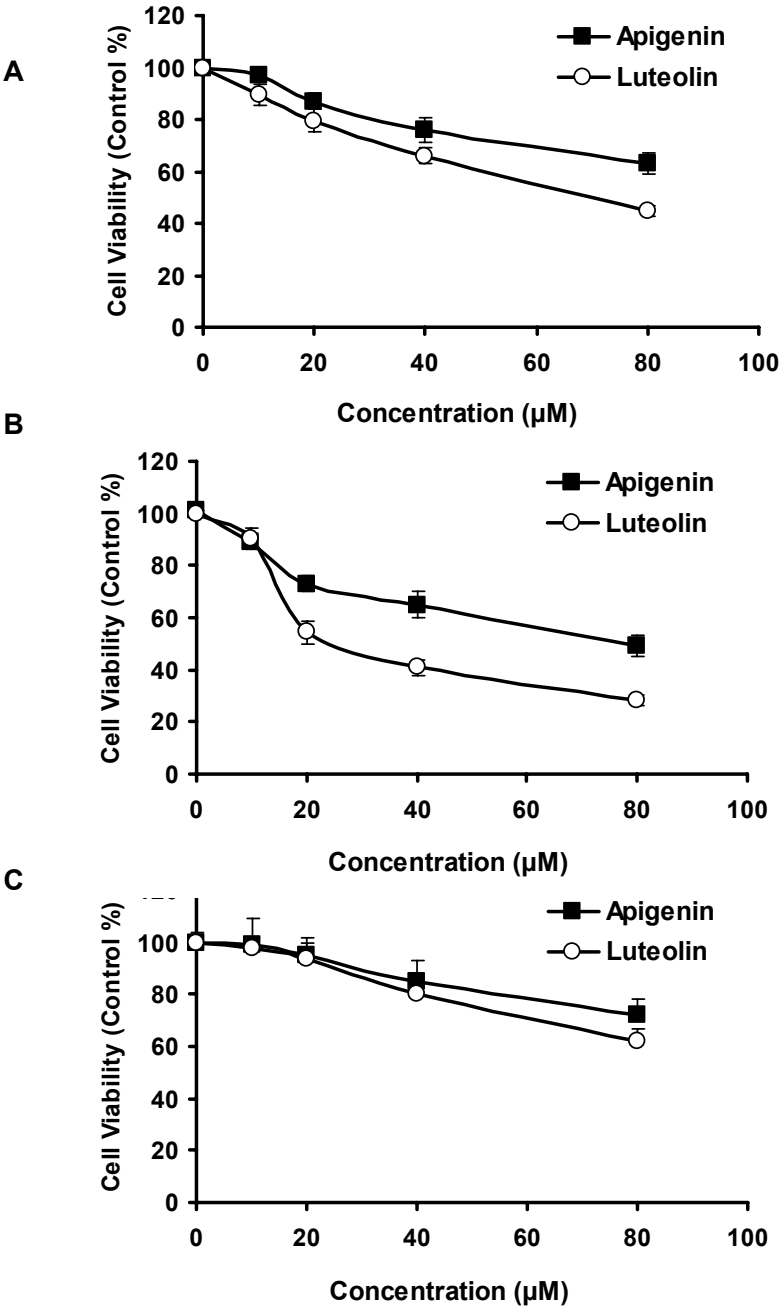


Figure 3.4 Cytotoxicities of luteolin and apigenin in human cancer cells
Human cancer cell HCT116 (A), COLO205 (B) and HT29 (C) were plated on 96-well plates. After 24 h, the cells were treated with indicated concentration of apigenin or luteolin for 24 h. At the end of treatment, cell viabilities were determined by MTT assay. Cell viability was expressed as means of three experiments ± SE

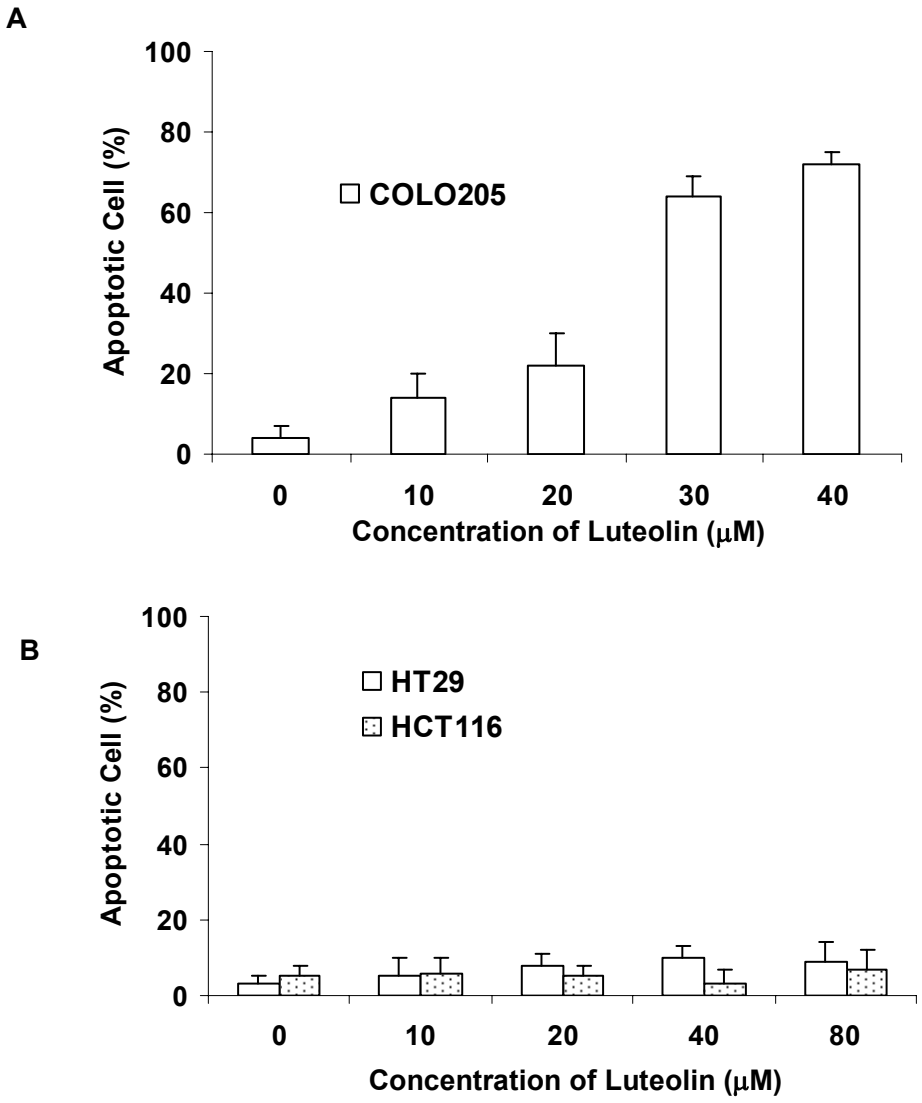


Figure 3.5 Luteolin induces apoptotic cell death in COLO205 cells but not in HCT116 or HT29 cells

COLO205, HCT116 and HT29 cells were plated on 24-well plates for 24 hours prior to various treatments by luteolin. At the end of treatment, cells were collected by trypsinization, fixed with ice-cold 70% ethanol and stained with PI solution. Samples were subject to flow cytometry and 10,000 cells were counted.

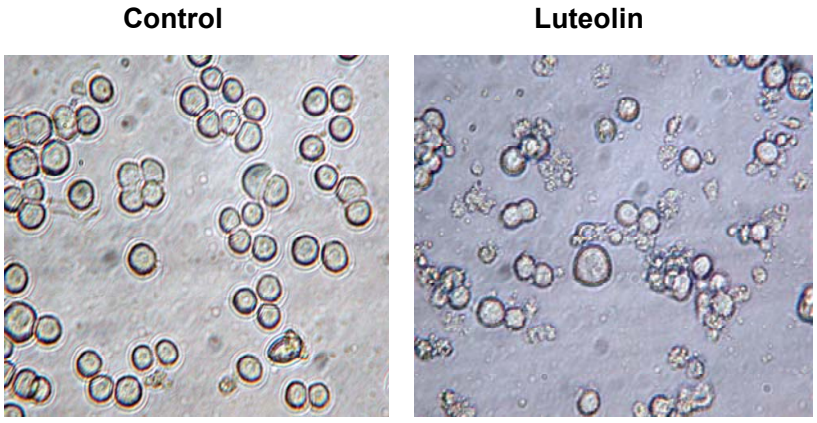


Figure 3.6 Morphological change of COLO205 after luteolin treatment

COLO205 cells were treated with 40 μM luteolin for 24 h, cells were observed under a light microscope.

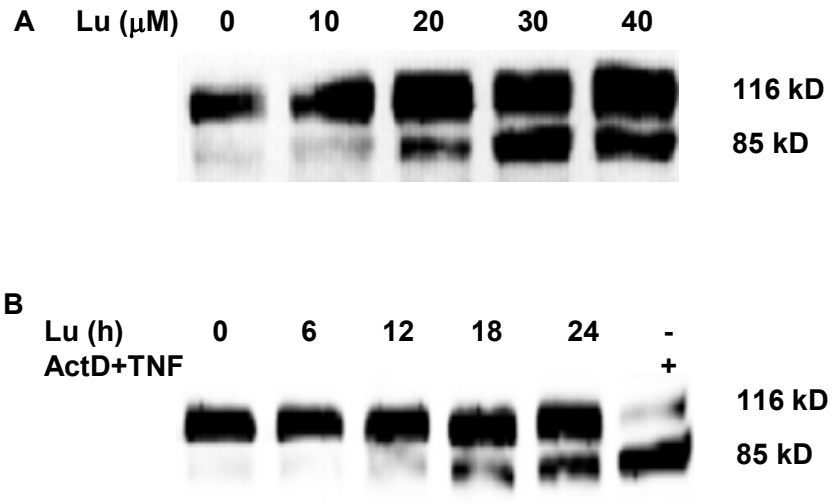


Figure 3.7 Luteolin induces PARP cleavage time- and dose-dependently in COLO205 cells

A, COLO205 cells were exposed to various concentration of luteolin for 24 h; B, COLO-205 Cells were exposed to 40 μM luteolin for indicated periods. Cells were collected for Western blotting. Anti-PARP antibody was used to detected both PARP (116 kD) and cleaved PARP (85 kD). ActD (1 μg/ml) with TNF (10 ng/ml) was used a positive control.

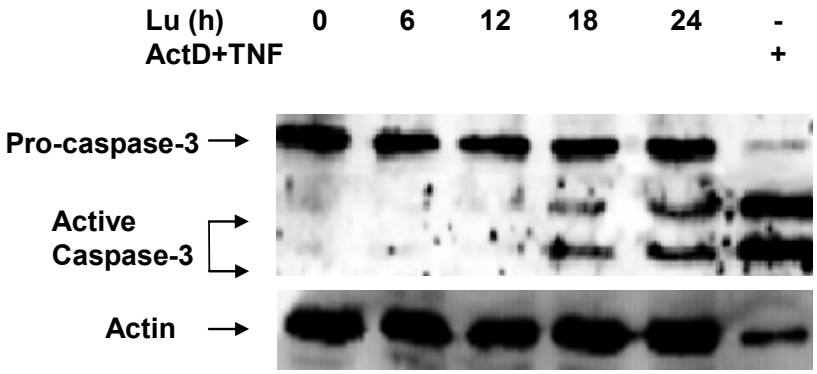


Figure 3.8 Luteolin induces caspase-3 cleavage in COLO205 cells

COLO205 cells were exposed to 40 μM luteolin for indicated periods and collected for Western blotting. Anti-caspase-3 antibody was used to detected both pro-caspase-3 PARP (32 kD) and cleaved caspase-3 (21and 17 kD). ActD (1 μg/ml) with TNF (10 ng/ml) was used a positive control. and actin was used as loading control.

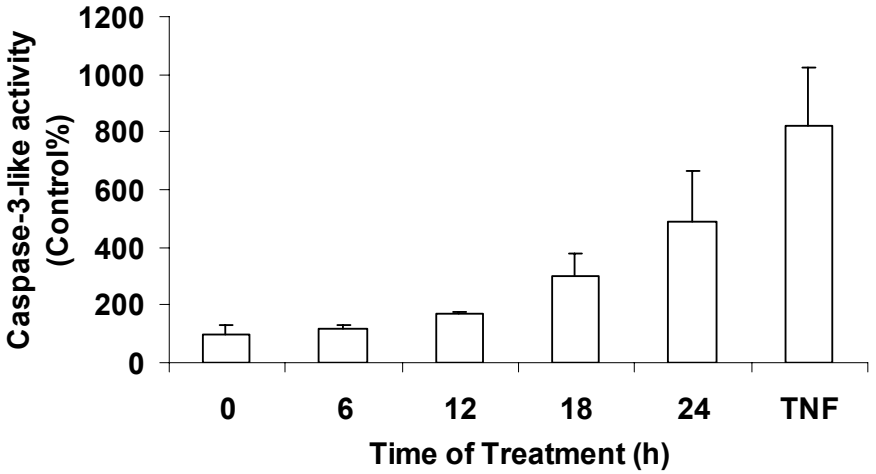


Figure 3.9 Luteolin activates caspase-3 like activity in COLO205

COLO205 cells were exposed to 40 μM luteolin for indicated periods and subject to caspase-3 like activity assay using Apo-one caspase-3 assay kit. Cells treated with ActD (1 μg/ml) and TNF (10 ng/ml) was used a positive control.

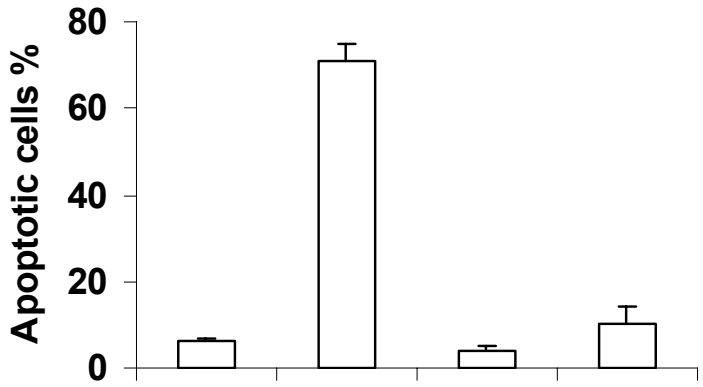


Figure 3.10 z-VAD-fmk inhibits cell death induced by luteolin in COLO205 cells

COLO205 cells were pretreated with z-VAD-fmk (25 μM) for 30 min before exposure to 40 μM luteolin for 24 h. Cells were then collected for detection of apoptosis using flow cytometry after PI staining.

3.4 DISCUSSION

In the previous Chapter, we found that flavonoids were the major cytotoxic components from water extract of chrysanthemum. In this Chapter, we further investigated their cytotoxicity in several cancer cell lines and the mechanisms involved.

We first tested the EtOAc extract (Fraction B), a mixture of flavonoids, on several colorectal cancer cells. We found that this fraction at 0.25 mg/ml or 0.5 mg/ml showed significant cytotoxicity in all three cancer cell lines studied (Figure 3.1). Its cytotoxicity is similar to that of several other plant flavonoid extracts, such as ginkgo biloba extract (EGb761) (Kim et al., 2005d) or grape seed extract (Ye *et al.*, 1999) and soybean extract (Kim *et al.*, 2004b), although their compositions are different. Further studies showed that the flavonoid extract induced apoptosis in these cancer cells, as evidenced by chromatin condensation and PARP cleavage (Figure 3.2). The inhibition of a caspase inhibitor on the apoptosis further proved that the apoptosis was through activating caspases (Figure 3.3).

To be noted, the EtOAc extract exerted higher cytotoxicity in wild type p53 cancer cells HCT116 and COLO205 than in mutant p53 cells HT29 (Figure 3.1). Similar results were found when luteolin and apigenin, the two major flavonoids of chrysanthemum, were tested (Figure 3.4). Therefore, it appears that the cytotoxic effect of chrysanthemum flavonoids requires the presence of a functional p53.

Although luteolin and apiginin are similar in their structure (luteolin contains one more hydroxyl group than apigenin, Figure 1.3), luteolin showed higher cytotoxicity than apignin in all three cell lines, especially in COLO205 cells (Figure 3.4). In addition, as suggest by Figure 2.3, the concentration of luteolin glycosides was higher that that of apigenin glycosides in chrysanthemum. Therefore, it is

believed that luteolin plays a more important role in the anti-cancer potential of chrysanthemum, a fact that leads us to focus on luteolin in our subsequent studies.

Data from this part of our study confirmed that luteolin is capable of inducing apoptosis in COLO205 cells as evidenced by DNA fragmentation, PARP cleavage and formation of apoptotic bodies (Figures 3.5, 3.6 and 3.7). The apoptosis was through activating caspases as it was completely inhibited by a pan-caspase inhibitor, z-VAD-fmk (Figure 3.8). Such a finding is basically consistent with some earlier reports in which luteolin can induce apoptosis in several cancer cell lines, including human epidermoid carcinoma A431(Huang et al., 1999b), human leukemia HL-60 (Ko *et al.,* 2002; Cheng *et al.*, 2005a) and U937 (Monasterio *et al.*, 2004), pancreatic tumor cell MiaPaCa-2 (Lee *et al*., 2002). Interestingly, luteolin fails to induce apoptosis in HCT116 or HT29 cells (Figure 3.5), although the growth of both cancer cells was inhibited by luteolin (Figure 3.4). It is thus believed that luteolin may exert anti-cancer effects in various cancer cells via different mechanisms.

In summary, we found that both the flavonoids extract of chrysanthemum and luteolin, one of the major flavonoid aglycones of chrysanthemum, can induce apoptosis in cancer cells by activating caspases. Such a finding lays a foundation for further study on the cancer therapeutic potential of luteolin.

CHAPTER FOUR

LUTEOLIN SENSITIZES TUMOR NECROSIS FACTOR-ALPHA-INDUCED APOPTOSIS IN HUMAN CANCER CELLS

4.1 INTRODUCTION

Tumor necrosis factor (TNF) is a proinflammatory cytokine with a wide spectrum of functions in many biological processes, including cell growth and cell death, development, oncogenesis, immunity, inflammatory and stress responses (Tracey and Cerami, 1993). The bioactivities of TNF are mainly elicited by TNF receptor 1 (TNFR1), via the following three distinct signaling pathways: (i) NF-κB, (ii) mitogen-activated protein kinase c-Jun N-terminal kinase (JNK), and (iii) a caspase cascade (Chen and Goeddel, 2002). Upon TNF binding, the trimerized TNFR1 first recruits a key adaptor protein TNF receptor-associated death domain (TRADD), which then binds to cytosolic proteins such as receptor-interacting protein (RIP), TNF receptor-associated factor 2 (TRAF2) and Fas-associated death domain (FADD). It has been well established that the recruitment of FADD into the signaling complex leads to the activation of a caspase cascade and apoptosis (Chinnaiyan *et al*., 1995; Yeh *et al*., 1998). On the other hand, the recruitment of RIP and TRAF2 results in the activation of NF-κB which mainly functions as a cell survival mechanism to protect cells against TNFα-induced apoptotic cell death (Ting *et al.*, 1996; Reinhard *et al.,* 1997; Kelliher *et al*., 1998). As TNFα activates both cell death and cell survival pathways simultaneously, most cancer cells are resistant to TNFα-induced apoptosis and thus inhibition of NF-κB activation becomes a popular strategy to enhance the sensitivity of cancer cells to apoptosis mediated by TNF family proteins (Baldwin, 2001; Yamamoto and Gaynor, 2001).

NF-κB is a ubiquitous transcription factor consisting of heterogenous dimeric proteins containing a Rel homology domain (Karin and Delhase, 2000). In resting cells, NF-κB binds to inhibitor of κB (IκB) proteins and localizes in cytoplasm. In response to TNFα-TNFR1 ligation, the activated IκB kinase (IKK) phosphorylates

IκB, which results in the degradation of IκB through ubiquitination. The released NF-κB then translocates from cytoplasm to nuclei and binds to the promoter regions of its target genes to regulate the gene expression. A number of genes such as A20, IAPs, cellular FLICE inhibitory protein (c-FLIP), TRAF1 and TRAF2 have been identified as NF-κB-regulated anti-apoptotic genes (Krikos et al., 1992; Wang et al., 1998; Micheau et al., 2001). In contrast to the well-established anti-apoptotic role of NF-κB, the exact function of JNK in TNF-mediated apoptosis remains largely controversial (Liu *et al*., 1996; Natoli *et al*., 1997). Recently it has been demonstrated that prolonged JNK activation by the suppression of NF-κB activity promotes TNFα-induced apoptosis (De Smaele *et al.,* 2001; Tang *et al*., 2001). It appears that the exact function of JNK in TNF-induced apoptosis depends on a number of factors such as cell type and/or the presence of other signaling pathways such as NF-κB activation (Karin and Lin, 2002).

Flavonoids are a group of natural polyphenolic compounds widely distributed in the plant kingdom. The bioactivities of flavonoids have been extensively studied, including their antioxidant, anti-inflammatory and anti-cancer activities (Ross and Kasum, 2002). The anti-cancer activity of flavonoids has been well proven in epidemiological investigation and in animal studies (Birt *et al*., 2001;Yang *et al*., 2001). Luteolin, 3', 4', 5, 7-tetrahydroxyflavone, is a flavonoid which is commonly found in many types of fruits and vegetables. We have previously identified luteolin as one of the major chrysanthemum flavonoids, which are responsible for the anticancer effects of chrysanthemum (Chapter 2). We then proved that luteolin can suppress the growth of several cancer cell lines (Chapter 3). It has been reported that luteolin can inhibit LPS-induced NF-κB activation in fibroblasts or macrophages (Xagorari et al., 2002; Kim et al., 2003b). As inhibition of NF-κB activation becomes

a popular strategy to enhance the sensitivity of cancer cells to the apoptosis mediated by TNF family proteins (Baldwin, 2001; Yamamoto and Gaynor, 2001), we naturally ask whether luteolin can synergistically enhance TNF-induced apoptosis in cancer cells.

In this chapter, we reported that luteolin significantly sensitizes TNFα-induced apoptosis in a number of human cancer cell lines. Such sensitization is closely associated with its inhibitory effect on NF-κB activation, resulting in down-regulation of some key anti-apoptotic genes such as *A20* and *c-IAP1*. Luteolin pretreatment also leads to augmented and prolonged JNK activation induced by TNFα, a process that is proven to be critical in the sensitization effect of luteolin to TNFα-induced apoptosis.

4.2 MATERIALS AND METHODS

4.2.1 Cell culture and treatment.

Human colorectal cancer cells COLO205, HCT116 were obtained from ATCC and maintained in RPMI1640 medium (Sigma) supplemented with 10% fetal bovine serum (FBS) (Hyclone) and antibiotics. Human cervix cancer cells HeLa (from ATCC) were maintained in Dulbecco's modified Eagle's medium (Sigma) containing 10 % fetal bovine serum and antibiotics. Treatment details with luteolin (Sigma) and TNFα (Sigma) were illustrated in figure legends.

4.2.2 Measurement of cell death and apoptosis

Percentage of the hypodiploid cells or sub-G1 cells were measured as a general parameter for cell death as described previously (Yang et al., 2000a). Various approaches were used to assay the specific parameters of apoptosis, including (i) poly(ADP-ribose) polymerase (PARP) cleavage by Western blot; (ii) DNA fragmentation using agarose gel electrophoresis (McGahon *et al.*, 1995) and (iii)

morphological changes after cells were stained with acridine orange (AO) and ethidium bromide (EB) (Lin *et al.*, 1999).

4.2.3 Caspase 3-like and caspase-8 activity assay

Apo-one homogeneous caspase-3/7 assay kit (Promega) was used to measure the caspase 3-like activity according to manufacturer's protocol. Briefly, cells were plated on 96-well microplates. At the end of designated treatments, z-DEVD-Rhodamine 110 was added into the cells as substrate and continued the incubation at 37°C for 1 h. The fluorescence intensity was then recorded using a spectrofluorimeter (Tecan) at excitation 485 nm and emission 535 nm. For the measurement of caspase 8 activity, cells were collected, pelleted and lysed in lysis buffer (10 mM Hepes, pH 7.4, 42 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, and 0.1% Triton X-100). The whole cell lysate containing 50 μg protein was incubated with 5 μM Ac-IETD-AMC (Biomol) in a total volume of 200 μl of reaction buffer (50 mM HEPES, pH 7.0, 10% glycerol, 0.1% CHAPS, 2 mM EDTA, 2 mM dithiothreitol) at 37°C for 1 h. The fluorescence intensity of released AMC was then quantitated using a spectrofluorimeter (excitation 390 and emission 510 nm). Caspase activities were presented as relative values of the fluorescence intensity over the control group.

4.2.4 Transient transfection

HeLa cells were transiently transfected with either pcDNA (Clontech), myc-A20 (kindly provided by Dr. A Ting, Mount Sinai School of Medicine, NY), HA-c-IAP1, CrmA, or dominant negative forms of both HA-JNKK1 and HA-JNKK2 (kindly provided by Dr. ZG Liu, NIH), using the Lipofectamine transfection reagent (Invitrogen). A red fluorescent protein expression vector (pDsRed, Clontech) was co-transfected as a transfection marker. After 24 h of transfection, the cells were pretreated with luteolin (40 μM × 2 h) followed by TNFα (15 ng/ml × 24 h). Cell

death was determined by morphological changes examined under an inverted fluorescent microscope.

4.2.5 NF-κB luciferase reporter assay

COLO205 cells were transiently transfected with the NF-κB-dependent luciferase reporter construct and β-gal construct (Clontech) using the Lipofectamine and PLUS transfection reagent according to the manufacturer's protocol (Invitrogen). Luciferase activity was determined using the luciferase assay system (Promega) and normalized with the-galactosidase enzyme activity for transfection efficiency.

4.2.6 Preparation of whole cell lysate, cell fractionation, immunoprecipitation and western blot

Whole cell lysate was obtained using M2 lysis buffer (20 mM Tris, pH 7.4, 0.5% NP-40, 250 mM NaCl, 3 mM EDTA, 3 mM EGTA, 2 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 20 mM β-glycerol phosphate, 1 mM sodium vanadate, and 1 μg/ml leupeptin) (Wu *et al.*, 2002). Cytosol and nuclear fractions were prepared based on a protocol described previously (Kim et al., 2003b). The immunoprecipitation experiment was performed using CBP antibody for detecting CBP–p65 interaction, based on a report method (Hehner *et al.*, 1998). For Western blot, equal amount of proteins were fractionated on SDS-polyacrylamide gel in the Mini-PROTEAN II system (Bio-Rad) and blotted onto PVDF membrane (Millipore). After blocked with 5% nonfat milk in TBST (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.1% Tween 20), the membrane was probed with various antibodies and developed with enhanced chemiluminescence (Pierce) using a Kodak Image Station (Kodak).

4.2.7 Electrophoretic Mobility Shift Assay (EMSA)

The DNA binding activity of nuclear proteins was tested according to established method with modifications (He and Ting, 2002). NF-κB consensus

oligonucleotides (5'-AGTTGAGGGGACTTTCCCAGGC-3' and 3'-TCAACTCCCCTGAAAGGGTCCG-5') (Promega) were labeled with p32-γATP using T4 kinase (Invitrogen). Equal amounts of nuclear protein (5 μg) were incubated with 100,000 cpm labeled NF−κB oligonucleotides in 5 × reaction buffer (100 mM HEPES/KOH, pH 7.9, 20% glycerol, 1 mM dithiothreitol, and 300 mM KCl) for 30 min at room temperature, in the presence of 2 μg poly(dI-dC) and 2 μg bovine serum album in a total volume of 20 μl. The DNA-protein complexes were resolved on a 5% polyacrylamide gel. Gels were then dried and exposed to an X-ray film (Kodak) at –80°C overnight.

4.2.8 RNA extraction and RT-PCR

RNA extraction was carried out using a total RNA extraction kit (Purescript), following the instructions from the manufacturer. Five μg of total RNA from each sample were subjected to reverse transcription using M-MLV reverse transcriptase (Promega). For PCR, the amplification reaction was carried with 200 pmol of each primer, 200 μM of each dNTPs, and 0.5 units of Tag DNA polymerase II (Promega). The PCR conditions were optimized to achieve exponential amplification in which the PCR product formation is proportional to the starting cDNA. The primers of human A20 (Sonoda *et al.*, 2000), c-IAP1, c-IAP2 (Petak *et al.*, 2000), c-FLIPL, c-FLIPS (Mafune *et al.*, 1999) and glyceraldehydes-3-phosphate dehydrogenase (G3PDH) (Denecker *et al.*, 2001) were based on literature. After PCR, products were size fractionated using 1.8% agarose gel and visualized by ethidium bromide staining.

4.2.9 Statistical Analysis

The numeric data are presented as mean ± SD from at least three sets of independent experiments and were examined using student's t test. $p < 0.05$ was considered statistically significant.

4.3 RESULTS

4.3.1 Luteolin sensitizes TNFα-induced cell death in cancer cells

Majority of cancer cells are resistant to TNFα without the blockage of gene transcription and *de novo* protein synthesis. In this study, human colorectal cancer COLO205 cells were found to be resistant to TNFα-induced cytotoxicity, as demonstrated by the lack of sub-G1 cells determined using flow cytometry (Figure 4.1). Similarly, no evident cell death was observed when cells were treated with luteolin alone (40 μM × 12 h). We next tested the combined effect of luteolin and TNFα and discovered that pretreatment with luteolin (40 μM × 2 h) greatly sensitized and accelerated COLO205 cells to TNFα-induced cell death. As shown in Figure 4.1, more than 60% of cell death occurred as early as 6 h with luteolin plus TNFα. The sensitizing effect of luteolin on TNFα-induced cell death was also found to be dose-dependent. Similar sensitization effect by luteolin was also found in another human colorectal cancer cells HCT116 and human cervix cancer HeLa cells (Figure 4.1), suggesting that the sensitization of luteolin to TNFα -induced cell death may apply to a wide spectrum of cancer types. Furthermore, we noted that the sequence of treatment is important. No sensitization was found when luteolin was added 2 h after TNFα exposure (luteolin post-treatment) (Figure 4.2), indicating that the sensitization effect by luteolin is probably achieved through the blockage of certain cell survival signals elicited by TNFα.

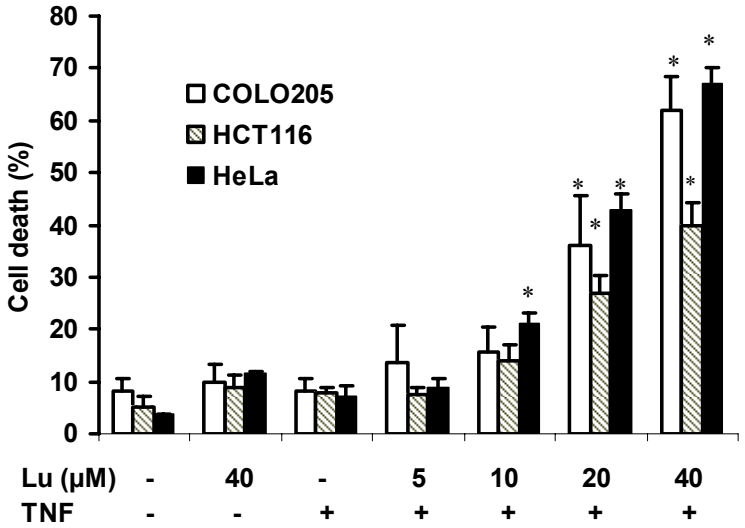


Figure 4.1 Luteolin pretreatment sensitizes TNFα-induced cell death in cancer cells.

Cells were pretreated with indicated concentrations of luteolin for 2 h, followed by TNFα (15 ng/ml) for another period of time (6 h for COLO205, or 24 h for HeLa and HCT116 cells). Cell death was determined by the percentage of sub-G1 cells as described in Materials and Methods. Data are presented as means ± SD from at least 3 independent experiments. * $p<0.05$ comparing to their respective non-treated control group (student's t test).

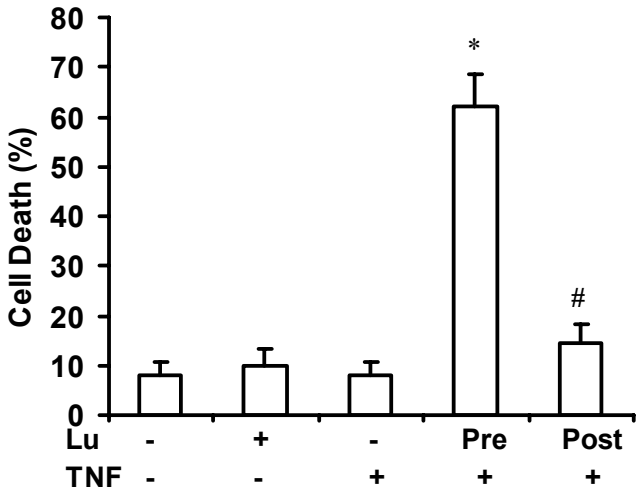


Figure 4.2 Effect of luteolin treatment sequence on sensitization.

In COLO205 cells, luteolin was administered either 2 h before (pre-treatment) or after (post-treatment) TNFα (15 ng/ml) exposure, then continued the culture for another 4 h. Cell death was determined by the percentage of sub-G1 cells as described in Materials and Methods. Data are presented as means ± SD from at least 3 independent experiments. * $p<0.05$ comparing to their respective non-treated control group; # $p<0.05$ comparing to the pre-treatment group.

4.3.2 Luteolin sensitizes TNFα-induced cell death through apoptosis

To further investigate the apoptosis induced by luteolin and TNFα, we then examined the morphological changes using AO/EB staining under a fluorescent microscope (Salghetti *et al.*, 1999). In COLO205 cells combined treatment of luteolin and TNFα resulted in evident cell shrinkage, cell membrane blebbing, chromatin condensation, and formation of apoptotic body at the early stage of cell death (data not shown), indicating that luteolin plus TNFα induces typical apoptotic cell death in this cell line. To confirm the above observations, we further analyzed two biochemical hall-markers of apoptosis: PARP cleavage and DNA fragmentation. While neither luteolin nor TNFα alone caused PARP cleavage or DNA fragmentation, luteolin pretreatment caused PARP cleavage (Figure 4.3A) and DNA fragmentation (Figure 4.3B) in cells treated with TNFα in a dose-dependent manner. The extent of these changes was comparable to cells treated with actinomycin D (ActD) plus TNFα. It is well known that ActD and cycloheximide (CHX) are general gene transcription and *de novo* protein synthesis inhibitors, respectively, which enhance TNFα-induced cell death via unspecific blockage of anti-apoptotic gene expression. To test whether luteolin acts through a similar mechanism, we examined the changes of the protein level of c-myc, a short half-life protein in the cell (Strasser and Newton, 1999). As shown in Figure 4.4, ActD and CHX but not luteolin reduced the c-myc protein content, indicating that luteolin is not a general gene expression inhibitor, but may act via other mechanisms.

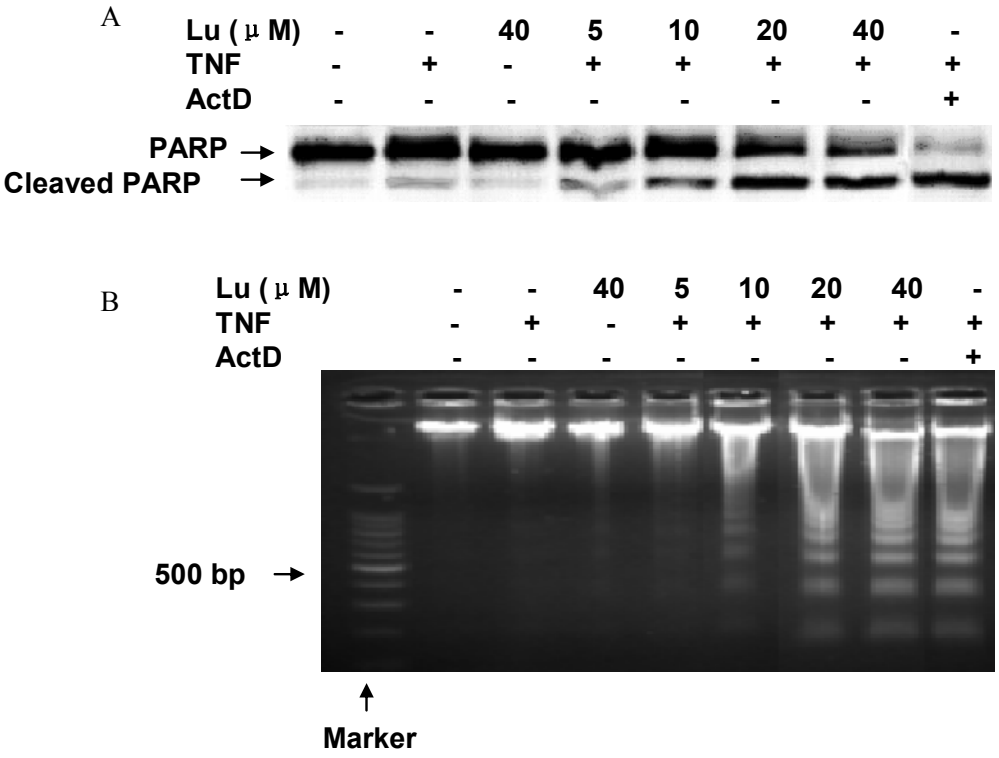


Figure 4.3 Luteolin and TNFα induce typical apoptosis in COLO205 cells.

COLO205 cells were pre-treated with indicated concentrations of luteolin for 2 h followed by TNFα (15 ng/ml) treatment for additional 6 h. ActD (1 μg/ml) pretreatment (1 h) was used as a positive control in both experiments. PARP cleavage was detected by Western blot (A). DNA fragmentation was examined by agarose gel electrophoresis (B).

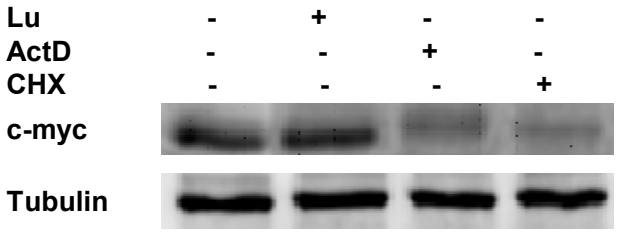


Figure 4.4 Effect of luteolin on c-myc protein level in COLO205 cells.

Changes of c-myc protein level in COLO205 cells treated with luteolin (40 μ M), ActD (1 μ g/ml) or CHX (10 ng/ml) for 6 h was detected by Western blot. The content of tubulin was used as a loading control.

4.3.3 Luteolin-induced sensitization to TNFα is associated with enhanced caspase-8 activation

Caspase activation is the central machinery in apoptosis and TNFα mediates apoptotic cell death via the cell death receptor pathway initiating from caspase-8 activation (Baldwin, 2001; Yamamoto and Gaynor, 2001). In this study, no caspase-8 activation was found in COLO205 cells treated with either luteolin or TNFα alone, while luteolin pretreatment markedly enhanced caspase-8 cleavage as well as caspase-8 activity in TNFα-treated cells (Figures 4.5 and 4.6). Similar pattern was also observed in subsequent activation of caspase-3. To confirm the role of such a caspase cascade in apoptosis mediated by luteolin plus TNFα, we tested the effect of a specific caspase-8 inhibitor (z-IETD-fmk) as well as a general caspase inhibitor (z-VAD-fmk) on cell death induced by luteolin plus TNFα. Both inhibitors effectively blocked cell death (Figure 4.7), PARP cleavage (Figure 4.8 upper panel) and DNA fragmentation (Figure 4.8 lower panel) in cells treated with luteolin plus TNFα, in concomitant with caspase-8 and caspase-3 inhibition (Figure 4.5). Similar results were also found in HeLa cells and HCT116 cells treated with luteolin plus TNFα (data not shown). These results thus indicate that the apoptosis induced by luteolin plus TNFα is mediated via a caspase cascade initiated from caspase 8 activation.

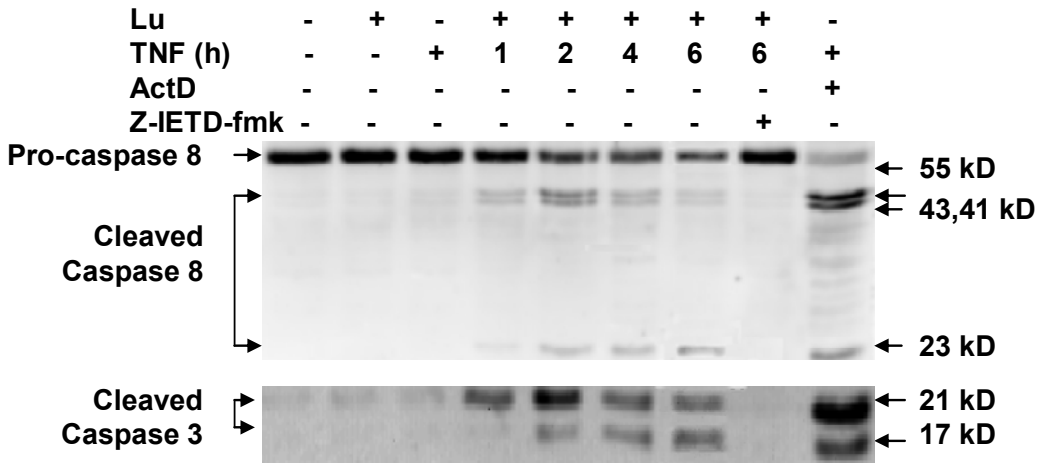


Figure 4.5 Effect of luteolin and TNFα on caspase.

COLO205 cells were pretreated with 40 μM luteolin for 2 h and then treated with TNF (15 ng/ml) for indicated hours. Cells were collected for detection of caspase-8 and caspase-3 activation by specific anti-caspase-8 and caspase-3 antibodies (Cell Signaling), recognizing the pro- and cleaved caspase-8, and the cleaved caspase-3, respectively. Cells treated with ActD and TNFα were used as a positive control.

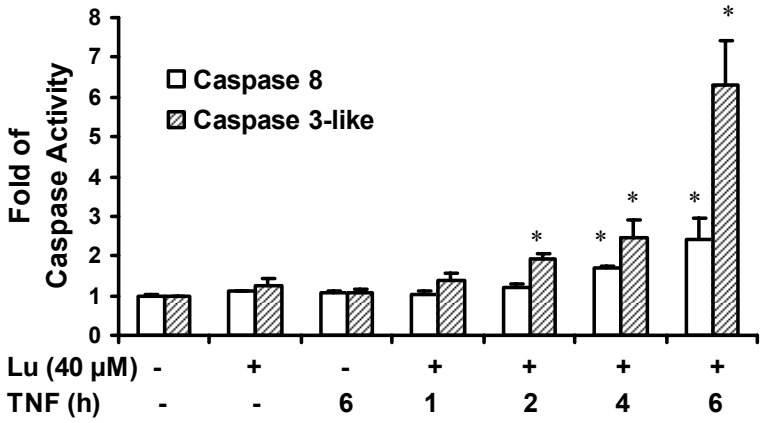


Figure 4.6 Effect of luteolin and TNFα on caspase activity.

COLO205 cells were pretreated with 40 µM luteolin for 2 h and then treated with TNF (15 ng/ml) for indicated period. Cells were collected for detection of caspase-8 and caspase-3 activity using their respective substrates (Ac-IETD-AMC and z-DEVD-Rhodamine 110). * $p<0.05$ comparing to their respective non-treated control group (student's t test).

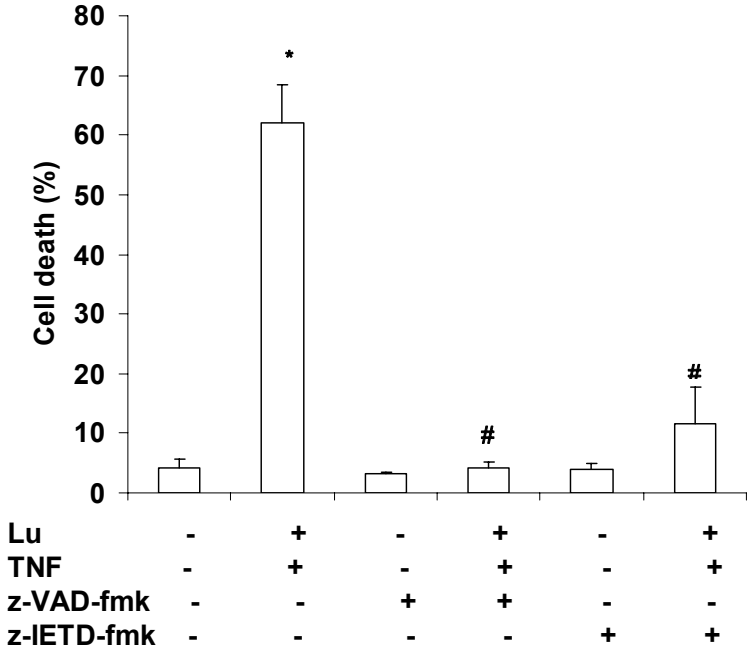


Figure 4.7 Effect of caspase inhibitors on luteolin and TNFα-induced apoptosis.

COLO205 cells were first treated with either z-VAD-fmk (25 μM) or z-IETD-fmk (25 μM) for 30 min, followed by luteolin (40 μM) for 2 h, and then TNFα (15 ng/ml) for another 6 h. Cell death was evaluated by flow cytometry after PI staining. * $p<0.05$ comparing to their respective non-treated control group (student's t test). # $p<0.05$ comparing to luteolin and TNF-treated group (student's t test).

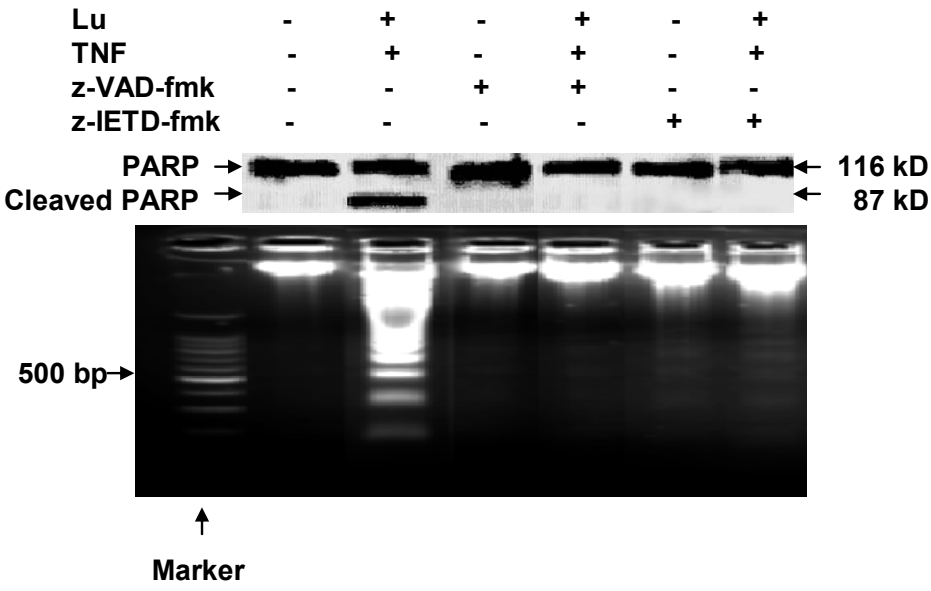


Figure 4.8 Effect of caspase inhibitors on luteolin and TNFα-induced apoptosis.

COLO205 cells were first treated with either z-VAD-fmk (25 μM) or z-IETD-fmk (25 μM) for 30 min, followed by luteolin (40 μM) for 2 h, and then TNFα (15 ng/ml) for another 6 h. Cells were collected for detection of PARP cleavage by Western blot (upper panel) and DNA fragmentation by agarose electrophoresis (lower panel).

4.3.4 TNFα-induced NF-κB activation is inhibited by luteolin

NF-κB is the main cell survival pathway elicited by TNFα. It has been well established that inhibition of NF-κB signaling pathway sensitizes TNFα-induced cell death (Kim *et al*., 2003b). Luteolin has recently been shown to inhibit LPS-induced NF-κB activation in fibroblasts, a mechanism involved in its anti-inflammatory activity (Hehner *et al.*, 1999). Here in order to understand the underlying mechanism responsible for the sensitization effect of luteolin to TNFα-induced apoptosis, we systematically tested the effects of luteolin on various phases of NF-κB signaling pathways triggered by TNFα. First, we examined the inhibitory effect of luteolin on NF-κB transcriptional activity in COLO205 cells by using the NF-κB luciferase reporter assay. As shown in Figure 4.9, treatment with TNFα significantly enhanced NF-κB transcriptional activity and luteolin pretreatment markedly suppressed the transactivation of NF-κB induced by TNFα. A similar inhibitory effect was found with parthenolide, a known specific inhibitor of IKK (Karin and Ben-Neriah, 2000). In order to exclude the possibility that the reduced luciferase activity by luteolin is due to its direct inhibition on luciferase enzyme activity, we performed luteolin post-treatment: cells were first treated with TNFα (15 ng/ml) for 2 h followed by luteolin (40 μM) treatment for another 2 h. It is rather interesting to find that luteolin post-treatment failed to inhibit the transactivation of NF-κB induced by TNFα, suggesting that luteolin does not suppress NF-κB post-transcriptionally and pose no direct inhibition to luciferase enzyme activity.

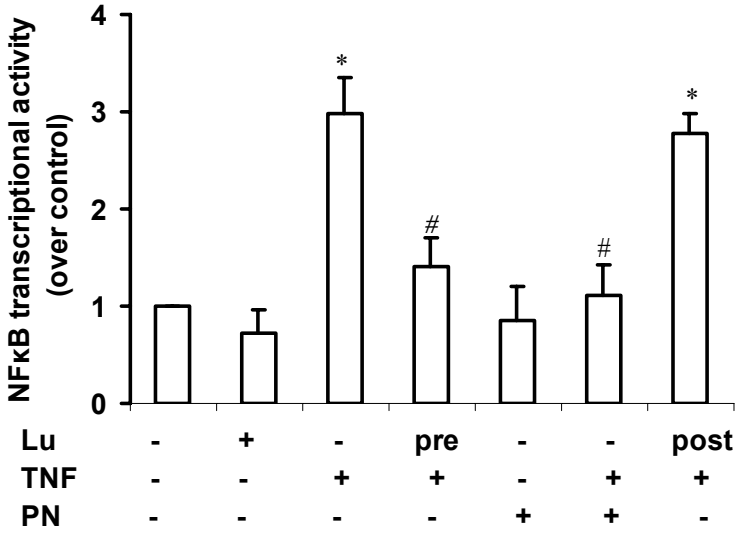


Figure 4.9 Luteolin inhibits TNFα-induced NF-κB transcriptional activity.

COLO205 cells were co-transfected with NF-κB-dependent luciferase reporter construct and β-galactosidase construct. The cells were then treated with luteolin pretreatment or post-treatment (40 μM × 2 h), followed by TNFα (15 ng/ml) for 2 h. Luciferase activity was expressed as fold increased over control after normalized with β-galactosidase enzyme activity. Pretreatment with pathenolide (PN, 20 μM × 2 h) was used as a positive control. Data are presented as means ± SD from at least 3 independent experiments. * $p<0.05$ comparing to the non-treated control group (student's t test). # $p<0.05$ comparing to the TNF-treated group (student's t test).

The activation of NF-κB requires a series of upstream events including degradation of IκBα, NF-κB nuclear translocation and NF-κB-DNA binding (Kim *et al*., 2003b). To define the mechanism by which luteolin inhibits NF-κB activation, we sought to define whether luteolin affects these upstream events. As shown in Figure 4.10, IκBα degradation and p65 nuclear translocation induced by TNFα in COLO205 cells were inhibited by parthenolide but not by luteolin. As shown in Figure 4.11, p65 was found to be main NF-κB component in TNFα-stimulated cells (supershift assay). Luteolin pretreatment also failed to influence NF-κB-DNA binding activity detected using EMSA (Figure 4.11), while it was completely abolished by parthenolide. Similar results were obtained when HeLa cells were tested (data not shown). It thus appears that luteolin may affect TNFα-induced NF-κB activation via interfering the transcriptional machinery, similar to its inhibitory effect on LPS-activated NF-κB transcription in fibroblasts (Gerritsen *et al.*, 1997).

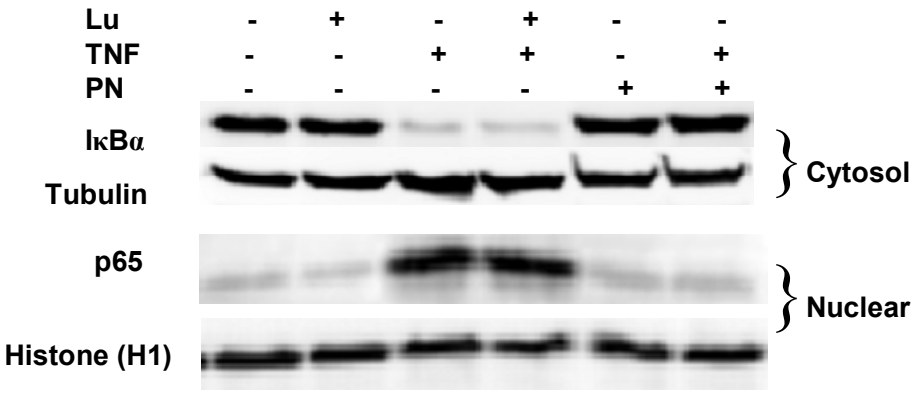


Figure 4.10 Effect of luteolin pretreatment on IκBα degradation and p65 nuclear translocation in COLO205 cells.

COLO205 cells were pretreated with luteolin (40 μM × 2 h) or PN (20 μM × 2 h), followed by TNFα (15 ng/ml) for 30 min. Cells were collected and fractioned to obtain cytosolic fraction and nuclear fraction. The levels of IκBα in cytosol and p65 in nuclear fraction were detected by Western blot. Tubulin and Histone were used as loading control for cytosol and nuclear fraction, respectively.

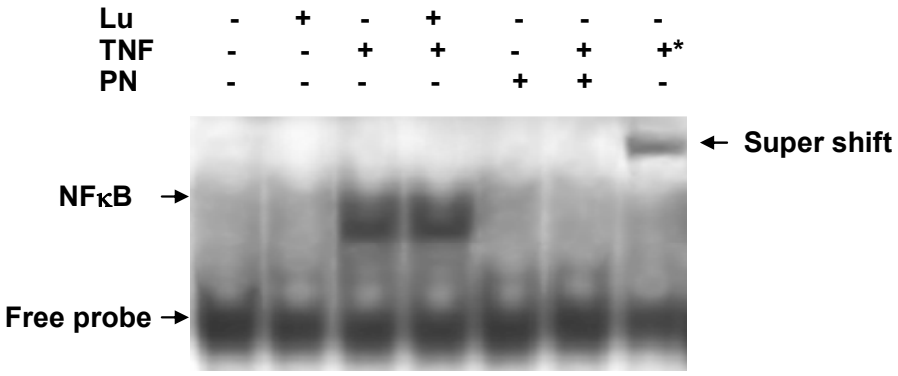


Figure 4.11 Effect of luteolin pretreatment on NF-κB-DNA binding activity.

COLO205 cells were pretreated with or without luteolin (40 μM) or PN (20 μM) for 2 h, followed by TNF (15 ng/ml) for 30 min. The cytosol and nuclear fraction were prepared as described in Materials and Methods. NF-κB-DNA binding activity was determined by EMSA.

4.3.5 Luteolin inhibits TNFα-activated NF-κB by interfering with CBP-p65 interaction

It is known that transcriptional activation of NF-κB requires participation of a number of coactivators including cAMP response element-binding protein (CBP) (Kim *et al.*, 2003b). We next examined whether luteolin affects the interaction between p65 and CBP. As shown in Figure 4.12, TNFα markedly enhanced CBP–p65 interaction and the interaction was significantly inhibited by luteolin pretreatment, suggesting that luteolin inhibits TNFα-induced NF-κB activation via interfering with p65-CBP interaction. Such action of luteolin was found to be similar to its inhibitory effect on LPS-activated NF-κB transcription in fibroblasts (Wang *et al*., 1998; He and Ting, 2002).

4.3.6 p65 expression protects the cell death induced by luteolin and TNFα

To further confirm the involvement of p65–CBP interaction in luteolin-mediated suppression on NF-κB activation, HeLa cells were transiently transfected with a p65-GFP expression vector. As shown in Figure 4.13A and B, p65 overexpression significantly overturned the sensitization effect of luteolin and protected against cell death. The above observation also suggests that NF-κB (p65) serves as the molecular target for the sensitization activity of luteolin on TNFα induced apoptosis.

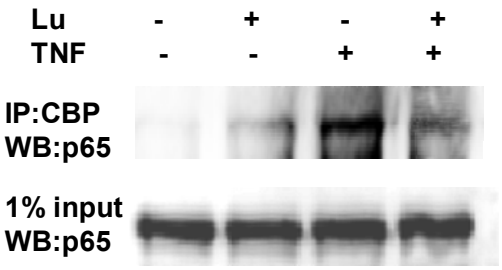


Figure 4.12 Effect of luteolin on CBP-p65 interaction

COLO205 cells were pretreated with 40 μM luteolin for 2 h followed by TNF (15 ng/ml) for 30 min. Cell lysate was co-immunoprecipited by anti-CBP antibody (Santa Cruz) and then detected by Western blot using anti-p65 antibody (Santa Cruz). 1% input was used as a proper control.

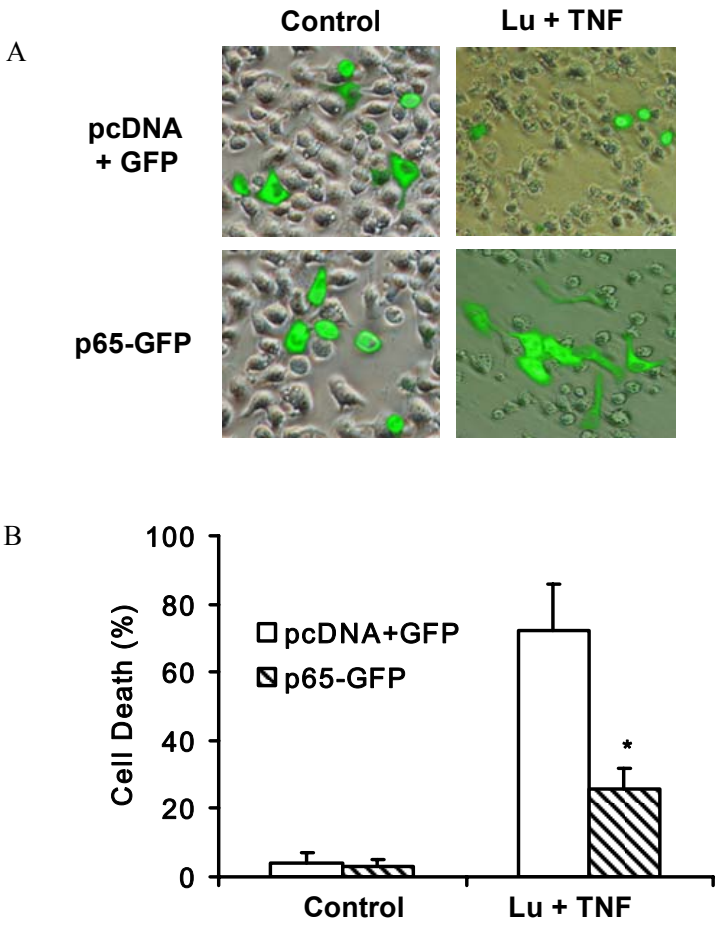


Figure 4.13 Effect of p65 overexpression on cell death induced by luteolin and TNF

A, HeLa cells were transiently transfected with pcDNA and GFP or p65-GFP for 24 h. Cells were then treated with luteolin (40 μM × 2 h) followed by TNF (15 ng/ml × 24 h). B, quantification of the cell death in A by counting the percentage of dead cells among those transfected cells in total 200 randomly selected transfected cells. Data are presented as means ± SD from 3 independent transfection experiments. * $p<0.05$ comparing to the group with pcDNA transfection.

4.3.7 Luteolin suppresses the expression of NF-κB anti-apoptotic target genes A20 and c-IAP1

The anti-apoptotic function of NF-κB is depending on the expression of its anti-apoptotic target genes. Here we further tested whether luteolin pretreatment influences the expression level of those genes. As shown in Figure 4.14, TNFα markedly upregulated the expression of *A20* and *c-IAP1*, while luteolin pretreatment significantly reduced their expression level. In contrast, no significant changes of c-IAP2, c-$FLIP_L$ and c-$FLIP_S$ were noted in cells treated with TNFα or luteolin. Both A20 and c-IAP1 are important anti-apoptotic molecules (De Smaele *et al*., 2001; Tang *et al.*, 2001) and their reduced expression levels caused by luteolin pretreatment are likely to contribute to the sensitization effect by luteolin to TNFα-induced apoptosis.

4.3.8 JNK activation contributes to the sensitization effect of luteolin to TNFα-induced apoptosis

Although the exact role of JNK in TNF-induced apoptosis is largely controversial, some recent studies have suggested that inhibition of NF-κB resulted in sustained JNK activation and apoptosis (Bennett *et al.*, 2001). Here we examined the effect of luteolin pretreatment on TNFα-induced JNK activation. TNFα alone caused a rapid and transient activation of JNK in COLO205 cells, demonstrated by the increased level of JNK (Figure 4.15, upper panel) and c-Jun phosphorylation (Figure 4.15, lower panel), with a peak level at 30 min. Although luteolin alone had little effect on JNK, luteolin pretreatment significantly augmented and prolonged JNK activation (both JNK and c-Jun phosphorylation). Similar augmentation effect of luteolin on TNFα-mediated JNK activation was also found in HeLa cells (data not shown).

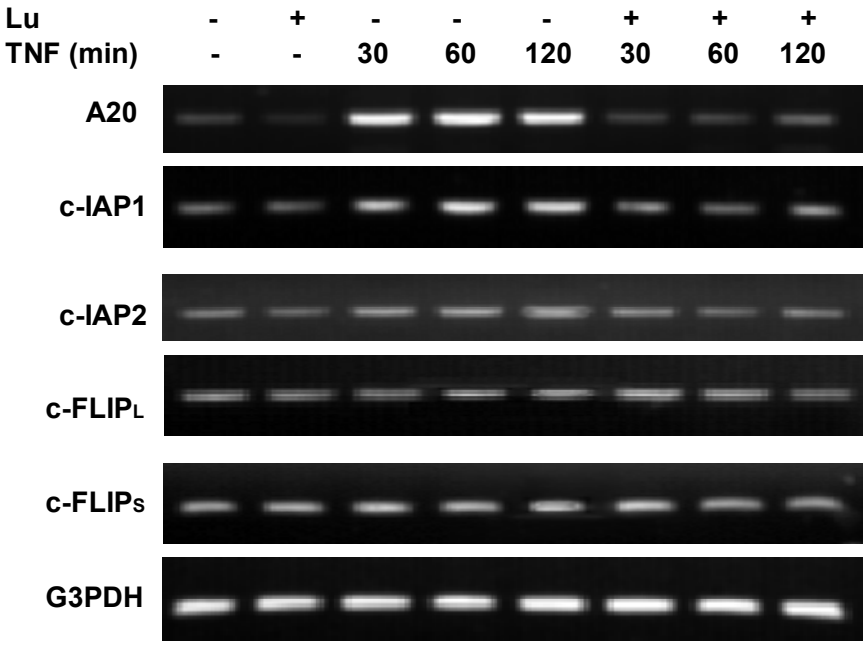


Figure 4.14 Luteolin pretreatment down-regulates expression of NF-κB anti-apoptotic target genes.

COLO205 cells were treated with TNFα (15 ng/ml) for 30, 60 or 120 min, with or without luteolin pretreatment (40 μM × 2 h). The mRNA level of various NF-κB target genes were examined using RT-PCR, as described in Materials and Methods.

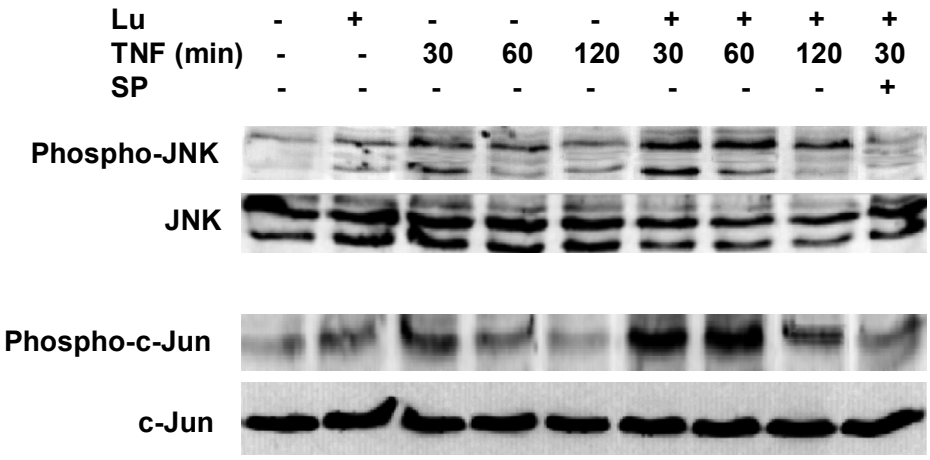


Figure 4.15 Luteolin pretreatment leads to augmented and prolonged JNK activation induced by TNF α .

COLO205 cells were first pretreated with SP600125 (20 μ M × 30 min), followed by luteolin (40 μ M × 2 h) and then TNF α (15 ng/ml) for indicated periods. Cells were collected for detection of JNK activation by enhanced level of both JNK and c-Jun phosphorylation by Western blot.

In order to understand the role of JNK activation in apoptosis induced by luteolin and TNFα, we next assessed the effect of a synthetic JNK inhibitor, SP600125 (Muzio *et al.*, 1997), on JNK activation and cell death in COLO205 cells treated with luteolin plus TNFα. As expected, pretreatment with SP600125 prevented luteolin plus TNFα induced JNK activation (Figure 4.15). More importantly, it almost completely blocked the catalytic cleavage of caspase-8 and its downstream effector caspase-3, as well as PARP cleavage (Figure 4.16), suggesting that JNK activation is required for caspase-8 activation and apoptotic cell death induced by luteolin plus TNFα.

4.3.9 Ectopic expression of A20, c-IAP1 and dominant negative forms of JNKK1 and JNKK2 prevents apoptosis induced by luteolin plus TNFα

The above data collectively demonstrate that the reduced expression of anti-apoptotic genes *A20* and *c-IAP1*, as well as the augmented activation of JNK may contribute to the apoptosis induced by luteolin plus TNFα (Figures 4.14 and 4.16). We then used genetic approaches to further establish the causative link between these events. HeLa cells were transiently transfected with either myc-A20 or HA-c-IAP1 expression vector together with a red fluorescence protein construct (pDsRed) as a transfection marker. In addition, a vector expressing a viral protein cytokine response member A (CrmA), which is known to be a specific caspase-8 inhibitor (Davis, 2000), were included as a positive control. As shown in Figure 4.17, the successfully transfected cells emitted strong red fluorescence as seen under a fluorescence microscope. Following combined treatments with luteolin and TNFα, most of the cells transfected with pDsRed and pcDNA died. A quantitative analysis counting the percentage of cell death among transfected cells was also carried out (Figure 4.18). CrmA Over-expression offered a complete protection against apoptotic cell death

induced by the combined treatments of luteolin and TNFα, confirming the earlier finding that such apoptosis is mediated by caspase-8 activation (Figures 4.7 and 4.8). In addition, the over-expression of either A20 or c-IAP1 protein significantly protected cell death induced by luteolin plus TNFα, although to a lesser extent than that of CrmA (Figure 4.18).

It has been well established that TNFα-mediated JNK activation is regulated by two upstream MAPK kinases: JNKK1 and JNKK2 (Ueda *et al.*, 2003). The effectiveness of dominant negative forms of JNKK1 and JNKK2 were proven in HeLa cells when they successfully blocked TNF-mediated JNK activation using JNK kinase assay (data not shown). Here cells with successful HA-JNKK1(DN)+HA-JNKK2(DN) transfection became largely resistant to apoptosis induced by luteolin+TNFα. Such findings, together with the pharmacological evidence from SP600125 (Figure 4.15), strongly suggesting that JNK plays a critical role in the sensitization effect of luteolin on TNFα-induced apoptosis.

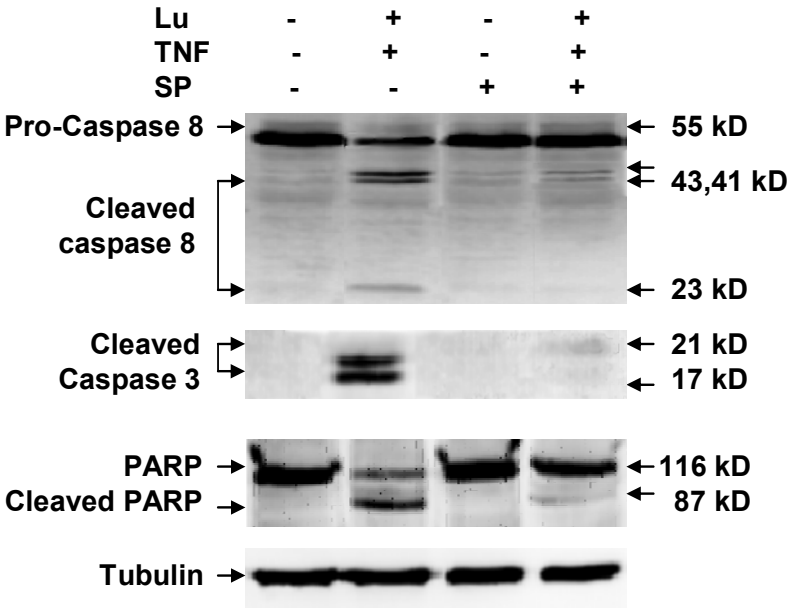


Figure 4.16 SP600125 Inhibits caspase 8 and caspase 3 activation and PARP cleavage in cells treated with luteolin and TNF α .

COLO205 cells were first pretreated with SP600125 (20 μ M × 30 min), followed by luteolin (40 μ M × 2 h) and then TNF α (15 ng/ml) for another 6 h. Western blotting was performed to detect caspase-8, caspase-3 and PARP cleavage. Tubulin was used as loading control

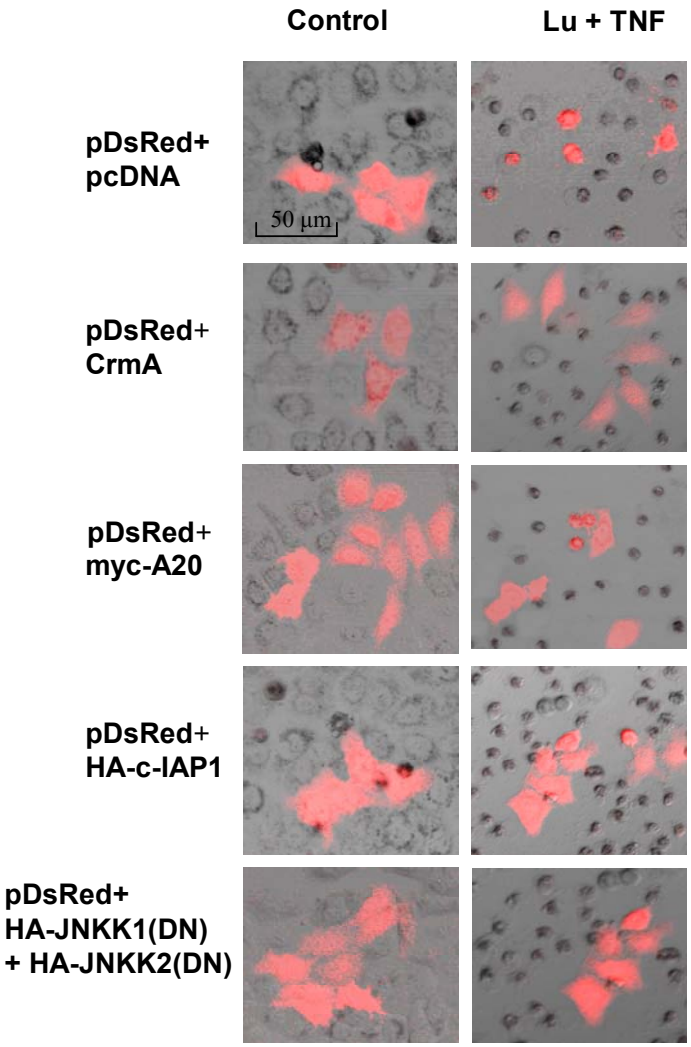


Figure 4.17 Ectopic expression of A20, c-IAP1 and JNKK1(DN)+JNKK2(DN) protects cell death induced by luteolin and TNF α .

HeLa cells were transiently transfected with either pcDNA, CrmA, HA-c-IAP1, myc-A20 or HA-JNKK1(DN)+JNKK2(DN), together with pDsRed as a transfection marker. After 24 h, the cells were treated with luteolin (40 μ M) for 2 h followed by TNF α (15 ng/ml) treatment for another 24 h. Cell death was then evaluated by morphological changes under a fluorescent microscope and those successfully transfected cells were in bright red.

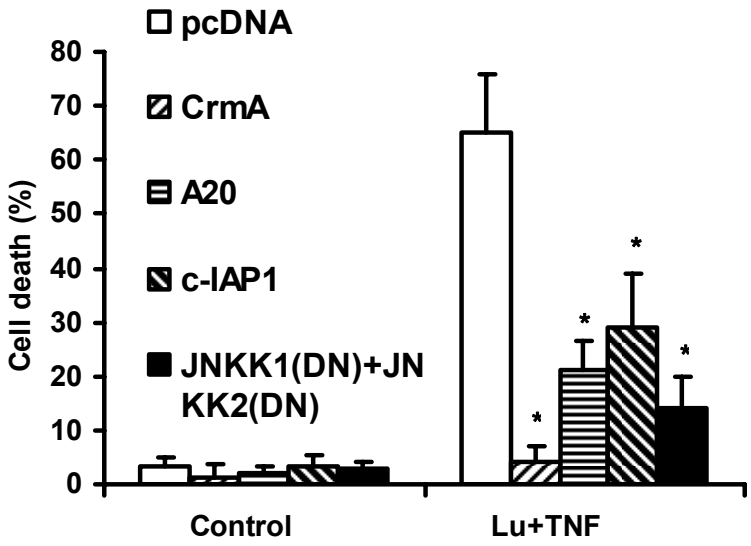


Figure 4.18 Ectopic expression of A20, c-IAP1 and JNKK1(DN)+JNKK2(DN) protects cell death induced by luteolin and TNF α (Quantification).

Cells were treated as in Figure 5.17, quantification of cell death was conducted by counting the percentage of dead cells among those transfected cells in a total of randomly selected 200 transfected cells. Data are presented as means ± SD from 2 independent transfection experiments. * $p<0.05$ comparing to the group with pcDNA transfection.

4.4 DISCUSSION

Luteolin is a common flavonoid found in human diet. Previous studies have demonstrated the anti-cancer property of luteolin. For instance: it is capable of preventing 7,12-dimethylbenz[a]anthracene (DMBA)- and 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced mouse skin cancer (Huang et al., 1999b; Casagrande and Darbon, 2001; Ko et al., 2002) or inducing cell cycle arrest and apoptosis in some human cancer cells *in vitro* (Strasser and Newton, 1999). In this study, we provided evidence that luteolin sensitizes TNFα-induced apoptosis in human colorectal and cervical cancer cells. Such sensitization is achieved via its inhibitory effect on NF-κB activation, which in turn results in reduced expression of anti-apoptotic NF-κB targets genes (*A20* and *c-IAP1*), as well as augmented and prolonged JNK activation. Data from this study thus reveal a novel function of luteolin and enhance the value of luteolin as a useful anti-cancer agent.

There are several features in the sensitization effect of luteolin to TNFα-induced cell death. First, the sensitization is mainly achieved through enhanced activation of caspase-8, the initial caspase in the death receptor signaling pathway to induce typical apoptosis (Muzio *et al.*, 1997). Such notion is supported by the strong inhibitory effects on cell death offered by z-IETD-fmk, a synthetic caspase-8 inhibitor (Figures 4.7 and 4.8) as well as by over-expression of CrmA (Figures 4.17 and 4.18), a powerful and specific caspase-8 inhibitor (Baldwin, 2001; Kucharczak *et al.*, 2003). In contrast, the contribution from mitochondria to the sensitization process by luteolin is believed to be insignificant based on the marginal inhibitory activity of a caspase-9 inhibitor on apoptosis (data not shown). Second, although luteolin sensitized TNFα-induced apoptosis in a similar pattern as ActD (Figure 4.3), they apparently act through different mechanisms. Luteolin did not block the expression level of c-myc as

ActD or CHX did (Figure 4.4), indicating that luteolin is not a general gene expression inhibitor, but rather works through a specific mechanism. Third, the sensitization effect of luteolin is depending on luteolin administration schedule and luteolin post-treatment failed to exert significant sensitization to TNFα-induced apoptosis (Figure 4.2), suggesting that the sensitization effect of luteolin may be associated with the blockage of anti-apoptotic gene expression elicited by TNFα.

NF-κB activation is the principal cell survival signaling triggered by TNFα through TNFR1. The anti-apoptotic function of NF-κB is achieved through up-regulation of its anti-apoptotic target genes (Kim *et al.,* 2003b). Therefore, we reasoned that luteolin sensitizes TNFα-induced apoptosis via its inhibitory effect on the NF-κB signaling pathway. Such a hypothesis is strongly supported by the finding that luteolin pretreatment efficiently blocked the transactivation of NF-κB determined by NF-κB luciferase assay (Figure 4.9). Meanwhile, post-treatment with luteolin did not inhibit NF-κB transactivation and subsequently failed to exert any sensitization effect (Figure 4.2). We also attempted to evaluate the effect of luteolin on upstream signaling events of NF-κB activation. To our surprise, luteolin did not affect IκBα degradation, p65 nuclear translocation and p65-DNA binding (Figures 4.10 and 4.11). Such findings are basically similar to the effect of luteolin on LPS-induced NF-κB activation in rat fibroblasts (Xagorari *et al*., 2001). In contrast, Dhanalakshmi *et al*. reported that, in macrophages, luteolin inhibited LPS-induced NF-κB activation via preventing IκBα degradation (Dhanalakshmi *et al.*, 2002). Different cell types used in those studies may explain the different responses to the same stimulus. A number of flavonoids are capable of inhibiting the NF-κB signaling pathway but distinct mechanisms are involved. Silibinin can directly inhibit IKKα kinase activity and subsequently block phospho-IκBα degradation and NF-κB translocation (Farah *et al.*,

2003; Kim *et al*., 2003b). In contrast, both apigenin and flavopiridol suppress TNFα-stimulated NF-κB transcriptional activity, without affecting IκBα degradation (Gerritsen et al., 1997)**,** which is similar to the effect of luteolin found in this study.

Then how does luteolin inhibit NF-κB transactivation? Since transcriptional activation of NF-κB requires participation of a number of coactivators including cAMP response element-binding protein (CBP) (Gerritsen *et al*., 1997), we next examined the effect of luteolin on the CBP-p65 interaction, which is known to be required for p65 transcriptional activation (Kim *et al.*, 2003b). Luteolin significantly inhibited TNFα-induced CBP-p65 interaction (Figure 4.12), which is similar to its effect on LPS-induced CBP-p65 interaction (Kim *et al*., 2003b). At present, it is not clear how luteolin interrupts CBP-p65 interaction. In the context of LPS signaling, luteolin might induce JNK and c-jun activation and thus sequester transcriptional coactivators (Krikos *et al*., 1992; Wang *et al.,* 1998; Micheau *et al.*, 2001). In this study, luteolin alone had only marginal effect on JNK activation (Figure 4.16) Furthermore, SP600125 (specific JNK inhibitor) failed to restore the suppressed NF-κB transactivation by luteolin (data not shown), suggesting that JNK activation is unlikely to be an important factor contributing to the inhibitory effect of luteolin on NF-κB activation. Based on the observation that p65 overexpression could overturn the cell death induced by luteolin and TNF (Figure 4.13), it is possible that luteolin may inhibit p65-CBP interaction by targeting p65, although the exact mechanism responsible for such action remains to be further identified.

Among various anti-apoptotic genes regulated by NF-κB, a number of them such as c-*IAP1, c-IAP2, c-FLIP*$_L$*, c-FLIP*$_S$ and *A20* have been well characterized to specifically inhibit caspase-8 activation (Krikos *et al.*, 1992). As shown in Figures 4.5, 4.6, 4.7 and 4.8, luteolin sensitizes TNFα-mediated cell death via enhanced caspase-8

activation. We thus postulated that luteolin may augment caspase-8 activation by relieving the suppressive effects of those molecules. Among the NF-κB target genes tested, the expression of *A20* and *c-IAP1*, which was up-regulated by TNFα, were found to be markedly suppressed by luteolin (Figure 4.14). More importantly, the ectopic expression of either A20 or c-IAP1 conferred significant protection against apoptosis induced by the combined treatment of luteolin with TNFα (Figure 4.17). Zinc finger protein A20 is a NF-κB inducible gene (Song *et al.*, 1996; Lademann *et al.*, 2001). The important function of A20 includes its strong inhibitory effect on TNFα-mediated apoptosis (Lee *et al.*, 2000). The A20 deficient cells are highly susceptible to apoptotic cell death induced by TNFα (He and Ting, 2002). A recent study revealed that A20 is capable of interrupting the recruitment of TRADD and RIP into TNFR1, thus blocking both caspase-8 (cell death) and the NF-κB (cell survival) pathways (Li *et al.*, 2002). On the other hand, c-IAP1, but not c-IAP2, is also found to be an important modulator in the sensitization effect of luteolin to TNFα-induced apoptosis, although to a lesser extent than that of A20. Despite the high sequence similarity between c-IAP1 and c-IAP2, different specificities have been identified regarding their effects on apoptosis. For instance, c-IAP1, but not c-IAP2, is capable of directing the ubiquitylation of TRAF2 in TNF signaling (De Smaele *et al*., 2001; Tang *et al*., 2001).

One intriguing issue in TNF signaling is the role of JNK in TNF-mediated apoptosis. Recently, several lines of evidence have demonstrated that inhibition of NF-κB leads to prolonged JNK activation, which promotes TNFα-induced apoptosis (De Smaele *et al*., 2001; Tang *et al.,* 2001). In this study, luteolin pretreatment also augmented and prolonged TNFα-mediated JNK activation (Figure 4.15). Since luteolin alone has little effect on JNK, such augmentation is most likely due to the

potent inhibitory of luteolin on TNFα-induced NF-κB transactivation (Figure 4.9). Suppressed NF-κB activation and subsequently diminished XIAP or GADD45b expression in luteolin-treated cell could account for the enhanced JNK activation (Tournier *et al.*, 2000). By using both genetic and pharmacological approaches, we provided convincing evidence that the augmented and prolonged JNK activation plays a critical role in apoptosis triggered by luteolin and TNFα (Figures 4.16 and 4.17). The exact mechanism for JNK-mediated apoptosis may vary depending on the cellular context and the nature of stimuli. In UV-induced apoptosis, JNK is an important component in mitochondrial apoptotic pathway (Deng *et al.*, 2003). On the other hand, a recent study by Deng et al showed that JNK is required to relieve the inhibition imposed by TRAF2-c-IAP1 on caspase-8 activation triggered by TNFα. This newly identified role of JNK in TNFα-induced apoptosis helps to explain our observation that a specific JNK inhibitor (SP600125) prevented caspase-8 activation in cells treated with luteolin plus TNFα (Figure 4.17).

In conclusion, in this part of study we have highlighted a novel function of luteolin by demonstrating that luteolin interferes with the TNF signaling pathway and markedly sensitizes human cancer cells to TNFα-induced apoptosis. Such sensitization is closely associated with the inhibitory effect of luteolin on NF-κB activation, resulting in down-regulation of some key anti-apoptotic genes such as A20 and c-IAP-1, as well as augmented and prolonged JNK activation induced by TNFα. Therefore, the strong sensitization effect of luteolin to TNFα-mediated apoptosis in human cancer cells suggests that luteolin are valuable as chemopreventive and chemotherapeutic agents against cancer together with TNFα.

CHAPTER FIVE

LUTEOLIN SENSITIZES TUMOR NECROSIS FACTOR- RELATED APOPTOSIS INDUCING LIGAND (TRAIL)-INDUCED APOPTOSIS IN HUMAN CANCER CELLS

5.1 INTRODUCTION

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is a member of the TNF superfamily (Wang and El Deiry, 2003). TRAIL is an ideal therapeutic agent for cancer treatment because it has been shown to be a potent apoptosis inducer in a wide variety of cancer and transformed cells without damaging most normal cells. TRAIL induces apoptosis through binding to its receptors on cell surface. To date, four types of receptors have been identified: death receptor 4 (DR4) and death receptor 5 (DR5) as death receptors and DcR1 and DcR2 as decoy receptors (Wang and El Deiry, 2003). Ligation of TRAIL to its receptors results in trimerization of receptors and clustering of intracellular death domains (DDs), which then recruit Fas-associated death domain protein (FADD) and caspase 8 to form the death-inducing signaling complex (DISC). Caspase 8 activation within DISC subsequently activates executor caspase 3, which in turn cleaves its substrates and eventually induces apoptosis (Green, 2000a; Ashkenazi, 2002). On the other hand, activation of caspase 3 by caspase 8 can be greatly facilitated through mitochondrial amplification pathway, in which activated caspase 8 cleaves the proapoptotic Bcl-2 family member Bid into truncated Bid (tBid). Translocation of tBid together with other pro-apoptotic Bcl-family members promotes release of cytochrome C and SMAC/DIABLO from mitochondria, leading to caspase 3 activation and apoptosis (Green, 2000a; Jiang and Wang, 2004).

However, the potential application of TRAIL in cancer therapy is limited as many cancer cells are found to be resistant to the cytotoxicity of TRAIL. The resistance may be due to low expression of pro-apoptotic molecules (DRs or caspase 8) or high expression of anti-apoptotic molecules (DcRs, FLIP, IAPs, Bcl-2) (Wang and El Deiry, 2003). Thus, combination TRAIL with other agents has been a

promising strategy to potentiate the cytotoxicity of TRAIL and its therapeutic applications (Bagli et al., 2004; Huerta-Yepez *et al.*, 2004; Rosato *et al.*, 2004; von Haefen *et al.*, 2004).

In previous chapter, we have found that luteolin greatly sensitizes TNF-induced cell apoptosis via inhibition of NF-κB and sustained and augmented activation of c-jun N-terminal kinase (JNK). We here demonstrated that luteolin also sensitizes TRAIL-induced apoptosis in various human cancer cells. Interestingly, such sensitization is achieved via enhanced XIAP ubiquitination and proteasomal degradation. Furthermore, our study demonstrates that the enhanced XIAP ubiquitination and degradation are likely due to suppressed protein kinase C (PKC) activation by luteolin. Data from this study thus present a novel function of luteolin as a potential anti-cancer agent.

5.2 MATERIALS AND METHODS

5.2.1 Reagents and Plasmids

Luteolin, 4'-6-Diamidino-2-phenylindole (DAPI) were purchased from Sigma (St Louis, MO). Human recombinant TRAIL was from R&D Systems (Minneapolis, MN) and was dissolved in 1% BSA as stock solution (50 μg/ml). The following reagents were from Calbiochem (San Diego, CA): pan caspase inhibitor z-VAD-fmk, caspase 8 inhibitor z-IETD-fmk, caspase 3 inhibitor z-DEVD-fmk, phorbol-12-myristate-13-acetate (PMA), general PKC inhibitor bisindolylmaleimide I (BIM), PI3 kinase inhibitor LY-294002 (LY) and Wortmannin (Wort), and proteasome inhibitor MG132, PSI and PSII. Flag-XIAP expression vector was a generous gift from Dr. Colin Duckett (University of Michigan, Ann Arbor, MI). Anti-caspase-8, anti-caspase-3, anti-Bcl-2, anti-Bcl-xL, anti-Bid, and anti-phospho-substrate (Ser)-PKC

and anti-ubiquitin antibodies were from Cell Signaling Technology (Beverly, CA). The anti-XIAP and anti-PARP antibody were from BD Transduction Laboratories (San Diego, CA). Anti-FLIP, anti-c-IAP-1, anti-c-IAP-2, anti-Mcl-1, anti-mouse-FITC, and anti-tubulin antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-DR4, anti-DR5, anti-DcR1 and anti-DcR2 were from R&D.

5.2.2 Cell culture and treatments

Human cervical cancer cells HeLa, human liver cancer cells HepG2 and human colorectal cancer cells HT29 were from ATCC and human nasopharyngeal cancer cells CNE1 was obtained from Sun Yet-sat University (Guangzhou, China). HeLa, HepG2 and CNE1 were maintained in DMEM medium (Sigma) with 10 % FBS (Hyclone). HT29 were maintained in RPMI-1640 medium with 10 % FBS.

5.2.3 Apoptosis assessment-DAPI staining

The cells undergoing apoptosis were evaluated by chromatin condensation, nuclear shrinkage and formation of apoptotic bodies, all visualized with 4',6-diamidino-2phenylindole (*DAPI*) staining (Fuentes et al., 2003). After various designated treatments, medium was removed and cells were fixed with 70% ethanol at room temperature for 10 minutes. Cells were then stained with 0.3 μg/mL DAPI (in PBS) at room temperature for 10 minutes and visualized under an inverted fluorescence microscope and photographed.

5.2.4 Colony formation assay

Cancer cells (HT29, HeLa and HepG2) were plated on 6-well plates (5,000 cells/well) for 24 hours followed by various treatments. After 3 weeks, the survival clones were stained by 0.5 % crystal violet for 1 hour and photos were taken using digital camera (Mangan *et al.*, 2004).

5.2.5 Transient transfection

HeLa cells were transiently transfected with either pcDNA or Flag-XIAP, using the Lipofectamine transfection reagent (Invitrogen, Carlsbad, CA). A red fluorenscent protein expression vector (pDsRed, Clontech Inc. Palo Alto, CA) was co-transfected as a transfection marker. After 24 h of transfection, the cells were pretreated with luteolin (40 μM × 2 hours) followed by TRAIL (1 ng/ml × 6 hours). Cell death was determined by morphological changes examined under an inverted fluorescent microscope.

5.2.6 Immunoprecipitation and western blot

At the end of treatment, cells were collected by scrapping and then washed with ice-cold PBS twice. Cells were lysed in lysis buffer [50 mM Tris HCl (pH7.4), 150 mM NaCl, 1 mM EDTA and 1 % TRITON X-100] for 1 hour on ice. The supernatant was collected after centrifugation at 20,000 × g for 15 minutes. Each sample was added with 0.5 μg anti-XIAP body BD Biosciences Pharmingen (San Jose, CA) and 50 μL protein A/G agarose beads (Roche Molecular Biochemicals, Indianapolis, IN) and rotated overnight at 4°C. The beads were washed four times using ice-cold PBS buffer and then eluted using SDS-sample buffer before subject to western blot analysis. For Western blot, equal amount of proteins were fractionated on SDS-polyacrylamide gel in the Mini-PROTEAN II system (Bio-Rad, Hercules, CA) and blotted onto PVDF membrane (Millipore, Bedford, MA). After blocked with 5% nonfat milk in TBST [10 mM Tris-HCl (pH 7.5), 100 mM NaCl and 0.1% Tween 20], the membrane was probed with various antibodies and developed with enhanced chemiluminescence (Pierce, Rockfold, IL) using a Kodak Image Station (Kodak, Rochester, NY).

5.2.7 RNA extraction and reverse transcription-PCR

RNA extraction was carried out using a total RNA extraction kit Purescript (Gentra Systems Inc., Minneapolis, MN), following the instructions from the manufacturer. Five μg of total RNA from each sample were subjected to reverse transcription using M-MLV reverse transcriptase (Promega, Madison, IL). For PCR, the amplification reaction was carried with 200 pmol of each primer, 200 μM of each dNTPs, and 0.5 units of Tag DNA polymerase II (Promega). The PCR conditions were optimized to achieve exponential amplification in which the PCR product formation is proportional to the starting cDNA. The primers of human XIAP (Asselin *et al.*, 2001), DR4, DR5, DcR1, DcR2 (Abdollahi *et al.*, 2003), and glyceraldehydes-3-phosphate dehydrogenase (G3PDH) (Mafune et al., 1999) were based on literature. PCR products were size-fractionated using 1.8% agarose gel and visualized by ethidium bromide staining.

5.2.8 Immunostaining for detection of death receptors

Cell surface expression of DR4, DR5, DcR1, and DcR2 were analyzed by indirect staining with primary mouse anti-human DRs (R&D systems), followed by FITC-conjugated rabbit anti-mouse IgG (Kim *et al.*, 2004a). Briefly, cells were first stained with saturating amounts of anti-DR4, anti-DR5, anti-DcR1, or anti-DcR2 on ice for 30 min. After washing, cells were reacted with FITC-conjugated anti-mouse IgG on ice for another 30 min (Shao *et al.*, 2004). Negative control cells were stained with the same FITC-conjugated secondary antibody. Cells were then subject to flow cytometry analysis to determine the expression of these death receptors.

5.2.9 Statistical analysis

The numeric data are presented as mean ± SD from at least three sets of independent experiments. The differences among different groups were examined using a student's test and $p < 0.05$ was considered statistically significant.

5.3 RESULTS

5.3.1 Luteolin sensitizes cancer cells to TRAIL-induced apoptosis

We have recently shown that luteolin was able to sensitize TNF-induced apoptosis in human cancer cells (Chapter 4). Here we further assessed the effect of luteolin on TRAIL-induced cell death. First we tested the cytotoxicity of TRAIL on human cancer cell lines originated from various tissues, including human live cancer cell HepG2, human colorectal cancer cell HT29, human nasopharyngeal cancer cell CNE1 and human cervical cancer cell HeLa. Some cancer cells were found to be TRAIL-resistant. For instance, up to as high as 200 ng/mL TRAIL exerted no significant cytotoxicity on HepG2, HT29 or CNE1 cells even after 24 hours treatment. In contrast, HeLa cells were sensitive to as low as 5 ng/mL TRAIL (Figure 5.1). Luteolin (40 μM) alone did not induce cell death in any of these cells. However, when the cells were pretreated with luteolin for 2 hours followed by a non-cytotoxic concentration of TRAIL for as short as 6 hours, all the four cell lines tested underwent dramatic apoptotic cell death (Figure 5.2). Figure 5.3A shows the chromosome condensation in HeLa cells treated with luteolin and TRAIL. These data suggest that luteolin pretreatment not only markedly sensitizes TRAIL-resistant cancer cells, but also significantly expedites the cell death process. To test the long term effect of luteolin and TRAIL on cancer cell growth, the colony formation assay was performed using HT29 cells. As shown in Figure 5.3B, luteolin (40 μM) alone reduced HT29

colony size evidently, although it was not cytotoxic in the short term apoptosis assay (Figures 5.1 and 5.2). However, a combination of luteolin and TRAIL completely suppressed cancer cell growth and colony formation. Similar results were also found in HeLa and HepG2 cells (data not shown).

5.3.2 Luteolin facilitates TRAIL-initiated caspase-3 maturation

TRAIL-induced apoptosis is mainly executed by the extrinsic cell death receptor pathway, involving caspase-8 as the initiator caspase and caspase-3 as the executor. Here we examined the effect of luteolin on TRAIL-initiated caspase cascade. As shown in Figure 5.4, TRAIL alone induced obvious caspase-8 cleavage, producing both p44 and p23. While luteolin alone had no effect on caspase-8 activation, luteolin pretreatment greatly promoted TRAIL-induced caspase-8 activation, as evidenced by the enhanced cleavage of pro-caspase-8 p55 to its intermediate form p44 and further to its active form p23. We next examined the pattern of caspase-3 activation. In cells treated with TRAIL alone for 6 hours, there was only slight cleavage of caspase-3, producing its inactive fragment p21 (Figure 5.4). Although luteolin alone did not cause any change of caspase-3, its pretreatment followed by TRAIL led to the complete cleavage of caspase-3, resulting in formation of the active form p17. This result indicates that luteolin, in combination with TRAIL, facilitates the maturation of caspase-3. Similar caspase changes were also observed in CNE-1, HT29 and HepG2 cells (data not shown). Finally, we found that only combined treatment with luteolin and TRAIL resulted in evident PARP cleavage, downstream of caspase-3 activation and a hall marker for apoptosis (Figure 5.4), which is consistent to cell death results shown in Figure 5.2.

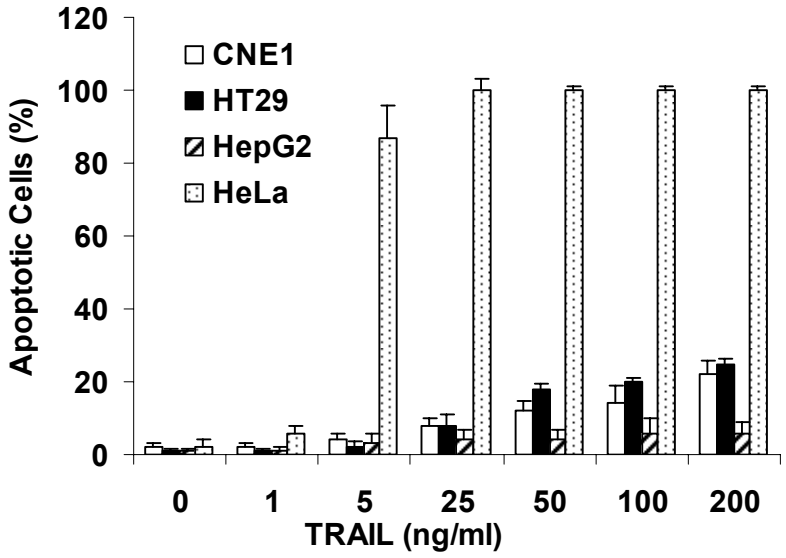


Figure 5.1 Sensitivity of human cancer cells to TRAIL-induced apoptosis.

CNE1, HT29, HeLa and HepG2 cells were treated with various concentrations of TRAIL for 24 h. At the end of treatment, cells were stained with DAPI and examined under an inverted fluorescent microscope. The result was presented as the percentage of cells with evident nuclear condensation in 200 randomly selected cells.

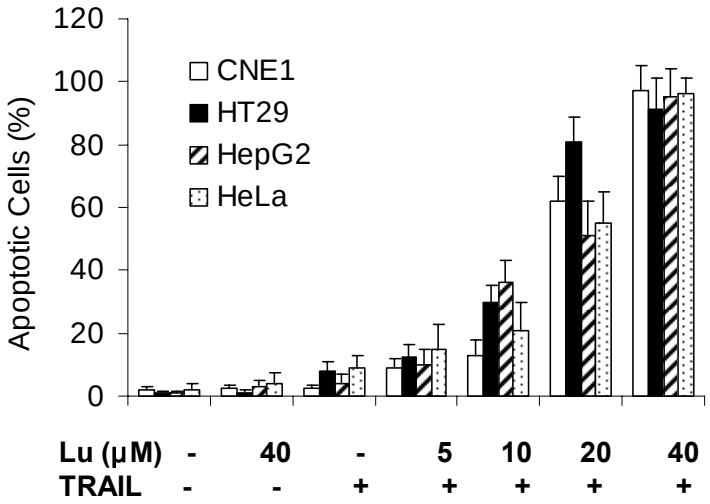


Figure 5.2 Luteolin sensitizes human cancer cells to TRAIL-induced apoptosis

Cells were first pretreated with indicated concentration of luteolin for 2 h, followed by treatment with a subtoxic concentration of TRAIL for another 6 h (1 ng/ml for HeLa and CNE1, 5 ng/ml for HT29 and HepG2). At the end of treatment, cells were stained with DAPI and examined under an inverted fluorescent microscope. The result was presented as the percentage of cells with evident nuclear condensation in 200 randomly selected cells.

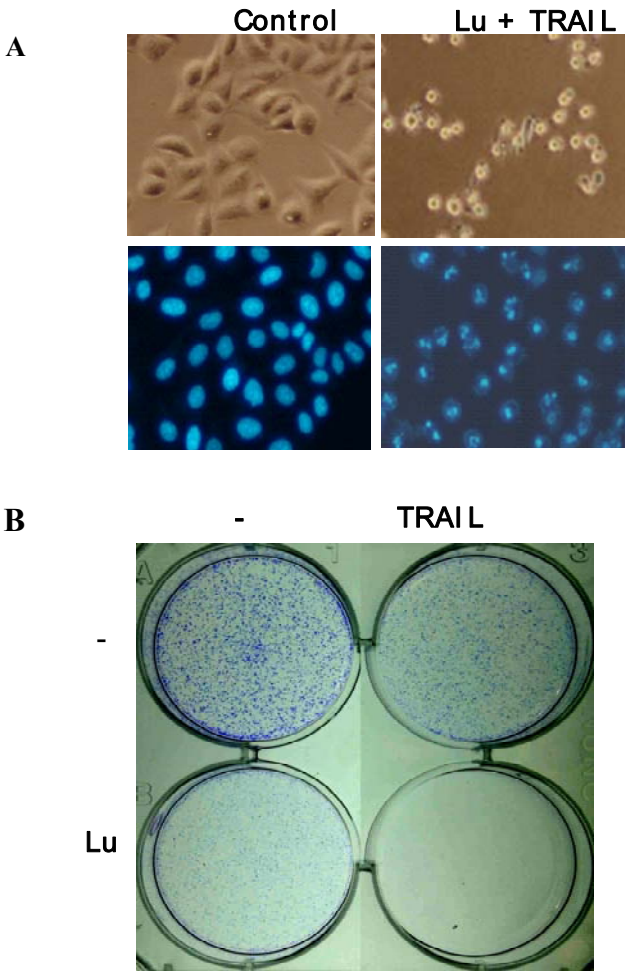


Figure 5.3 Luteolin sensitizes human cancer cells to TRAIL-induced apoptosis.

A, apoptotic morphological changes in HeLa cells with combined treatment of luteolin (40 μM× 8 h) and TRAIL (1 ng/ml× 6 h). Top: cells pictured under a normal light microscope; bottom: the cells with DAPI staining under an inverted fluorescence microscope. B, colony formation assay. HT29 cells were plated on six-well plates (5,000 cells/well) and treated with luteolin alone (40 μM), TRAIL alone (1 ng/mL), or their combination for 3 weeks. The survival clones were stained with 0.5 % crystal violet.

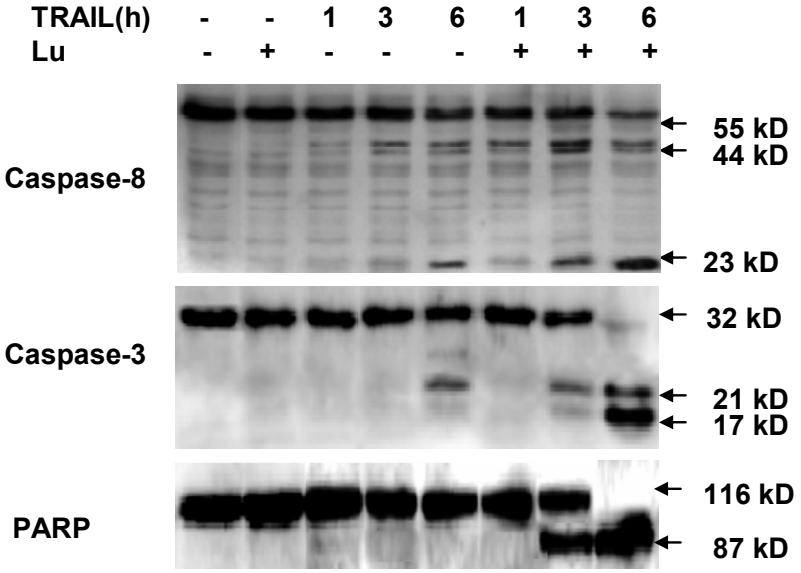


Figure 5.4 Luteolin and TRAIL induces caspase activation.

HeLa cells were treated with TRAIL (1 ng/ml) for the indicated periods with or without the presence of luteolin pretreatment (40 μM × 2 h). Cells were collected and subjected to Western blot for detection of the cleavage of caspase-8, caspase-3 and PARP.

We then used various caspase inhibitors to confirm the role of the observed caspase cascade in the cell death induced by luteolin and TRAIL. Figures 5.5 and 5.6 show that z-DEVD-fmk (a caspase-3 inhibitor), z-IETD-fmk (a caspase-8 inhibitor) and z-VAD-fmk (a pan caspase inhibitor) completely blocked caspase-3 activation and cell death induced by luteolin and TRAIL. One interesting finding here is that z-DEVD-fmk, the specific inhibitor of caspase-3, also abrogated caspase-8 cleavage in cells treated with luteolin and TRAIL, indicating the presence of a caspase-8 and caspase-3 positive feed back loop (Shi, 2002).

In certain cells, TRAIL has been demonstrated to induce apoptosis via the intrinsic mitochondrial pathway via caspase-8-mediated Bid cleavage (Shi, 2002). However, in this study we found that a caspase-9 inhibitor did not offer significant protection against luteolin and TRAIL-induced apoptosis (data not shown). Therefore, it is believed that luteolin enhances TRAIL-induced apoptosis mainly by utilizing the cell death receptor pathway.

5.3.3 Luteolin does not alter expression of death receptors

It has been reported that modulation of surface expression of death receptors could sensitize cells to TRAIL-induced apoptosis (Gibson *et al.*, 2000; Nagane *et al.*, 2000). We then tested the changes of various TRAIL death receptors after luteolin treatment by using immunofluorescence staining for the cell surface protein level and RT-PCR for their mRNA level. However, it was found that luteolin treatment did not alter the surface expression of death receptors (DR4, DR5, DcR1 or DcR2) (Figure 5.7). mRNA level of death receptors does not change either after luteolin treatment (Figure 5.8), suggesting that luteolin promotes caspase activation via other mechanisms

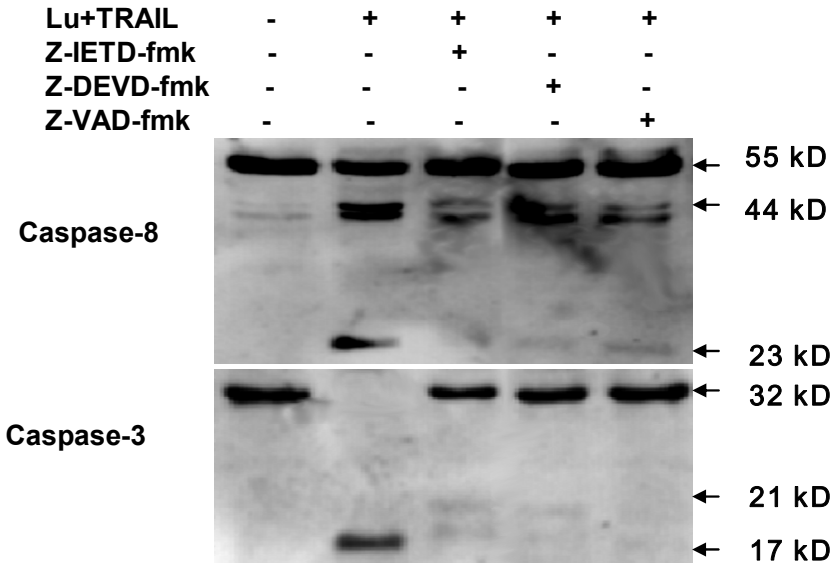


Figure 5.5 Effect of caspase inhibitors on caspase activation induced by luteolin and TRAIL.

HeLa cells were pretreated with z-IETD-fmk (25 μM), z-DEVD-fmk (25 μM) or z-VAD-fmk (25 μM) for 30 min, then cells were treated with a combination of luteolin (40 μM × 8 h) and TRAIL (1 ng/ml × 6 h). Cells were collected for measuring caspase-3 and caspase-8 cleavage by Western blot.

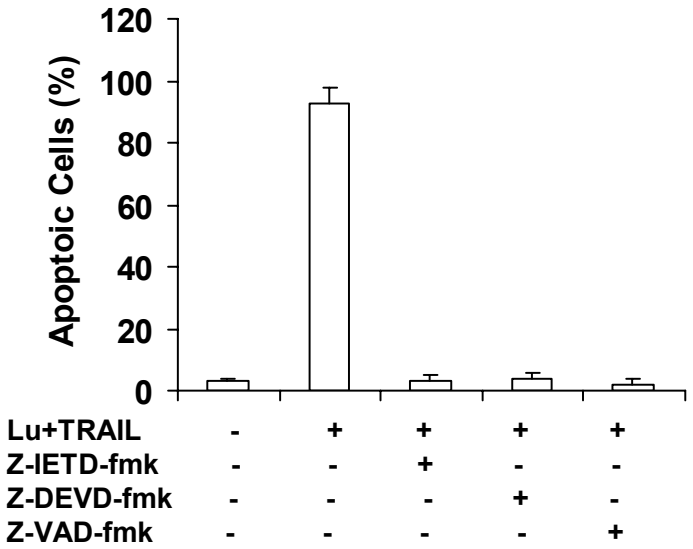


Figure 5.6 Effect of caspase inhibitors on cell death induced by luteolin and TRAIL.

HeLa cells were pretreated with z-IETD-fmk (25 μM), z-DEVD-fmk (25 μM) or z-VAD-fmk (25 μM) for 30 min, then cells were treated with a combination of luteolin (40 μM × 8 h) and TRAIL (1 ng/ml × 6 h). The percentage of apoptosis was evaluated using DAPI staining as described in Figure 5.1.

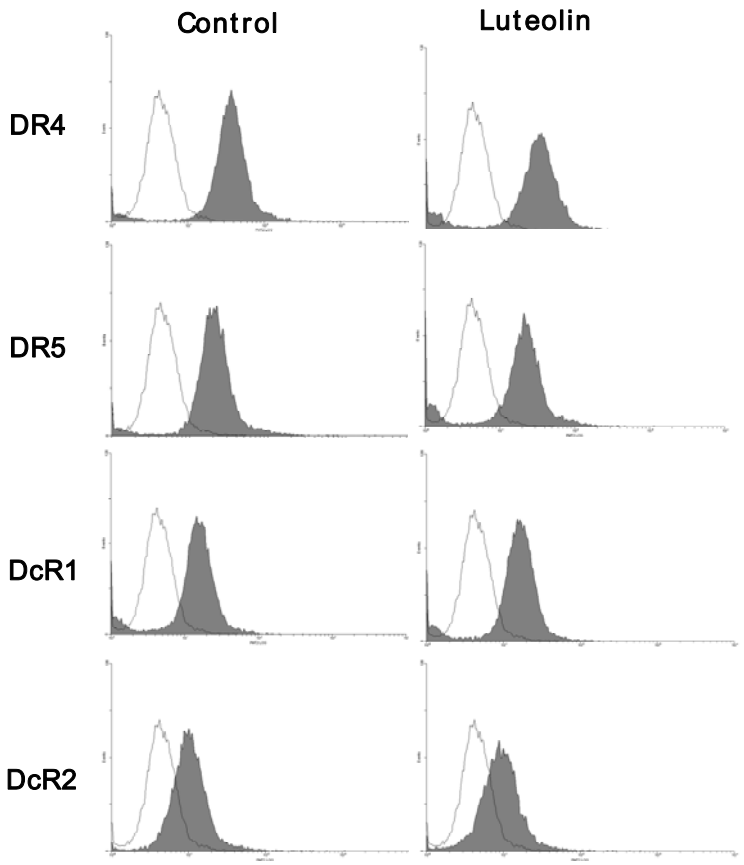


Figure 5.7 Effect of luteolin on expression level of various TRAIL death receptors.

HeLa cells were treated with 40 μM luteolin for 6 h, then collected and washed prior to immunostaining using respective first antibody to DR4, DR5, DcR1 and DcR2, followed by FITC-conjugated secondary antibody. Cells were analyzed by flow cytometry and the histogram were representative from 3 independent experiments. Open frame stands negative control and the closed frame stands for cells with immunostaining.

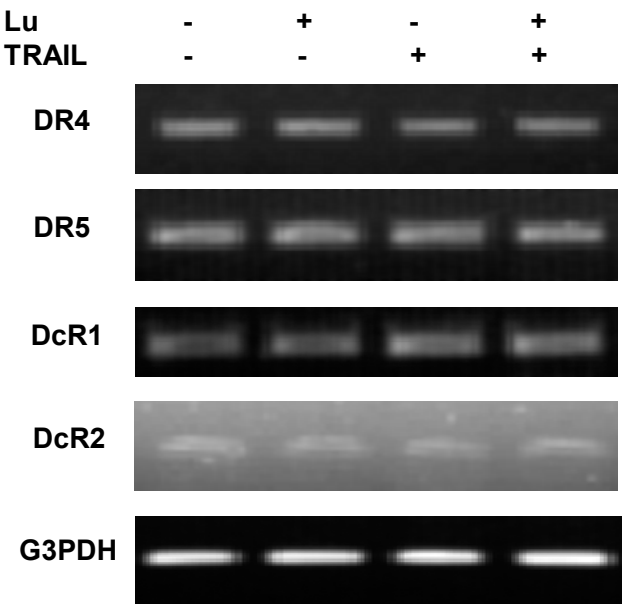


Figure 5.8 Effect of luteolin and TRAIL on death receptor mRNA level.

HeLa cells were treated with luteolin (40 μM) for 2 h followed by TRAIL (1 ng/ml) 2 h. Or cells were treated with luteolin alone 4 h or TRAIL alone 2 h. Cells were then collected for detection of mRNA level of DR4, DR5, DcR1 and DcR2 using RT-PCR.

5.3.4 NF-κB is not involved in the sensitization of luteolin

On the other hand, NF-κB is a potent anti-apoptotic factor in TNF-induced apoptosis (Wang *et al.*, 1998; Yamamoto and Gaynor, 2001). In previous chapter, we have found that luteolin sensitized TNF-induced cell death through inhibition of NF-κB. Although TRAIL-induced NF-κB activation has been observed in certain cells (Zauli *et al.*, 2004), in this study NF-κB is unlikely to be important in the sensitization activity of luteolin on TRAIL-induced-apoptosis, based on the finding that either TRAIL or luteolin did not change NF-κB luciferase activity (Figure 5.9). In contrast, luteolin pretreatment successfully blocked the TNF-induced NF-κB transcriptional activation. Such a finding is consistent with an earlier report that sensitivity to TRAIL-induced apoptosis is not significantly modulated by transfection of dominant negative mutants of IKKβ or IκBα (Leverkus *et al.*, 2003).

5.3.5 XIAP down-regulation contributes to the sensitized cell death

It has been well documented that a number of cellular proteins are important regulators in apoptosis via inhibition of the caspase cascade. Those proteins include FLIP, c-IAP, Bcl-2, Bcl-xL and XIAP, which are known to be regulated by NF-κB at the transcriptional level (Deveraux and Reed, 1999; Micheau *et al*., 2001; Yamamoto and Gaynor, 2001). In search of the molecular mechanisms which may be involved in the sensitization activity of luteolin, we tested the changes of these proteins in cells treated with TRAIL with or without luteolin pretreatment. The protein levels of FLIP, c-IAP1, c-IAP2, Bcl-2 and Bcl-xL remained constant among various treated groups (Figure 5.10). This finding is basically consistent with the earlier observation that TRAIL or luteolin is unable to affect NF-κB transcriptional activity in HeLa cells (Figure 5.9). Interestingly, the protein levels of two anti-apoptotic proteins, Mcl-1 and XIAP, significantly decreased as cells undergoing apoptosis (Figure 5.11A). However,

the decrease of Mcl-1, but not XIAP, was reversed in the presence of z-VAD-fmk, a pan caspase inhibitor, indicating that the reduction of Mcl-1 protein level is the result of caspase activation (Herrant *et al.*, 2004), while XIAP down-regulation is caspase-independent. Figure 5.11B demonstrates the dose-dependent pattern of XIAP down-regulation in cells treated with luteolin and TRAIL, which is consistent with the dose-dependent pattern of cell death observed above (Figure 5.2). Luteolin-dependent reduction of XIAP protein level was also observed in two other TRAIL-resistant cell lines (HT29 and HepG2) (data not shown).

To further confirm the role of XIAP in the cell death induced by luteolin and TRAIL, we examined whether XIAP overexpression will protect the cell death. HeLa cells were transiently transfected with either wild-type XIAP plasmid (Flag-XIAP) or an empty vector (pcDNA). Red fluorescence protein plasmid (pDsRed) was used as a transfection marker. In pcDNA-transfected cells, almost all cells died after luteolin and TRAIL treatment based on the morphological changes. In contrast, most XIAP-overexpressing cells remained alive while those non-transfected cells underwent cell death (Figures 5.12 and 5.13). The above results thus strengthen our argument that XIAP down-regulation plays a critical role in luteolin and TRAIL induced cell death.

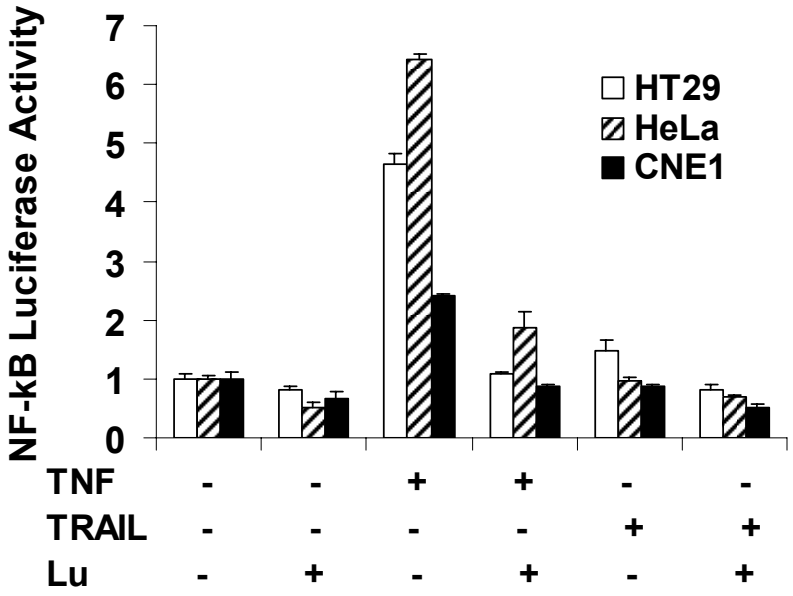


Figure 5.9 Effect of TRAIL and luteolin on NF-κB transcriptional activity.

Three human cancer cells were transfected with NF-κB-luciferase construct and β-galactosidase construct for 24 h, followed by TNF (15 ng/ml × 2 h) or TRAIL (1 ng/ml × 2 h for HeLa and CNE1, 25 ng/ml × 4 h for HT29) in the presence or absence of luteolin pretreatment (40 μM × 2 h). NF-κB luciferase activity was normalized by β-galactosidase activity and expressed as folds over the control.

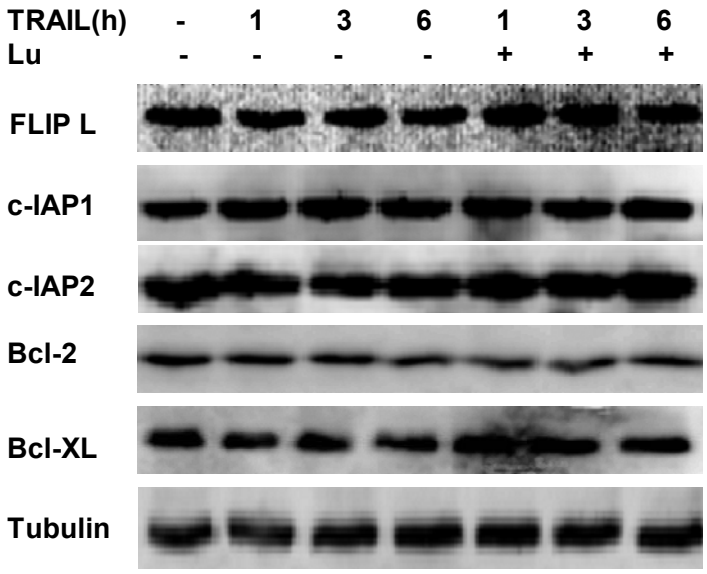


Figure 5.10 Effect of luteolin and TRAIL on expression of anti-apoptotic proteins.

HeLa cells were treatment with TRAIL (1 ng/ml) for indicated periods with or without presence of luteolin pretreatment (40 μM × 2 h). Cells were collected for detection of FLIPL, c-IAP1, c-IAP2, Bcl-2 and Bcl-xL protein level by Western blot. Tubulin was used as a loading control.

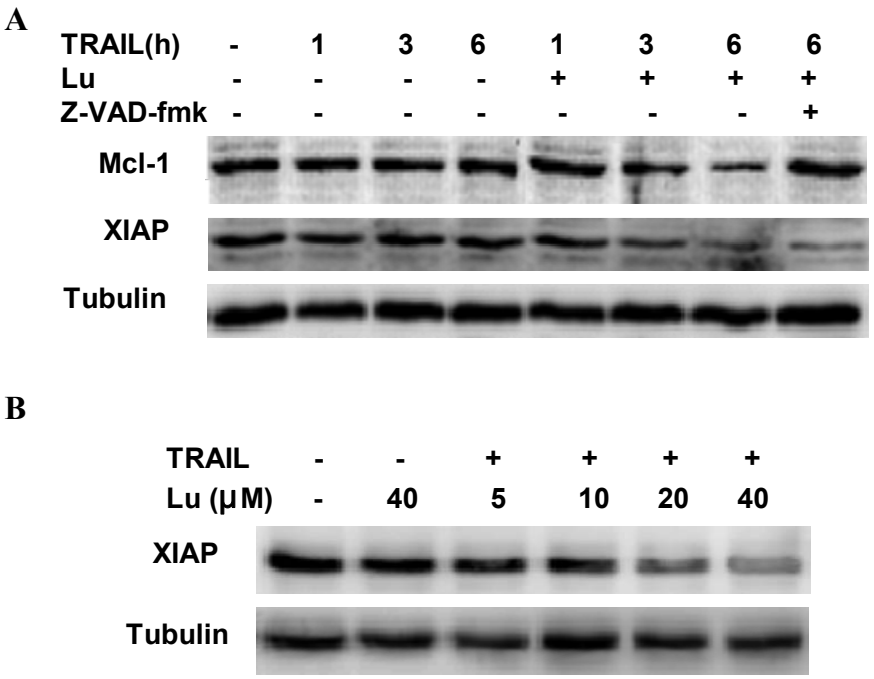


Figure 5.11 Down-regulation of XIAP in cells treated with luteolin and TRAIL.

A, HeLa cells were first pretreated with z-VAD-fmk (25 μM × 30 min), then cells were treated with TRAIL (1 ng/ml) for indicated periods with or without luteolin pretreatment (40 μM × 2 h). Cells were collected for detection of Mcl-1 and XIAP by Western blot. **B**, HeLa cells were treated with indicated concentrations of luteolin for 2 h, followed by TRAIL (1 ng/ml) for additional 6 h and then cells were collected for detection of XIAP protein level by Western blot. Tubulin was used as a loading control.

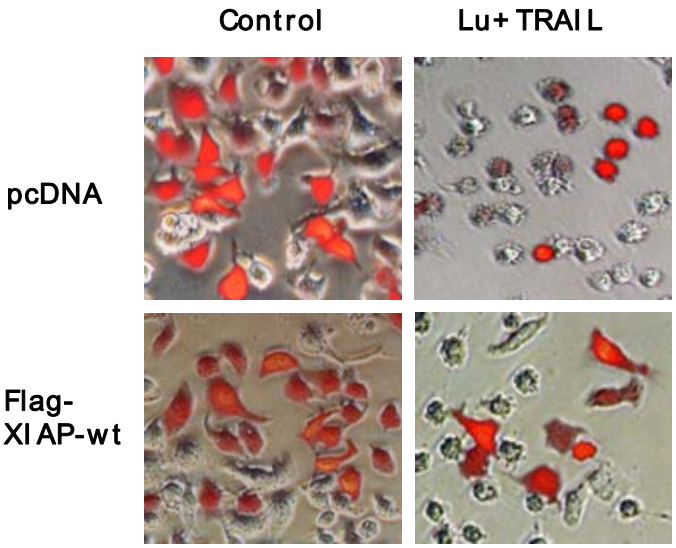


Figure 5.12 Ectopic expression of XIAP protects cell death induced by luteolin and TRAIL.

HeLa cells were transiently transfected with either pcDNA or Flag-XIAP-Wt, together with pDsRed as a transfection marker. After 24 h, the cells were treated with a combination of luteolin (40 μM × 8 h) and TRAIL (1 ng/ml × 6 h). Cell death was then evaluated by morphological changes under a fluorescent microscope and those successfully transfected cells were in bright red.

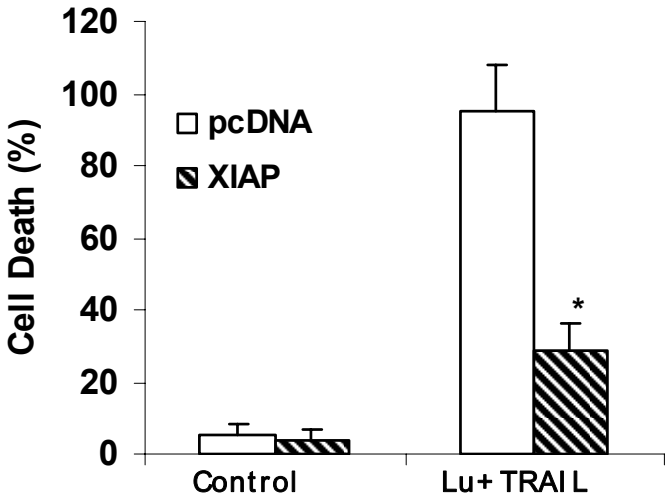


Figure 5.13 Ectopic expression of XIAP protects cell death induced by luteolin and TRAIL (Quantification).

Cells were treated as described in Figure 6.12. Cell death was quantified by counting the percentage of dead cells among 200 randomly selected transfected cells. Data are presented as means ± SD from 3 independent transfection experiments. * $p<0.05$ comparing to the group with pcDNA transfection (One-way ANOVA with Scheffe's test).

5.3.6 XIAP down-regulation is mediated by ubiquitination and proteasomal degradation

The down-regulation of XIAP could be due to modulation at either transcriptional or post-transcriptional level. In order to elucidate the molecular mechanism involved, we first measured the XIAP mRNA level using RT-PCR. As shown in Figure 5.14, either luteolin, TRAIL, or their combined treatment did not alter the XIAP mRNA level up to 4 h, suggesting that the XIAP is mainly regulated post-transcriptionally.

In order to determine whether the decreased XIAP level is due to proteasomal degradation, here we tested the effects of proteasome inhibitors on XIAP protein level. As shown in Figure 5.15A, MG132 (1 μM), PSI (5 μM) or PSII (5 μM) completely abolished the XIAP down-regulation induced by luteolin and TRAIL. The effect of MG132 was also found to be dose-dependent; and a low concentration of MG132 (0.1 μM) only partially prevented XIAP degradation (data not shown). Since XIAP is probably the most potent apoptosis inhibitor, the stabilization of XIAP would render cells resistant to apoptosis induced by luteolin and TRAIL. Such a hypothesis was supported by the results shown in Figure 5.15B that the three proteasome inhibitors were able to completely prevent cell death induced by luteolin and TRAIL. It is thus believed that the down-regulation of XIAP protein through proteasomal degradation is the underlying mechanism in the sensitization effect of luteolin on TRAIL-induced apoptosis. Similar results were also found in other cell lines such as HT29 and HepG2 (data not shown).

It is known that XIAP has ubiquitin protease ligase (E3) activity and the autoubiquitination and degradation is an important mechanism for regulating the XIAP function in apoptosis (Yang *et al.*, 2000b; Zhang *et al.*, 2004). Here we further

examined whether treatment with luteolin and TRAIL promotes XIAP ubiquitination by directly measuring XIAP ubiquitination. It was found that the combined treatment of luteolin and TRAIL significantly enhanced the level of ubiquitylated XIAP in HeLa cells in the presence of proteasome inhibitor MG132 (Figure 5.16). In consistent, the level of total protein ubiquitination was also increased by luteolin and TRAIL in the presence of MG132. Similar results were also in HT29 and HepG2 cells (data not shown). The above results thus clearly demonstrate that luteolin and TRAIL promotes XIAP degradation by enhancing its ubiquitination.

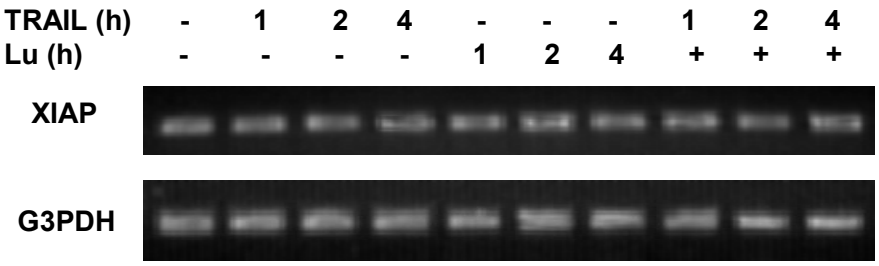


Figure 5.14 Effect of luteolin and TRAIL on XIAP mRNA level.

HeLa cells were treated with luteolin (40 μM), or TRAIL (1 ng/ml) or a combination of both for indicated periods. Cells were collected for detection of XIAP mRNA level using RT-PCR. G3PDH was used as a loading control.

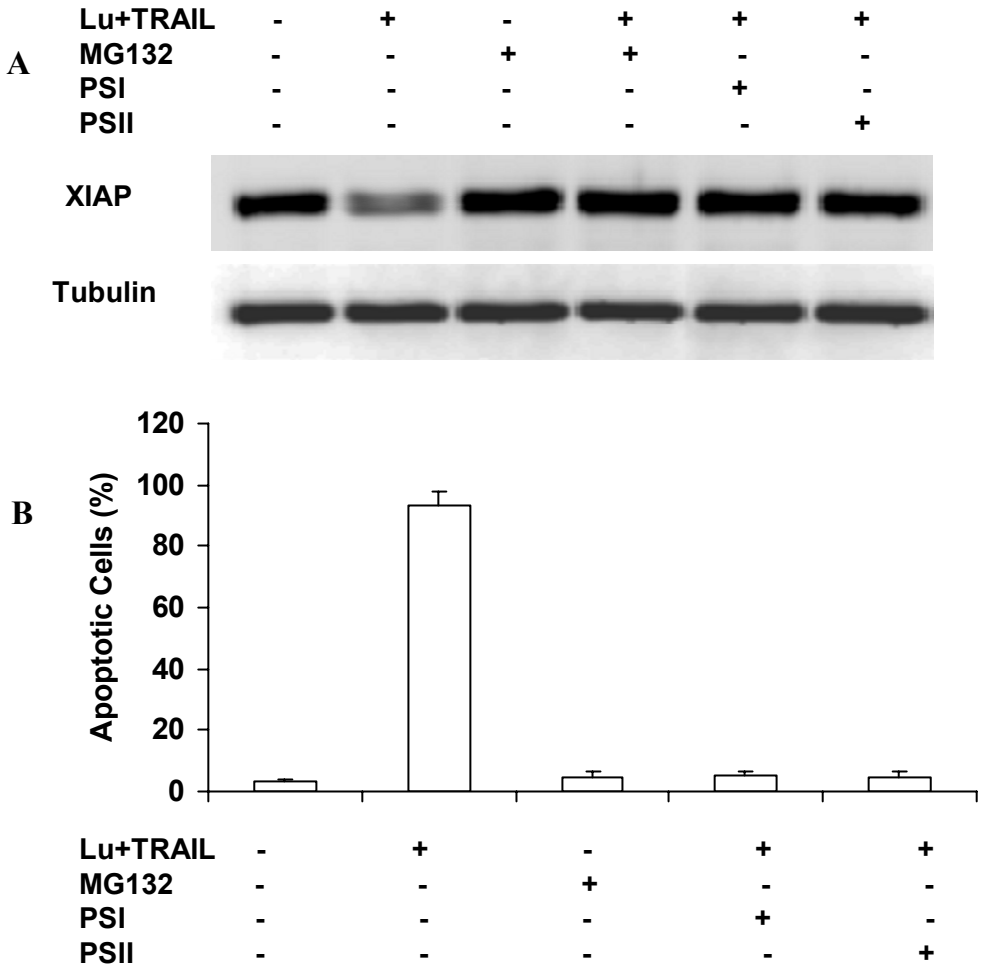


Figure 5.15 XIAP down-regulation is through proteasomal degradation in cells treated with luteolin and TRAIL.

A, HeLa cells were pretreated with proteasome inhibitor MG132 (1 μM), PSI (5 μM), or PSII (5 μM) for 1 h, followed by combined treatment of luteolin (40 μM × 8 h) and TRAIL (1 ng/ml × 6 h). XIAP protein level was determined by Western blot. B, HeLa cells were treated as described in A and the percentage of apoptotic cell death was evaluated by DAPI staining.

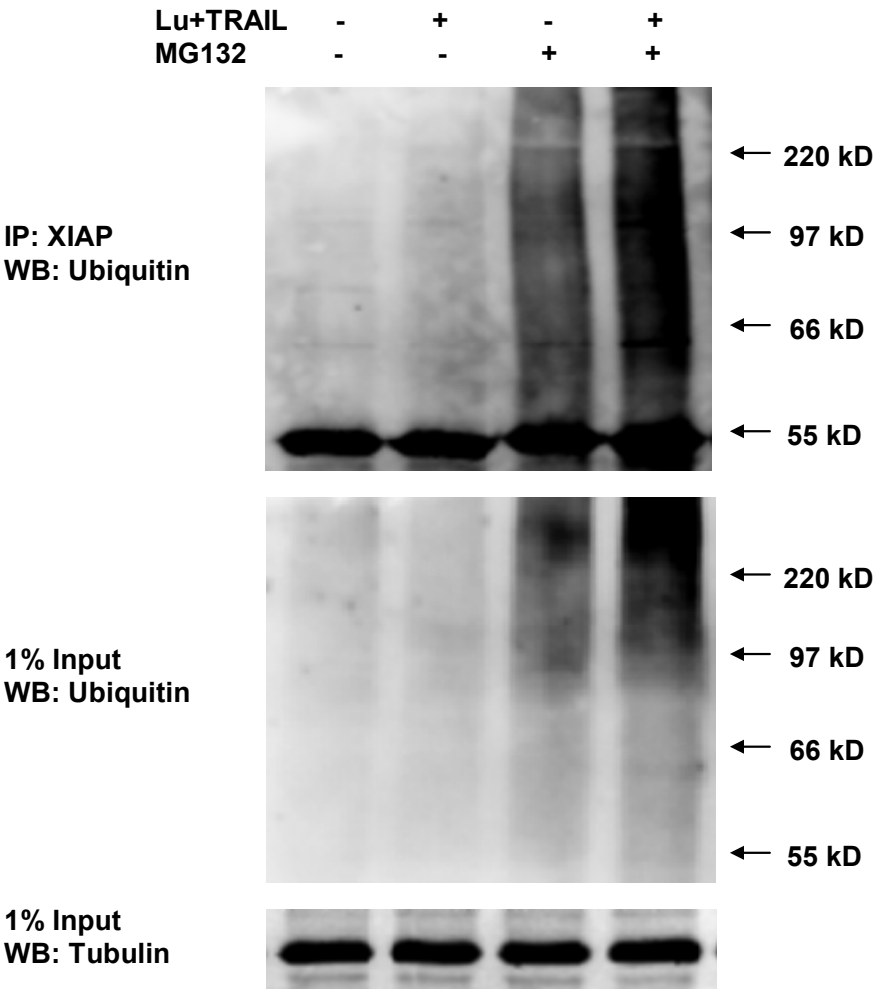


Figure 5.16 A combination of luteolin and TRAIL promotes XIAP ubiquitination.

HeLa cells were pretreated with proteasome inhibitor MG 132 (1 μM) for 2 h before combined treatment with luteolin (40 μM) and TRAIL (1 ng/mL) for another 2 h. Cell lysate was used for immunoprecipitation with anti-XIAP antibody, followed by Western blot using anti-ubiquitin antibody.

5.3.7 PI3K/AKT is not involved in cell death induced by luteolin and TRAIL

Previous studies have shown that the PI3K-AKT pathway plays a protective role in TRAIL-induced apoptosis (Thakkar *et al.*, 2001) and one of the mechanisms is that AKT phosphorylates and stabilizes XIAP by inhibiting its ubiquitination (Dan *et al.*, 2004). On the other hand, it is known that phorbol 12 myristate 13 acetate (PMA) is capable of protecting cells from TRAIL-induced apoptosis (Harper *et al.*, 2003b). In our study, PMA pretreatment also completely prevented luteolin and TRAIL induced cell death (Figure 5.17).

It has been well established that PMA stimulates a series of downstream signals including PI3K-AKT, MAPK and PKC (Thakkar et al., 2001; Harper et al., 2003b). We examined the involvement of each signaling pathway in the protective activity of PMA using various specific inhibitors. The two PI3K inhibitors (LY and Wort) failed to reverse the protective effect of PMA (Figure 5.17). Similar negative results were also found with a JNK inhibitor (SP600125), a p38 inhibitor (SB203580), or an ERK inhibitor (PD98059) (data not shown). The effectiveness of these two inhibitors on the PI3K/AKT pathway was confirmed in PMA-stimulated cells (Figure 5.18A). We also found that either TRAIL or luteolin alone or their combination has no effect on AKT activation (Figure 5.18B). Therefore, the above data indicate that neither the PI3K-AKT nor the MAPK pathway plays a critical role in the protective effect of PMA against the apoptosis induced by luteolin and TRAIL.

5.3.8 PKC activation blocks XIAP degradation and prevents the cell death induced by luteolin and TRAIL

It has been reported that PKC activation plays a protective role against TRAIL-induced apoptosis (Harper *et al.*, 2003b). Here we attempted to explore the

possible role of PKC in luteolin and TRAIL-induced apoptotic cell death. First, BIM, a general PKC inhibitor, is capable of abolishing the protective effect of PMA on luteolin and TRAIL-induced cell death (Figure 5.19A), suggesting that the protective effect of PMA is mediated via PKC activation. Second, we asked whether PKC activation is associated with changes of XIAP protein level. As shown in Figure 5.19B, PMA pretreatment completely prevented XIAP degradation in cells treated with luteolin and TRAIL. Moreover, such an effect by PMA on XIAP was completely abolished by BIM, thus suggesting that PMA-mediated PKC activation is able to stabilize XIAP and subsequently prevent apoptosis. The effectiveness of BIM on PKC activation was confirmed by the overall PKC activity which was assessed using an anti-phospho (Ser)-PKC substrate antibody by Western blot (Tanaka *et al.*, 2003). As expected, PMA readily activated PKC and this activation was completely blocked by BIM but not LY and Wort (Figure 5.20), clearly suggesting that the protective effect of PMA is mediated via PKC activation.

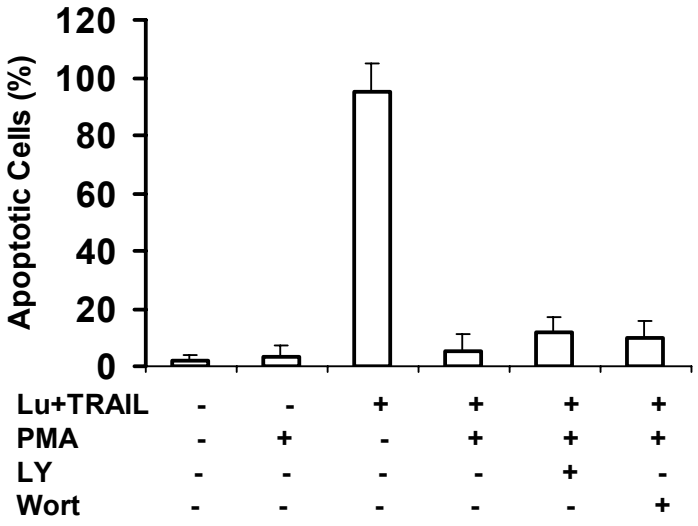


Figure 5.17 Effect of PMA on the cell death induced by luteolin and TRAIL.

HeLa cells were pretreated with either 10 μM LY or 0.5 μM Wort for 30 min, followed by treatment with PMA (80 ng/ml × 30 min) and finally with a combination of luteolin (40 μM × 8 h) and TRAIL (1 ng/ml × 6 h). Cell death was evaluated by DAPI staining.

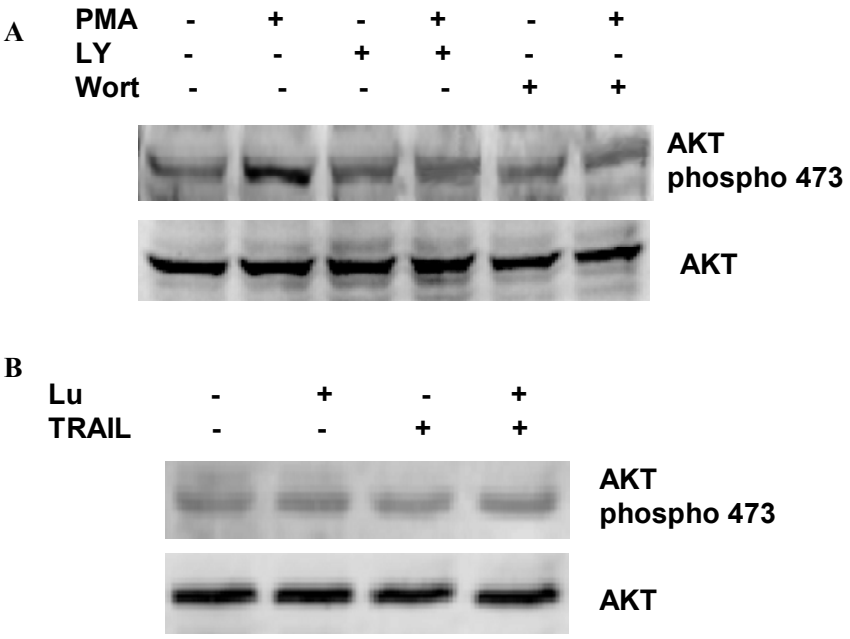


Figure 5.18 Effect of luteolin and TRAIL on PI3K/AKT pathway.

A, HeLa cells were pretreated with LY (10 μM) or Wortmannin (1 nM) for 60 min followed by PMA (80 nM) treatment for 1 h. Cells were collected for detection of activation of AKT by Western blot using anti-phospho 473-AKT. Total AKT level was used as loading control. **B**, HeLa cells were pretreated with luteolin for 2 h followed by TRAIL for 1 h. Cells were collected for detection of activation of AKT by Western blot using anti-phospho 473-AKT. Total AKT level was used as loading control.

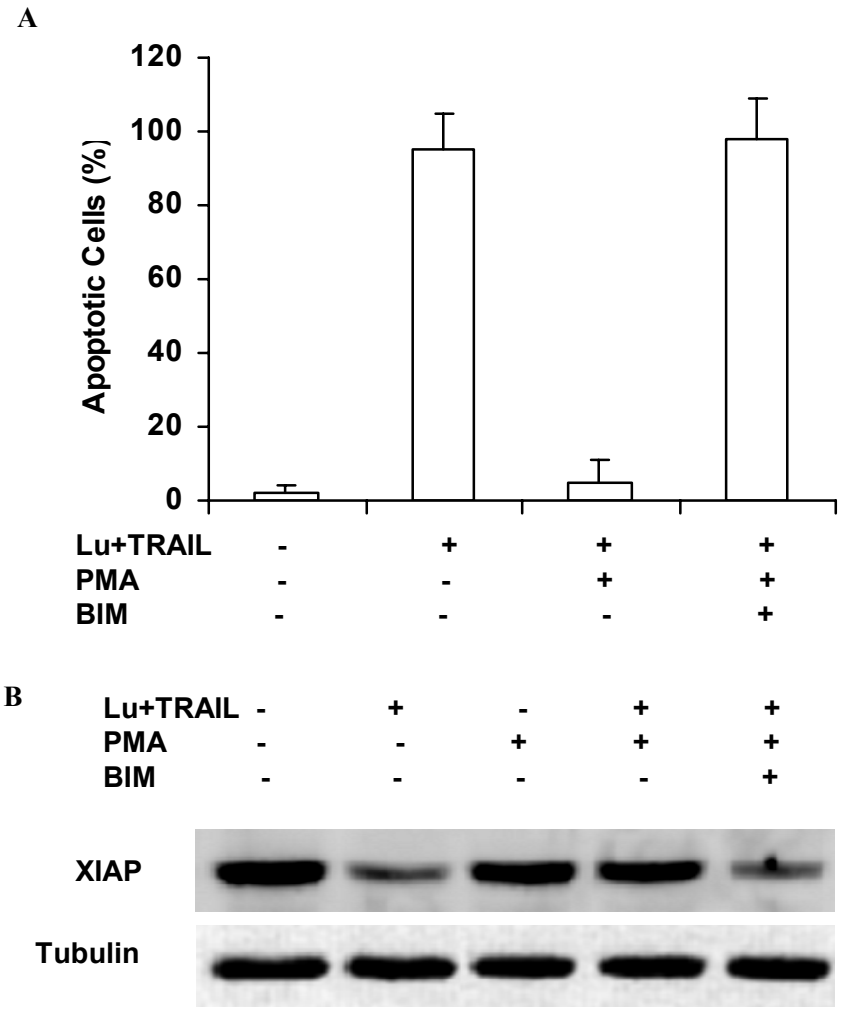


Figure 5.19 PKC activation protects cell death and XIAP down-regulation induced by luteolin and TRAIL.
A, HeLa cells were first pretreated with 10 μM BIM for 30 min, followed by treatment with PMA (80 ng/ml × 30 min), and finally with combined treatment of luteolin (40 μM × 8 h) and TRAIL (1 ng/ml × 6 h). Cell death was evaluated by DAPI staining. **B**, HeLa cells were treated as in panel A and XIAP protein level was detected by Western blot. Tubulin was used as a loading control.

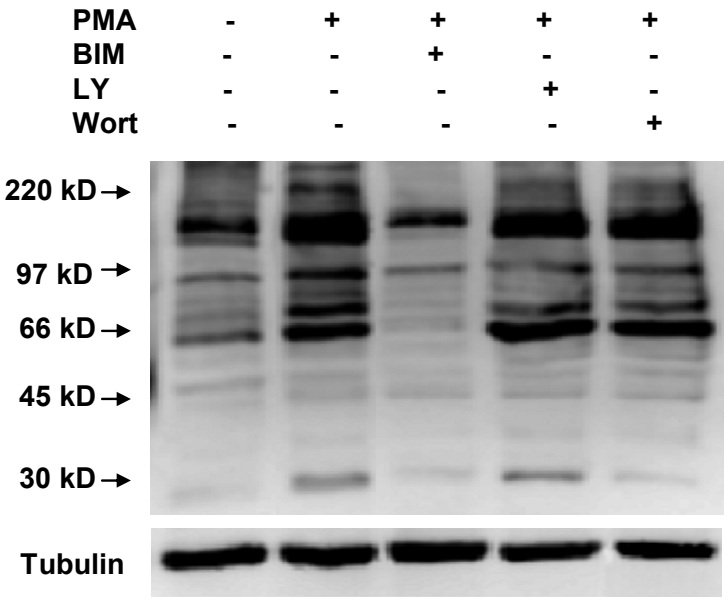


Figure 5.20 Effect of LY, Wort and BIM on PMA-induced PKC activation.

HeLa cells were pretreated with either 10 μM BIM, 10 μM LY or 0.5 μM Wort for 30 min, followed by treatment with PMA (80 ng/ml × 30 min). Cells were collected for detection of PKC activation by Western blot using anti-phospho (Ser)-PKC substrate antibody. Tubulin was used as a loading control.

5.3.9 PKC inhibition promotes XIAP down-regulation and apoptosis in TRAIL-treated cells

As the above data on PKC were all obtained from cells stimulated with PMA, next we examined whether the sensitization activity of luteolin also involves PKC without the presence of PMA. An earlier report showed that TRAIL activates PKC in one pancreatic adenocarcinoma cell line (PancTuI) but marginally in another (Colo357 cells) (Trauzold *et al.*, 2001), suggesting the effect of TRAIL on PKC is cell-type specific. In this study, there was considerable degree of basal PKC activation in the control HeLa cells and marginal PKC activation by TRAIL. In contrast, luteolin pretreatment markedly reduced both the basal PKC and PKC activation by TRAIL, which is similar to the effect of BIM (Figure 5.21). These data thus support the hypothesis that luteolin sensitizes TRAIL-induced apoptosis through PKC inhibition.

To confirm the role of PKC in XIAP stability and the possible mechanisms involved, we further tested the effect of BIM on XIAP protein level in cells treated with TRAIL. Combined treatment of BIM and TRAIL significantly down-regulated XIAP level, a process not affected by z-VAD-fmk (data not shown), but prevented by MG132 (Figure 5.22A), which is similar to effect of luteolin on TRAIL-induced XIAP down-regulation as shown earlier (Figures 5.11A and 5.15A). Furthermore, similar to the sensitization activity of luteolin, BIM also significantly enhanced TRAIL-induced cell apoptosis (Figure 5.22B). Similar results were found in TRAIL-resistant cells (HT29 and HepG2, data not shown). Therefore, data from this part of our study demonstrate that PKC activation plays a protective role in TRAIL-induced apoptosis via stabilization of XIAP and that luteolin may act as a PKC inhibitor to sensitize cancer cells to TRAIL-mediated apoptotic cell death.

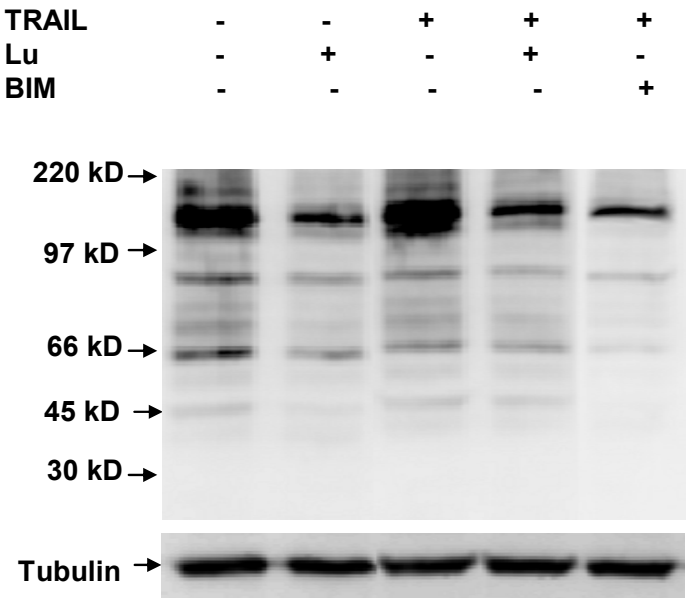


Figure 5.21 Effect of luteolin on PKC activation.

HeLa cells were first pretreated with luteolin (40 μM) or BIM (10 μM) for 30 min, followed by TRAIL (1 ng/ml for 1 h). Cells were then collected for detection of PKC activation by Western blot using anti-phospho (Ser)-PKC substrate antibody. Tubulin was used as a loading control

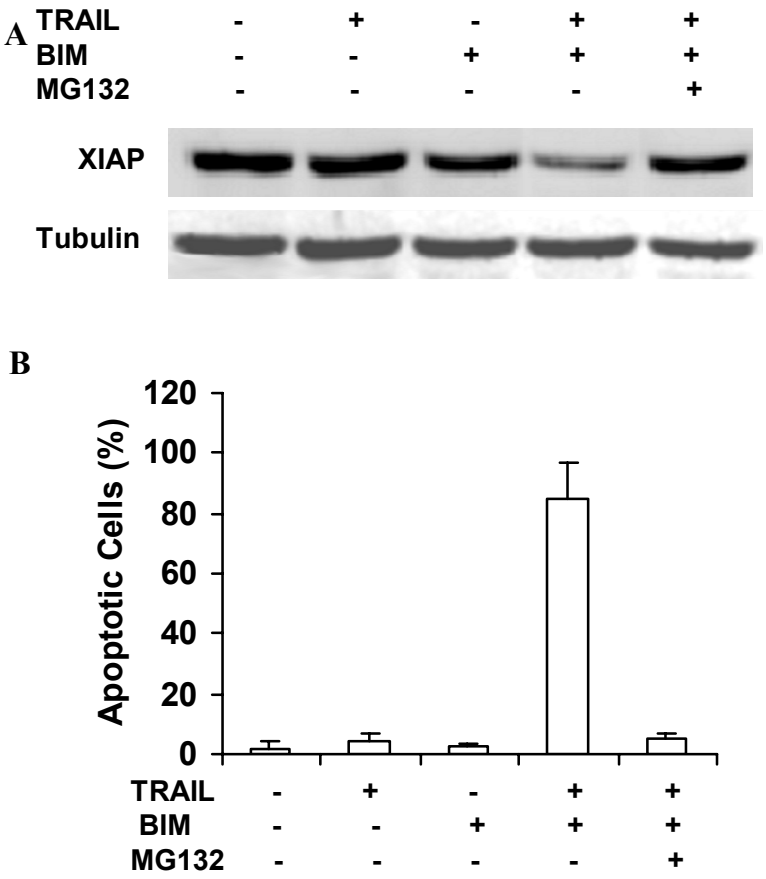


Figure 5.22 A combination of PKC inhibition and TRAIL enhances XIAP degradation and cell death.

A, HeLa cells were pretreated with BIM (10 μM), and/or MG132 (1 μM) for 30 min, followed by TRAIL (1 ng/ml × 6 h). XIAP protein level was detected by Western blot. Tubulin was used as a loading control. B, cells were treated as described in panel A and cell death was evaluated by DAPI staining.

5.4 DISCUSSION

TRAIL is a potent therapeutic agent due to its unique property of killing cancer cells by apoptosis but sparing normal cells. However, many cancer cells are found to be resistant to TRAIL, thus limiting its clinical application. A number of factors may be involved in the resistance, including modified expression of surface death receptors or changes of anti-apoptotic proteins (Wang and El Deiry, 2003). One effective strategy to overcome TRAIL resistance is to combine TRAIL with other anti-cancer agents (Gibson *et al*., 2000; Nagane et al., 2000; Huerta-Yepez *et al.*, 2004; Rosato *et al.*, 2004; von Haefen *et al*., 2004). We demonstrated in an earlier study that luteolin sensitized TNF-induced apoptosis in cancer cells (Chapter 4). We here reported that luteolin also sensitizes TRAIL-induced apoptosis in a number of cancer cells. Such sensitization effect of luteolin is probably achieved via inhibition on PKC activation, promotion of XIAP ubiquitination and proteasomal degradation, which then removes the blockage on caspase and enhances apoptosis.

Data from this study have demonstrated that the sensitization activity of luteolin on TRAIL-induced apoptosis is mainly executed through the cell death receptor pathway and luteolin sensitizes TRAIL-induced apoptosis via enhanced caspase-3 maturation (Figures 5.4 and 5.5). It was reported that luteolin upregulated DR5 protein level in HeLa cells only after 12 h (Horinaka *et al.*, 2005). This is consistent of our finding that luteolin treatment did not alter the expression level of TRAIL death receptors (DR4 and DR5) or its decoy receptors (DcR1 and DcR2) in 6 h (Figures 5.7 and 5.8), suggesting that the slow increase of DR5 level unlikely contributes to the rapid sensitized cell death (4 h). We then went on to screen the changes of other apoptosis regulatory proteins after luteolin and TRAIL treatment. Among many anti-apoptotic proteins tested, we found that XIAP was significantly

down-regulated by combined treatment of luteolin and TRAIL, but not by their individual treatment (Figure 5.11). More importantly the XIAP down-regulation was caspase-independent, indicating the down-regulated XIAP level is upstream of caspase-3 activation. XIAP, a member of IAP family, is probably the most potent apoptosis inhibitory protein and plays important roles in cell survival. XIAP is characterized by baculoviral IAP repeat (BIR) domains, which can inhibit caspase-3 and caspase-9 activity by direct binding (Deveraux *et al.*, 1997; Deveraux and Reed, 1999; Riedl *et al.*, 2001). In this study, it appears that luteolin sensitizes TRAIL-induced apoptosis by targeting XIAP to remove the blockage on caspase-3 activation and cell death. Such a hypothesis was further supported by the fact that over-expression of XIAP protein offers complete protection against luteolin and TRAIL-induced apoptosis (Figures 5.12 and 5.13).

We further examined the possible mechanisms contributing to the reduced XIAP protein level in cells treated with luteolin and TRAIL. Since either luteolin, TRAIL or their combined treatment had no effect on the XIAP mRNA level (Figure 5.14), the reduced XIAP protein level is most probably the result of enhanced post-transcriptional degradation. The effect of luteolin is apparently different from that of flavopiridol which acts synergistically with TRAIL by suppression of XIAP gene transcription (Rosato et al., 2004). It is known that the RING finger domain of XIAP has ubiquitin protease ligase (E3) activity and is responsible for its autoubiquitination and degradation after an apoptosis stimulus (Yang *et al.*, 2000b). Here we tested whether luteolin and TRAIL promote XIAP ubiquitination and subsequent proteasomal degradation., the three proteasome inhibitors offered complete protection against both XIAP degradation (Figure 5.15A) and cell death (Figure 5.15B) in cells treated with luteolin and TRAIL. By performing XIAP immunoprecipitation and

ubiquitin western blot analysis, we then provided direct evidence showing that the level of ubiquitylated XIAP was enhanced in cells treated with luteolin and TRAIL. It is thus believed that the decreased XIAP protein level is mediated via ubiquitination and proteasomal degradation, a process crucial for deciding the susceptibility to apoptosis induced by luteolin and TRAIL in cancer cells.

One interesting finding in this study is that PMA pretreatment completely blocked the XIAP down-regulation and apoptotic cell death induced by luteolin and TRAIL (Figure 5.17). PMA is a potent inducer for a number of important cell signaling pathways, including the PI3K-AKT pathway (Hah *et al.*, 2003). It has been reported that AKT activation stabilizes XIAP through enhanced phosphorylation and suppressed ubiquitination and proteasomal degradation (Dan *et al*., 2004). It has also been noted that over-expression of active AKT render TRAIL-sensitive cells to be resistant (Chen *et al.*, 2001; Nesterov *et al.*, 2001). In this study, pretreatment with the two PI3K inhibitors (LY or Wort) did not alter the cell sensitivity to TRAIL-induced apoptosis (Data not shown). No evident change of AKT activation was found in cells treated with either luteolin, TRAIL alone or their combination (Figure 5.18B). Moreover, LY or Wort failed to abrogate the protection effect of PMA against apoptosis induced by luteolin and TRAIL (Figure 5.17). Therefore, it appears that the PI3K-AKT pathway is not an important factor in luteolin and TRAIL-mediated XIAP down-regulation and apoptosis.

We next turned our attention to the possible involvement of PKC in luteolin and TRAIL-mediated XIAP and apoptosis since PMA is also known to be a potent stimulus for both classical and novel PKC activation (Tanaka *et al.*, 2003). In this study we first confirmed that PKC activation contributes to the protective effect of PMA against luteolin and TRAIL-induced apoptosis by the following observations: (i)

PMA activates PKC (Figure 5.20) and (ii) a general PKC inhibitor (BIM) prevents PKC activation and abolishes the protective effect of PMA (Figure 5.22). It has been reported that PKC activation plays a protective role in TRAIL-induced apoptosis (Harper *et al*., 2003b). The protective effects of PKC against TRAIL-induced apoptosis could be achieved through interfering with DISC formation (Harper *et al*., 2003b) or disrupting proteolytic cleavage of procaspase-8 (Meng *et al.*, 2002) or affecting caspase-8-mediated Bid cleavage (Sarker *et al.*, 2002). Here, we propose a novel mechanism to illustrate the anti-apoptotic function of PKC: PKC activation is associated with decreased XIAP proteasomal degradation and increased stability. Such a hypothesis is supported by the findings that PMA pretreatment prevents XIAP down-regulation while BIM reverses the effect of PMA on XIAP protein level in cells treated with luteolin and TRAIL (Figure 5.22). The suggested close linking between PKC activation and XIAP level indicates that there might be a positive correlation between the basal PKC level and XIAP level among cells with different sensitivity. However, we did not find any correlation after comparing the basal PKC and XIAP level among both TRAIL-sensitive cells and TRAIL-resistant cells (data not shown). It is possible that the basal level PKC activity and XIAP are not the only determining factors in the cellular response to TRAIL. It is known that phosphorylation of XIAP by some other protein kinases such as AKT protects XIAP from ubiquitination and proteasomal degradation (Dan et al., 2004). It is thus possible that PKC acts through a similar mechanism to stabilize XIAP. Thus, a number of important questions remain to be further investigated. For instance, does XIAP serve as the direct substrate for PKC? If so, which specific PKC subunit is involved? Further studies on these topics will certainly shed lights on the underlying mechanisms controlling TRAIL resistance and sensitivity in cancer cells.

It was reported that certain flavonoids, including luteolin, inhibited PKC activity in some *in vitro* cell-free systems (Ferriola *et al*., 1989; Agullo *et al.*, 1997). In this study, luteolin was found to significantly block both the basal PKC activation in control cells and the enhanced PKC activation in TRAIL-treated cells (Figure 5.21). Furthermore, the general PKC inhibitor (BIM) mimics the effect of luteolin: pretreatment with BIM down-regulated XIAP protein level in the presence of TRAIL (Figure 5.22A) and greatly caused apoptosis (Figure 5.22B). Therefore it is likely that luteolin acts as a PKC inhibitor to facilitate XIAP degradation and to promote TRAIL-mediated apoptosis. However, it is still not clear how luteolin inhibits PKC activity. Through the structure analysis, it has been hypothesized that flavonoids might interfere with the binding of PKC to calcium or diacylglycerol (DAG), but not directly interacting with the functional domain of PKC protein (Ferriola *et al.*, 1989). Interestingly, although either luteolin or BIM could effectively suppress the basal PKC activation in control cells, they failed to cause any evident reduction of basal XIAP protein level. It thus suggests that PKC-induced modification of XIAP has no effect on its stability or expression, but likely acts on the recognition process by the proteosomal degradation pathway. Further study on PKC activation and XIAP ubiquitination and degradation is obviously required to address this issue.

Taken together, we discovered a novel anti-cancer function of luteolin. Luteolin sensitizes TRAIL-induced apoptosis in human cancer cells via inhibition on PKC activation and promotion of XIAP degradation (Figure 5.23). Understanding of such an effect of luteolin supports its potential therapeutic application in overcoming TRAIL resistance, especially in those cancers with elevated level of PKC activation. In addition, we also reveal a novel function of PKC in TRAIL-mediated apoptosis: PKC may protect the cell death by blocking XIAP ubiquitination and degradation

although a firm biochemical link between PKC and XIAP ubiquitination and degradation remains to be further established.

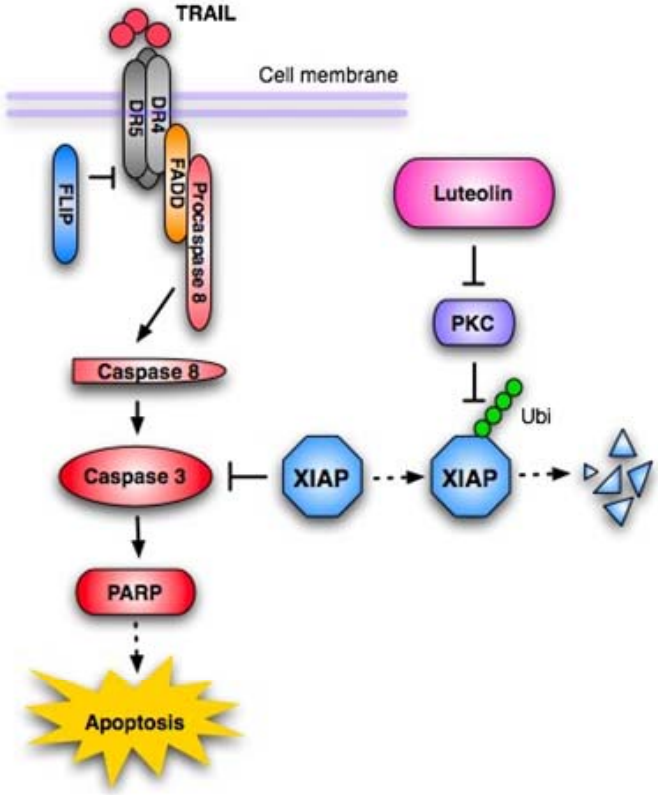


Figure 5.23 Illustration of the pathways involved in the sensitization activity of luteolin on TRAIL-induced apoptosis in cancer cells.

CHAPTER SIX

LUTEOLIN ENHANCES THE ANTI-CANCER EFFECT OF CISPLATIN VIA STABILIZING P53

6.1 INTRODUCTION

Cisplatin (cis-diamminediachloroplatium) and its derivatives are among one of the most effective anticancer drugs used clinically in the treatment of solid tumors, including ovarian, testicular, cervical, and small cell lung cancers (Loehrer, 1984). The anti-cancer effect of cisplatin is mainly depending on its DNA damaging activity, via its direct interaction with DNA to form DNA adducts (Dijt, 1988). Subsequently, several signaling transduction pathways are activated, including ataxia telangiectasia mutated kinase (ATM); ataxia telangiectasia and rad3-related kinase (ATR), p53, mitogen-activated protein kinases (MAPKs). Among them, p53 activation is one of major factors responsible for the apoptosis induced by cisplatin (Siddik, 2003). It has been observed that cisplatin can cause apoptosis in wild type p53 cancer cells but not in p53 deficient or mutant cancer cells, suggesting that p53 is the key regulator for cisplatin-mediated apoptosis in cancer cells (Song *et al.*, 1998; Kanata *et al.*, 2000; Tang and Grimm, 2004; Beuvink *et al*., 2005).

p53 is a short-lived tumor suppressor protein (Ko, 1996; Levine, 1997). Its stability is mainly regulated by its interaction with its transcriptional target mouse double minute 2 (MDM2). Acting as an ubiquitin E3 ligase, MDM2 interacts with p53 directly and promotes its ubiquitination and proteasomal degradation. Hence, p53 level is kept at a low level in p53 wild type cancer cells (Kubbutat, 1997). Upon the DNA damage caused by cisplatin, p53 is up-regulated via increased p53 stability which is achieved through disrupting the interaction between MDM2 and p53. For example, DNA damage-activated ATM, ATR and DNA-PK can phosphorylate p53 on Ser 15 and Ser 37 (Shieh, 1997); Chk2 contributes to phosphorylation of p53 on serine 20 (Shieh, 1999); whereas JNK phosphorylates p53 on tyrosine 81 (Buschmann *et al.*,

2001). Phosphorylation on p53 affects its interaction with MDM2 or its binding to DNA or its transcriptional activity (Steegenga *et al.*, 1996).

One important role of p53 as a tumor suppressor is its involvement in apoptosis. p53 activates caspase cascade and apoptosis mainly via an intrinsic pathway that involves mitochondria, a central regulator of apoptosis. The integrity of outer mitochondrial membrane is tightly regulated by Bcl-2 family proteins. Pro-apoptotic members of the Bcl-2 family, such as Bax, Bak and Bid, form channels in membranes to regulate the release of pro-apoptotic proteins from mitochondria. On the other hand, anti-apoptotic members of the Bcl-2 family, such as Bcl-2 and Bcl-X_L, tend to block the above process. (Kelekar and Thompson, 1998). Activation of p53 can perturb the balance of Bcl-2 family proteins and favour the release of cytochrome C, SMAC, AIF, and EndoG from mitochondria to cytosal through openings on outer mitochondria membrane (Green, 2000b; Schuler and Green, 2001).

In Chapter 4 and 5, we have discovered that luteolin was capable of sensitizing apoptotic cell death induced by TNF or TRAIL in various human cancer cells, suggesting the potential therapeutic value of luteolin in cancer therapy. The resistance by tumor cells to cisplatin is one of the major limitations in cisplatin chemotherapy (Kartalou and Essigmann, 2001; Siddik, 2003). Furthermore, it would be of interest to find out whether luteolin, a common flavonoid that could be found in plants, could have a synergistic anti-cancer effect with a commonly used chemotherapeutic agent. In this part of our study, we aimed to evaluate the effect of luteolin on the chemotherapeutic efficacy of cisplatin, using both *in vitro* cell culture and *in vivo* cancer cell xenograft model. We reported here that luteolin significantly enhanced the anti-cancer effects of cisplatin by sensitizing cisplatin-induced apoptosis. The molecular mechanism responsible for this enhancement activity is found to be closely

related to the p53-controlled cell death pathway: luteolin is capable of stabilizing p53 and promoting the activity of the pro-apoptotic Bcl-2 family member Bax at the site of mitochondria. Results from this study provide new evidence for the potential application of luteolin as a chemosensitizer in cancer therapy.

6.2 MATERIALS AND METHODS

6.2.1 Reagents and chemicals

Luteolin, 4'-6-Diamidino-2-phenylindole (DAPI), cisplatin, camptothecin, doxorubicin and anti-Bax 6A7 antibody were all purchased from Sigma (St Louis, MO). Pan-caspase inhibitor z-VAD-fmk was from Calbiochem (San Diego, CA). Anti-caspase 3, anti-Bcl-2, anti-Bcl-xL, anti-Bid, anti-p53 and anti-ubiquitin antibodies were from Cell Signaling Technology (Beverly, CA). Anti-Bax antibody was from Chemicon (Temecula, CA). Anti-XIAP antibody was from BD transduction laboratories (San Diego, CA). Anti-tubulin and anti-MDM2 antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Fluorescin (FITC)-labeled synthetic siRNA (scrambled siRNA and p53 siRNA) were from QIAGEN (Valencia, CA).

6.2.2 Cell culture and treatments

Human liver cancer cells HepG2 and Hep3B, and human colorectal cancer cells HT29 and HCT116 were from American Type Culture Collection (ATCC, Manassas, VA) and human nasopharyngeal cancer cells CNE1 was obtained from Sun Yet-sat University (Guangzhou, China). HepG2, HCT116 and CNE1 were maintained in DMEM medium (Sigma) with 10 % FBS (Hyclone, Logan, UT). HT29 cells were maintained in Mcoy5A medium with 10 % FBS.

6.2.3 Apoptosis assessment-4',6-diamidino-2phenylindole staining

The cells undergoing apoptosis were evaluated by chromatin condensation, nuclear shrinkage and formation of apoptotic bodies, all visualized with 4',6-diamidino-2phenylindole (*DAPI*) staining (Fuentes *et al.*, 2003). After various designated treatments, medium was removed and cells were fixed with 70% ethanol at room temperature for 10 minutes. Cells were then stained with 0.3 μg/mL DAPI (in PBS) at room temperature for 10 minutes and visualized under an inverted fluorescence microscope and photographed.

6.2.4 RNA interference

HCT116 cells were transfected with scrambled siRNA labeled with FITC or p53 siRNA using the Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA). The knockdown efficiency was confirmed by western blotting.

6.2.5 Immunoprecipitation, cell fractionation and Western blot

Cells were lysed in Chaps lysis buffer [150 mM NaCl, 10 mM HEPES (pH 7.4) and 1 % Chaps] for 1 hour on ice. The supernatant was collected after centrifugation at 20,000 × g for 15 minutes. Each sample was added with 0.5 μg anti-Bax 6A7 antibody or anti-MDM2 antibody and 50 μL protein A/G agarose beads (Roche Molecular Biochemicals, Indianapolis, IN) and rotated overnight at 4°C. The beads were washed four times using ice-cold PBS buffer and then eluted using SDS-sample buffer before subject to western blot analysis (Izeradjene *et al.*, 2005). For cell fractionation, treated cells were suspended in 100 μl of buffer (20 mM HEPES, 10 mM KCl, 1.5 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyluoride, 250 mM sucrose, pH 7.5), homogenized by a syringe with a 27-guage needle for 15–20 times. The lysate was centrifuged at 1000 g for 10 min to spin down the intact cells and nuclear. The supernatant was further centrifuged at 15 000g for 1 h to obtain cytosolic fraction. For Western blot, equal amount of

proteins were fractionated on SDS-polyacrylamide gel in the Mini-PROTEAN II system (Bio-Rad, Hercules, CA) and blotted onto PVDF membrane (Millipore, Bedford, MA). After blocked with 5% nonfat milk in TBST [10 mM Tris-HCl (pH 7.5), 100 mM NaCl and 0.1% Tween 20], the membrane was probed with various antibodies and developed with enhanced chemiluminescence (Pierce, Rockfold, IL) using a Kodak Image Station (Kodak, Rochester, NY).

6.2.6 RNA extraction and real time-PCR

RNA extraction was carried out using a total RNA extraction kit Purescript (Gentra Systems Inc., Minneapolis, MN), following the instructions of the manufacturer. Five μg of total RNA from each sample were subjected to reverse transcription using M-MLV reverse transcriptase (Promega, Madison, IL). Real-time PCR was carried out with QuantiTect SYBR Green PCR kit (QIAGEN, Valencia, CA) using 2 μL cDNA in a 20 μL final volume. Quantitative PCR was performed using an Opticon real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA) for 45 cycles. A threshold cycle (C_T) value, corresponding to the PCR cycle number at which fluorescence was detected above threshold, was calculated using Opticon analysis software (Bio-Rad Laboratories, Hercules, CA). The data was expressed as the fold increase after normalization using G3PDH. The primers of p53, glyceraldehydes-3-phosphate dehydrogenase (G3PDH) (Mafune *et al*., 1999) and human MDM2 (Wu *et al.*, 2004a) were based on literature.

6.2.7 *In vivo* nude mice xenograft experiment

To assess the effect of luteolin on the anti-cancer efficacy of cisplatin *under in vivo* condition, we used a nude mice xenograft model. The protocol was approved by the University Institutional Animal Care and Use Committee (IACUC). Briefly, female Balb/c nude mice of 5-6 weeks old, about 20 g were purchased from the

Animal Resources Centre (Murdoch, Australia) and maintained in SPF facility. The mice were inoculated subcutaneously in the two sides of flank with 10^7 HCT116 cells in a volume of 100 μL of PBS (Brattain *et al.*, 1981). One week post-inoculation, mice bearing with visible tumors were randomly assigned to four experimental groups (six mice per group): vehicle (PBS), luteolin (40 mg/kg body wt), cisplatin (1.25 mg/kg body wt) and luteolin (40 mg/kg body wt) plus cisplatin (1.25 mg/kg body wt). The dose of cisplatin we used was much lower than that in other studies ranging from 3 mg/kg to 6 mg/kg (Hofmann *et al.*, 1990; Okamoto *et al.*, 2001; Goto *et al.*, 2004). The dose of luteolin was based on our preliminary study and was comparable with that of other flavonoids used *in viv*o (Hofmann *et al*., 1990). The treatment was administered through i.p. three times per week (every Monday, Wednesday and Friday) with close monitoring of the general conditions of the animals. After three weeks, all mice were sacrificed by CO2 inhalation, the tumors were isolated and tumor weight and size were measured.

6.2.8 Immunohistochemistry for p53 staining

p53 expression in tumor was evaluated by immunohistochemical staining. The tumor tissues obtained above were fixed in buffered formalin (10%) for paraffin sectioning. The p53 protein level was detected by immunohistochemistry with light counterstaining using haematoxylin. The cell thus stains brown in a positive and blue in a negative case.

6.3 RESULTS

6.3.1 Luteolin enhances cisplatin-induced caspase-dependent apoptosis in human cancer cells

Many cancer cells are resistant to cisplatin-induced apoptosis (Kartalou and Essigmann, 2001; Siddik, 2003). As shown in Figure 6.1, all three cancer cells, HepG2, CNE-1 and HCT116 cells were rather refractory to cisplatin: only small fraction of cells (<5%) were apoptotic when treated with 10 μg/mL of cisplatin for 24 h. Luteolin alone at 40 μM did not induce evident apoptosis in any of these cells. However, pretreatment with luteolin for 2 h significantly enhanced cisplatin-induced apoptosis in all three cancer cells. Apoptotic cells were evaluated by DAPI staining which shows typical chromatin condensation in apoptotic cells, as illustrated in Figure 6.2.

The apoptosis induced by luteolin and cisplatin was further examined by detection of PARP cleavage and caspase activation, the two hall markers of apoptosis. Caspase-3 is the main downstream effector caspase and PARP is one of its major substrates (Boulares *et al.*, 1999). In HCT116 cells, either luteolin or cisplatin alone caused no caspase-3 or PARP cleavage (Figure 6.3). However, their combination led to evident cleavage of caspase-3 and PARP, which is consistent with the cell death evaluated by DAPI staining (Figure 6.1). Furthermore, the cleavage of PARP and caspase 3 were inhibited by a pan-caspase inhibitor, z-VAD-fmk (Figure 6.3), suggesting that the apoptosis induced by luteolin and cisplatin is caspase-dependent.

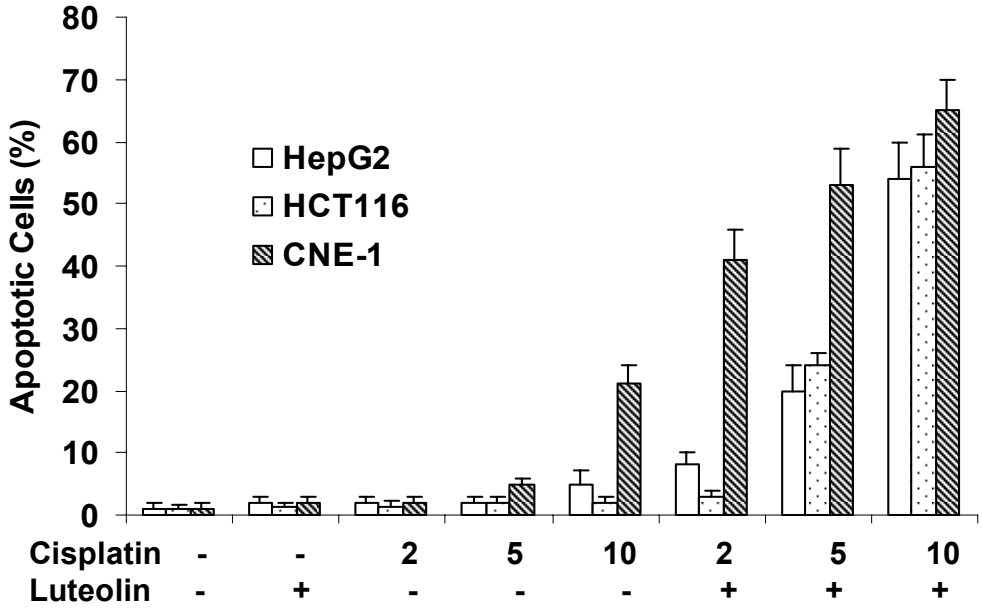


Figure 6.1 Luteolin enhances cisplatin-induced apoptosis in cancer cells.

Cells were pre-treated with luteolin for 2 h (40 μM for HCT-116 and CNE-1, 20 μM for HepG2) and then treated with indicated concentrations of cisplatin (μg/ml) for another 24 h. Cells were fixed with 70% ethanol for 5 min and then stained with 0.3 μM DAPI for 10 min. Apoptotic cells were counted according to their morphologic changes under a fluorescence microscope. *Column*: means of three independent experiments; *bar*: SD.

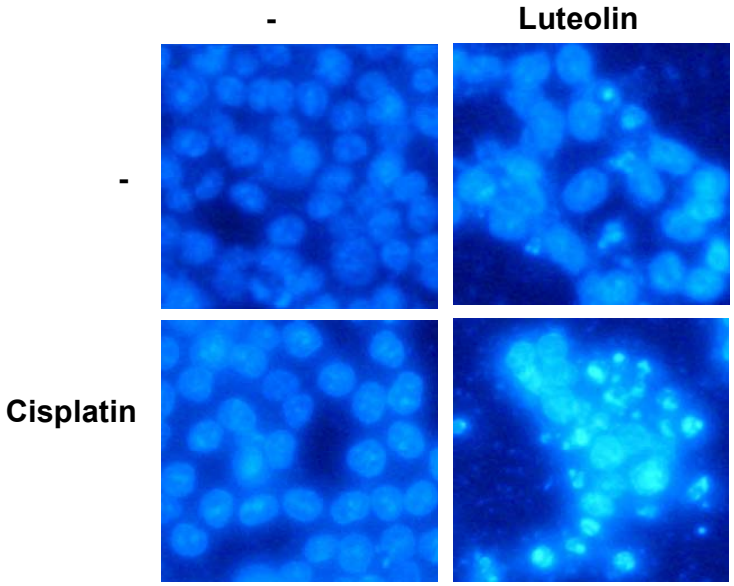


Figure 6.2 Luteolin enhances cisplatin-induced apoptosis in HCT116 cells.

HCT116 cells were treated with cisplatin (10 μg/ml) for 24 h with or without luteolin (40 μM × 2 h) pretreatment. Cells were fixed with 70% ethanol for 5 min and then stained with 0.3 μM DAPI for 10 min. Pictures were taken under an inverted fluorescence microscope with UV stimulation

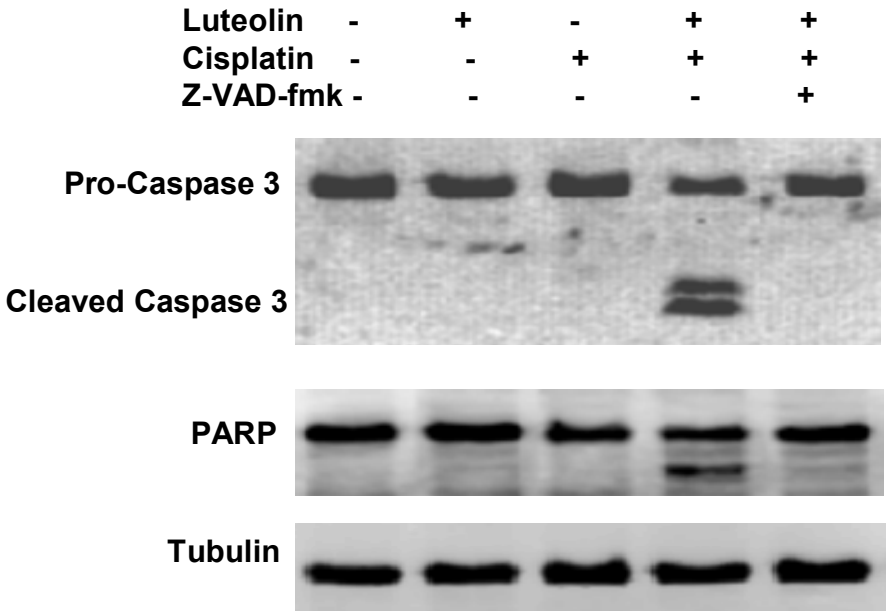


Figure 6.3 A combination of luteolin and cisplatin causes caspase activation

HCT116 cells were treated with luteolin (40 μM × 2 h) followed by cisplatin (10 μg/ml) for 24 h, with or without the presence of z-VAD-fmk (25 μM, 30 min pretreatment). Cells were then collected for detection of PARP and caspase 3 using Western blot. Tubulin was used as a loading control.

6.3.2 Luteolin and cisplatin elevate p53 protein level

To elucidate the possible mechanism involved in the sensitization effect of luteolin on cisplatin-induced apoptosis, we first examined the changes of several important apoptosis regulatory proteins, such as XIAP, Bcl-2, Bcl-X_L and Bax in cells treated with luteolin and cisplatin. However, none of them demonstrated any significant changes in HCT116 cells (Figure 6.4). Since cisplatin is known to activate p53 via DNA damage and p53 is the key regulator for cisplatin-mediated apoptosis in cancer cells (Siddik, 2003), we next examined the change of p53 protein in HCT116 cells treated with luteolin, cisplatin, or their combination. As shown in Figure 6.4, luteolin or cisplatin alone is capable of enhancing p53 protein level significantly. More importantly, combined treatment of luteolin and cisplatin further increased the p53 protein level, especially at 12 and 18 h after treatment, suggesting that p53 may play an important role in promoting luteolin and cisplatin-induced apoptosis.

6.3.3 Luteolin does not enhance cisplatin-induced apoptosis in mutant p53 cells

To further test the involvement of p53 in apoptosis induced by combined treatment of luteolin and cisplatin, we compared the responses of cells with different genetic features of p53. As shown in Figure 6.5, luteolin plus cisplatin induced significant apoptosis in HepG2 and HCT116 cells, both of them are with wild type p53 (Boyer *et al.*, 2004). In contrast, luteolin plus cisplatin failed to induce evident apoptosis in Hep3B or HT29 cells with a mutant p53. Therefore, it is believed that induction of apoptosis by luteolin and cisplatin is dependent on the presence of functional p53.

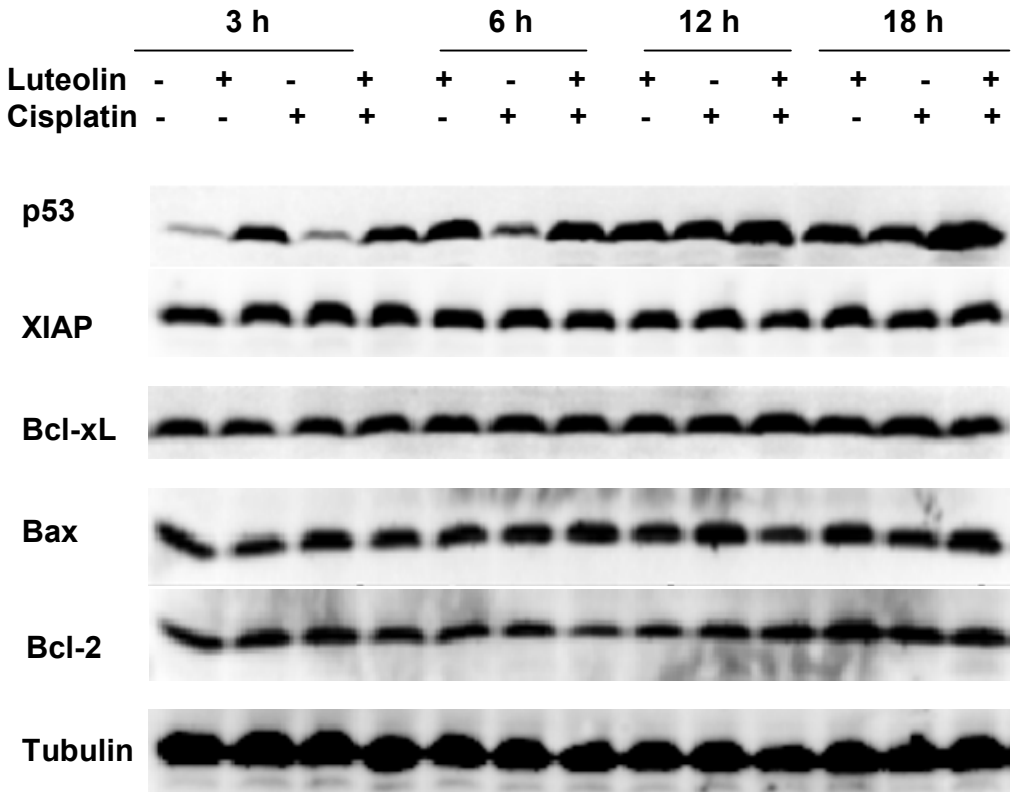


Figure 6.4 A combination of luteolin and cisplatin elevates p53 protein level

HCT116 cells were treated with cisplatin (10 μg/ml) for the indicated period with or without the presence of luteolin pretreatment (40 μM × 2 h). Cells were collected for detection of p53,XIAP, Bcl-xL, Bax and Bcl-2 protein levels by Western blot. Tubulin was used as loading control.

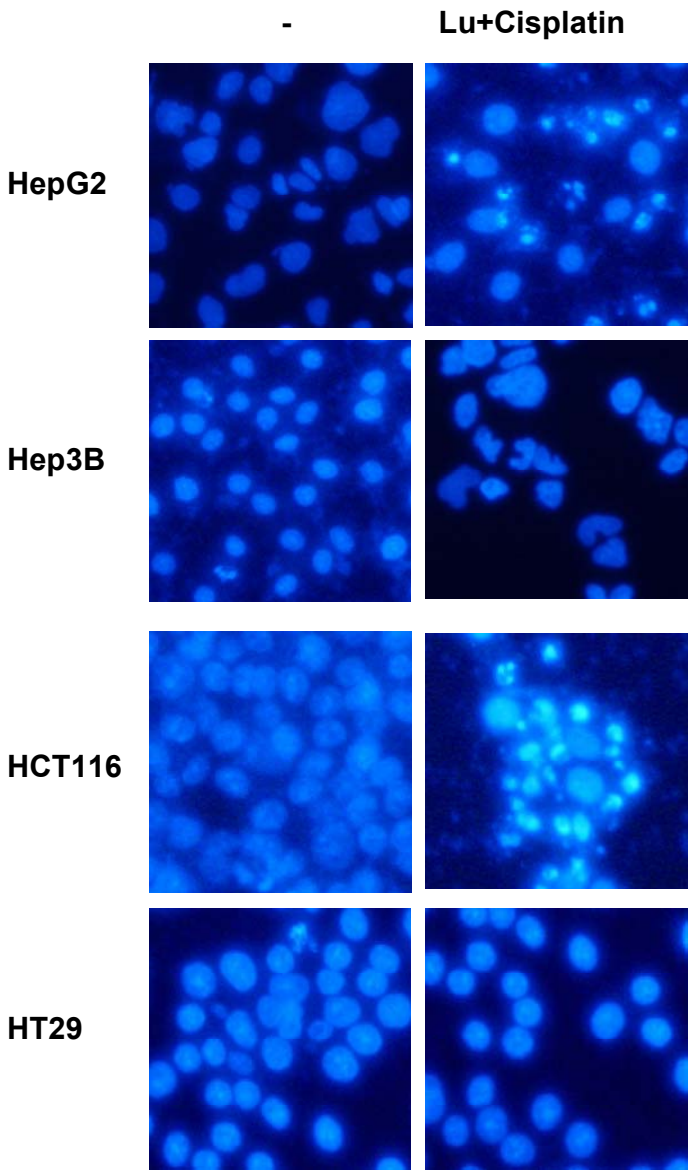


Figure 6.5 A combination of luteolin and cisplatin does not cause apoptosis in mutant p53 cancer cells

Cells were pre-treated with luteolin for 2 h (40 μM for HCT-116 and HT29, 20 μM for HepG2 and Hep3B) and then treated with cisplatin (10 μg/ml) for another 24 h. Cells were fixed with 70% ethanol for 5 min and then stained with 0.3 μM DAPI for 10 min. Pictures were taken under an inverted fluorescence microscope with UV stimulation.

6.3.4 p53 knockdown abolishes the apoptosis induced by luteolin and cisplatin

In order to further confirm the role of p53 in apoptosis induced by luteolin and cisplatin, we knocked down p53 protein in HCT116 cells using the technique of RNA interference. The transfection efficiency of siRNA was higher than 90%, as monitored by a FITC-labeled siRNA (data not shown). The knockdown efficiency was confirmed by Western blot. As shown in Figure 6.6, luteolin plus cisplatin elevated p53 protein level in cells transfected with scrambled siRNA, which does not target any gene. The p53 protein level was significantly reduced in cells transfected with p53 siRNA. We then examined the apoptosis rate induced by luteolin and cisplatin after various siRNA transfections. Luteolin plus cisplatin caused significant apoptotic cell death in cells transfected with scrambled siRNA (Figure 6.7), which is similar to the cell death in non-transfected cells (Figure 6.1). However, p53 knockdown significantly reduced the number of apoptotic cells induced by luteolin and cisplatin. This again confirms that p53 is required for the enhanced apoptosis by luteolin and cisplatin.

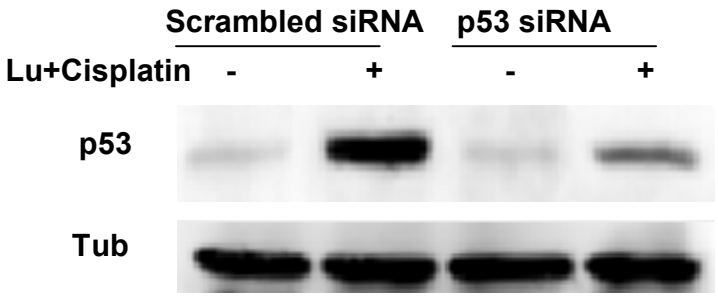


Figure 6.6 p53 RNA interference

HCT116 cells were transfected with scrambled siRNA labeled with FITC, or p53 siRNA for 24 h, then cells were treated with luteolin (40 μ M) for 6 h and cells were collected for detection of p53 by Western blot

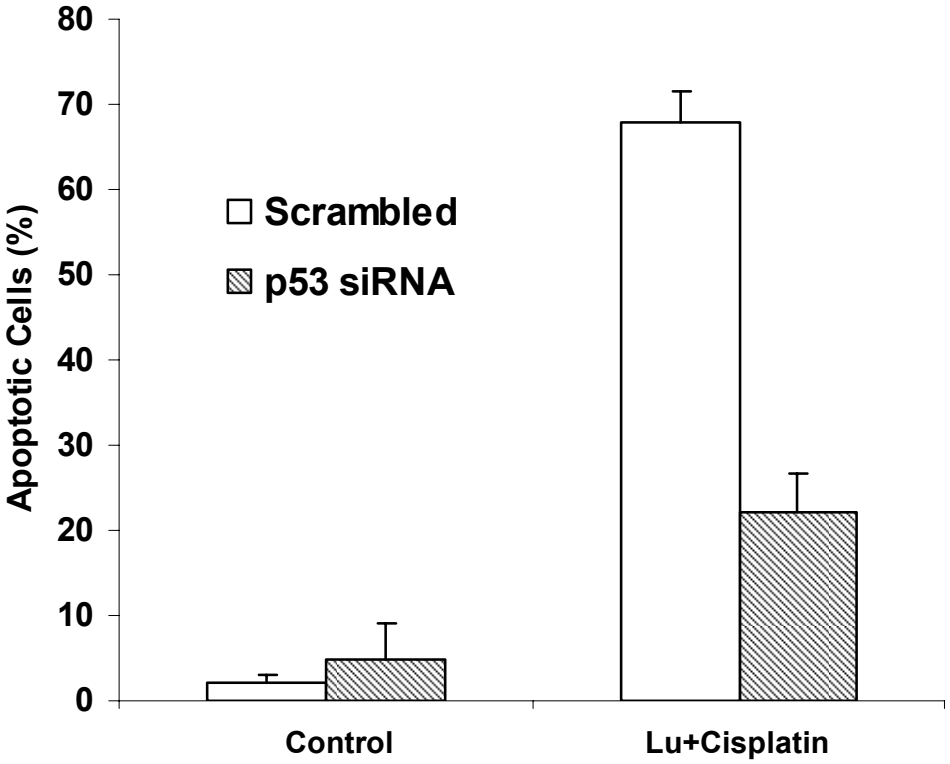


Figure 6.7 p53 RNA interference suppresses the apoptosis induced by luteolin and cisplatin in HCT116 cells

HCT116 cells were transfected with scrambled siRNA or p53 siRNA for 24 h, then were treated with luteolin (40 μM × 2 h) followed by cisplatin (10 μg/ml × 24 h). The cells were fixed with 70% ethanol and stained with 0.3 μM DAPI for 10 min. Apoptotic cells were counted. *Column*, mean of three independent experiments; *bar*, SD.

6.3.5 Luteolin elevates p53 by increasing its protein stability

One important finding from Figure 6.4 is that luteolin alone can elevate p53 protein level significantly and rapidly. In the presence of luteolin, significant increase of p53 protein was found as early as 3 h, which is much faster than the changes induced by cisplatin. Such a kinetic difference suggests that luteolin and cisplatin promotes p53 protein accumulation via different mechanisms. To elucidate the mechanisms of p53 elevation by luteolin, we first examined the effect of luteolin on p53 mRNA level. However, luteolin did not affect p53 mRNA level at 1, 3, or 6 h (Figure 6.8), when p53 protein was significantly elevated (Figure 6.4), suggesting that the rapid elevation of p53 protein level by luteolin is not regulated at transcriptional level.

p53 is a short half-life protein, which is degraded rapidly once it is synthesized. We then examined the p53 stability in the presence of luteolin. In cells treated with cycloheximide (CHX), a *de novo* protein synthesis inhibitor to block p53 protein synthesis, the p53 protein was rapidly degraded, showing a half-life of around 15 min (Figure 6.9). On the other hand, luteolin can significantly prolong the half-life of p53 to about 60 min, suggesting that luteolin promotes p53 protein stabilization.

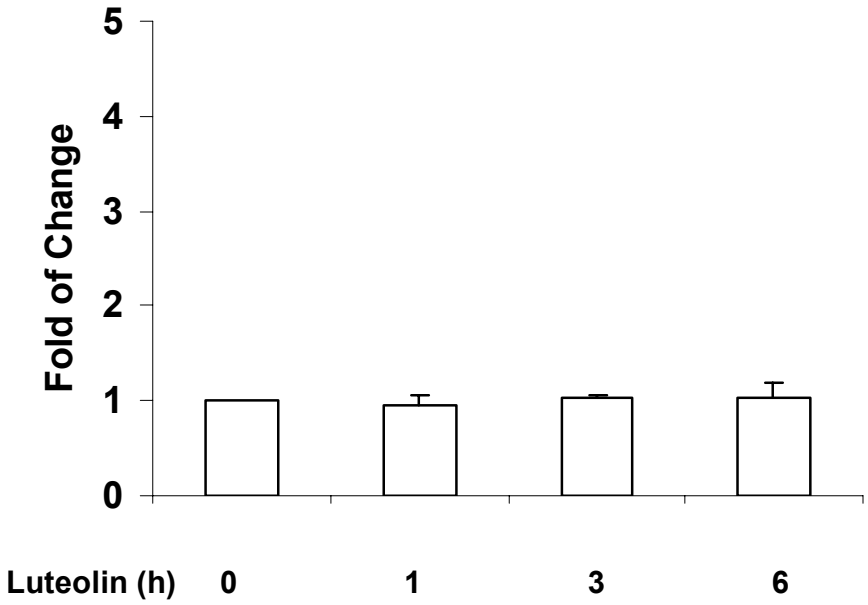


Figure 6.8 Luteolin does not affect p53 mRNA level in HCT116 cells

HCT116 cells were treated with luteolin (40 μ M) for indicated period. Cells were collected for detection of MDM2 mRNA level using real time PCR. G3PDH was used for normalization.

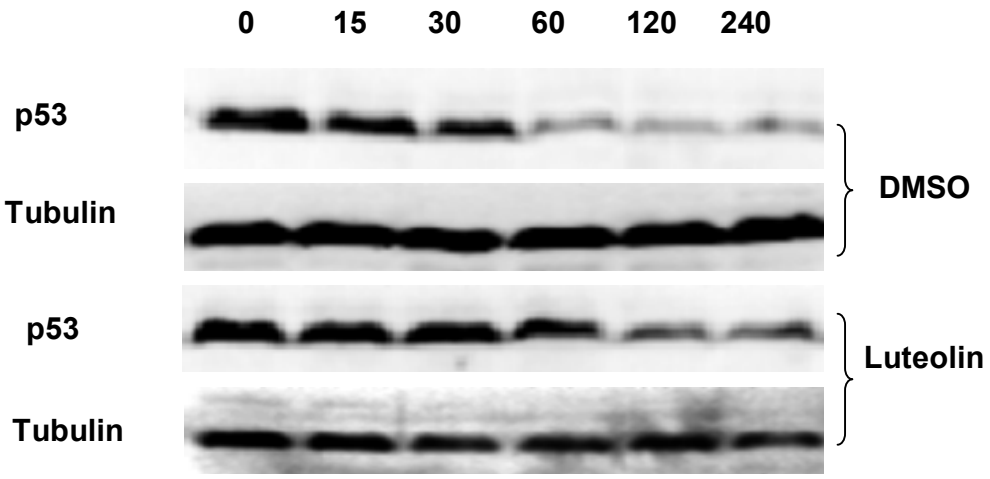


Figure 6.9 Luteolin elevates p53 stability in HCT116 cells

HCT116 cells were treated with luteolin (40 μ M) or vehicle for 30 min followed by cycloheximide (CHX) (1 μg/ml) for the indicated period. Cells were collected for detection of p53 and tubulin using Western blot.

6.3.6 Luteolin increases p53 protein stability by inhibiting MDM2 and disrupting their interaction

Stability of p53 protein is mainly regulated by its interaction with its transcriptional target mouse double minute 2 (MDM2), which acts as an ubiquitin E3 ligase and promotes p53 ubiquitination and proteasomal degradation (Kubbutat, 1997; Chi *et al.*, 2005). We then investigated the effect of luteolin on the interaction between p53 and MDM2 using immunoprecipitation. In vehicle-treated cells, there was an interaction between p53 and MDM2, which is supposed to mediate p53 ubiquitination and degradation. However, the interaction was significantly disrupted by luteolin (Figure 6.10).

One important finding from the data presented in Figure 6.10 is that luteolin treatment reduced the MDM2 protein level. We thus decided to examine the time course of MDM2 protein level change after luteolin treatment. Interestingly, MDM2 protein level decreased after 3 or 6 h treatment of luteolin (Figure 6.11), which occurred at the same when p53 protein was elevated (Figure 6.4). These findings suggest that luteolin stabilizes p53 protein level by decreasing MDM2 protein and disrupting its interaction with p53.

To elucidate the mechanism of MDM2 protein down-regulation by luteolin, we then tested whether MDM2 was regulated at transcriptional level. Figure 6.12 shows that luteolin significantly decreased MDM2 mRNA level at as early as 1 h, which was earlier than the protein level change (Figure 6.11). As expected, evident reduction of MDM2 mRNA level was found in cells treated with actinomycin D (ActD), a transcription inhibitor. However, cisplatin did not appear to affect the MDM2 mRNA level.

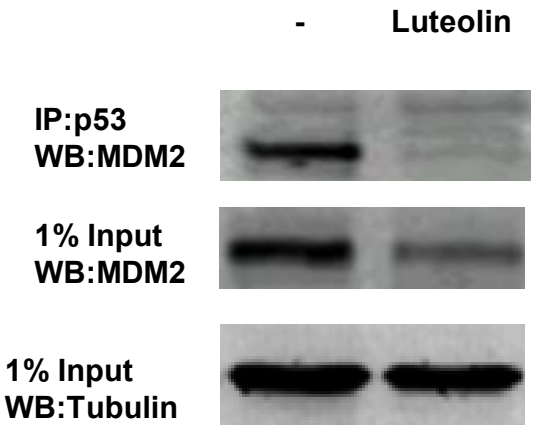


Figure 6.10 Luteolin disrupts the p53-MDM2 interaction in HCT116 cells

HCT116 cells were treated with luteolin (40 μ M) or vehicle for 6 h. Cell lysate was immunoprecipated with anti-p53 antibody, followed by Western blot using anti-MDM2 antibody. 1 % input lysate was subjected to Western blot to detect MDM2 and tubulin.

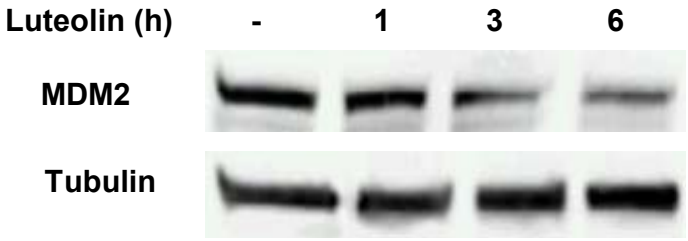


Figure 6.11 Luteolin decreases MDM2 protein level

HCT116 cells were treated with luteolin (40 μ M) for indicated period. Cells were collected for detection of MDM2 protein level using Western blot. Tubulin was used as a loading control.

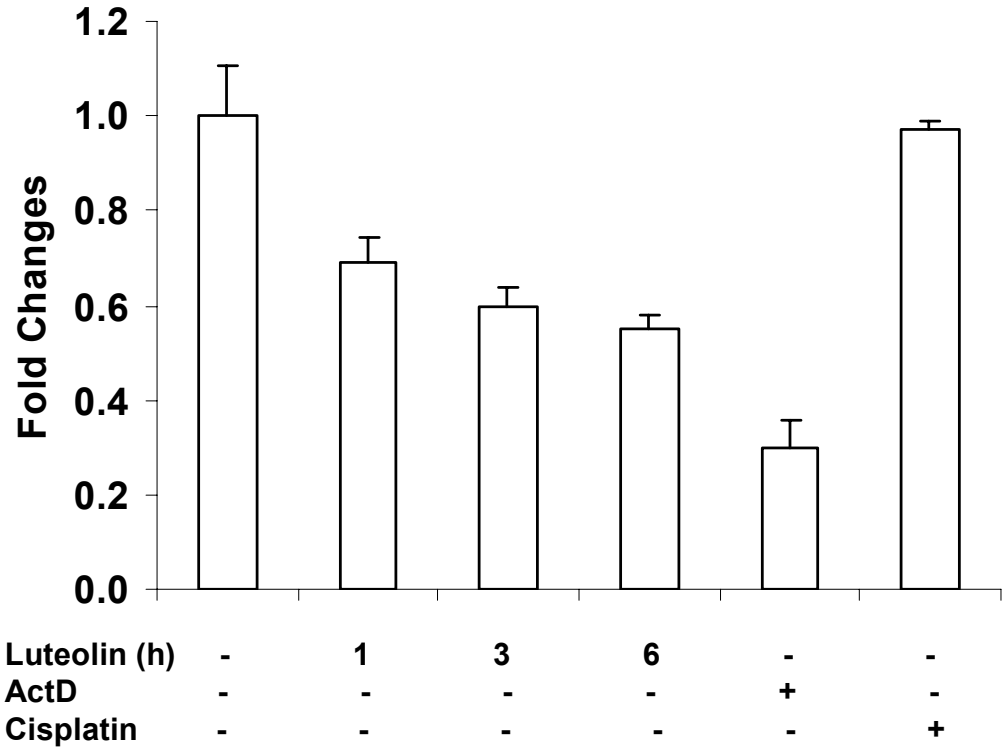


Figure 6.12 Luteolin decreases MDM2 mRNA level

HCT116 cells were treated with luteolin (40 μ M) for indicated period or actinomycin D (ActD 1 μ g/ml) for 6 h or cisplatin (μg/ml) for 12 h. Cells were collected for detection of MDM2 mRNA level using real time PCR. G3PDH was used for normalization.

6.3.7 Luteolin and cisplatin induces p53 and Bax mitochondrial translocation

Since the apoptosis induced by luteolin and cisplatin was not prevented by a specific caspase 8 inhibitor, z-IETD-fmk (data not shown), suggesting that the apoptosis is unlikely to be executed via the extrinsic death receptor pathway, but rather the intrinsic mitochondrial pathway. This speculation was confirmed by the finding that cytochrome c release to cytosol was caused by a combination of luteolin and cisplatin (Figure 6.13).

Recent evidence suggests that apoptosis mediated by p53 could involve its mitochondrial translocation (Chipuk *et al.,* 2003; Erster *et al.,* 2004). To test whether this is the case in luteolin and cisplatin combined treatment, we carried out mitochondrial fraction. The complete separation was confirmed by the absence of tubulin in mitochondria fraction. Figure 6.14 shows that both luteolin and cisplatin caused p53 translocation to mitochondria, whereas the combination treatment of luteolin and cisplatin induced an even more extensive p53 mitochondrial translocation.

The p53 mediated transcription-independent apoptosis requires the involvement of another partner, Bax (Chipuk *et al*., 2003). Although the total protein level of Bax was not increased by either luteolin, cisplatin or their combination (Figure 6.4), Bax translocation to mitochondria was significant in the presence of luteolin. For the combined treatment, the translocation was even more significant (Figure 6.14).

It is known that the pro-apoptotic function of Bax requires its transformational changes (Park *et al*., 2002). The activated Bax on mitochondria can be distinguished by a conformational change in the N-terminus that exposes the formerly buried 6A7 epitope (Desagher *et al*., 1999; Nechushtan *et al*., 1999). We thus used a specific antibody, anti-Bax 6A7, to pull down the active form of Bax and detect it using

Western blot. As shown in Figure 6.15, the Bax transformation was increased by either luteolin or cisplatin. More importantly, Bax was further activated when cisplatin was treated together with luteolin. The above observations suggest that the enhanced p53 and Bax mitochondrial translocation, as well as enhanced Bax transformation, contribute to enhanced apoptotic cell death induced by combined treatment of luteolin and cisplatin.

6.3.8 Luteolin enhances the anti-cancer effect of cisplatin *in vivo*

To evaluate the potential of luteolin as a sensitizer of cisplatin *in vivo*, we tested the combined anti-cancer effect of the two agents on HCT116 xenografted nude mice. As shown in Figures 6.16 and 6.17, the tumor in vehicle-treated mice grew very fast and increased to about 1.2 g after 3 weeks. Low dose of cisplatin (1.25 mg/kg body weight) alone slightly suppressed the tumor growth and luteolin (40 mg/kg body weight) alone did not affect much the tumor growth. However, tumor weight was significantly reduced by the combined treatment. In several mice, the tumor was almost invisible (Figure 6.16). Therefore the *in vivo* evidence confirms that luteolin can significantly enhance the anti-cancer effect of cisplatin.

6.3.9 Luteolin enhanced the anti-cancer effect of cisplatin *in vivo* by elevating p53

To explore the mechanisms involved in the *in vivo* anti-cancer effect by luteolin and cisplatin, the tumor tissue were fixed and stained with anti-p53 antibody using immunohistochemistry. In vehicle treated mice, the expression of p53 was only marginal (Figures 6.18 and 6.19). Although both cisplatin and luteolin could significantly increase the expression of p53 protein, their patterns are different. About 50% of the cells were found over-expressing p53 in cisplatin-treated mice and the p53 level was moderate in those cells. In contrast, the p53 protein level in luteolin-treated mice was much higher than that in cisplatin-treated mice, although the number of

positive cells was less. It is noted that in mice with combined treatment of luteolin and cisplatin, more than 80% cancer cells were stained positive and the p53 protein level was also significantly higher than the individual treatment. Such a finding is basically consistent with *in vitro* data (Figure 6.4).

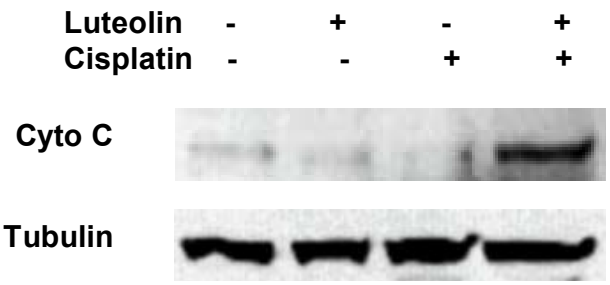


Figure 6.13 A combination of luteolin and cisplatin induces cytochrome c release to cytosol

HCT116 cells were treated with luteolin (40 μM × 2 h) followed by cisplatin (10 μg/ml) for 18 h. Cells were then collected and cytosol was fractioned for detection of cytochrome c using Western blot. Tubulin was used as a loading control.

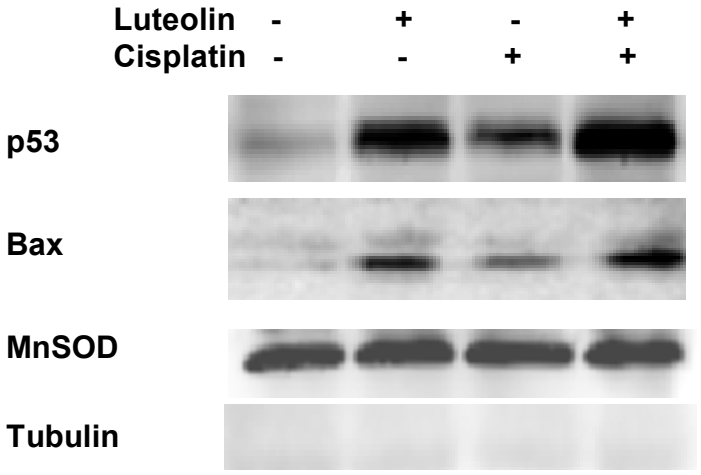


Figure 6.14 Luteolin and cisplatin induced p53 and bax mitochondrial translocation

HCT116 cells were treated with luteolin (40 μ M) or cisplatin (10 μ g/mL) or their combination for 12 h. Cells were collected and mitochondria fractions were obtained to detect p53 and Bax using Western blot. Anti-MnSOD was used as a loading control and anti-tubulin was used to prove the absence of cytosal proteins.

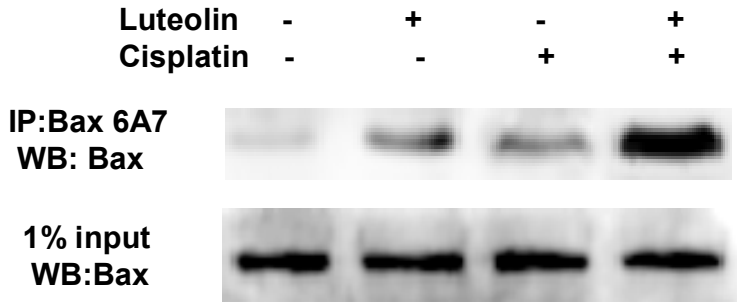


Figure 6.15 Luteolin and cisplatin induced bax mitochondrial translocation

HCT116 cells were treated with luteolin (40 μ M) or cisplatin (10 μ g/mL) or their combination for 12 h. Cells were collected and cell lysate was used for immunoprecipitation using anti-Bax (6A7) antibody, followed by Western blot using anti-Bax antibody. Bax in 1 % input lysate was used as a loading control.

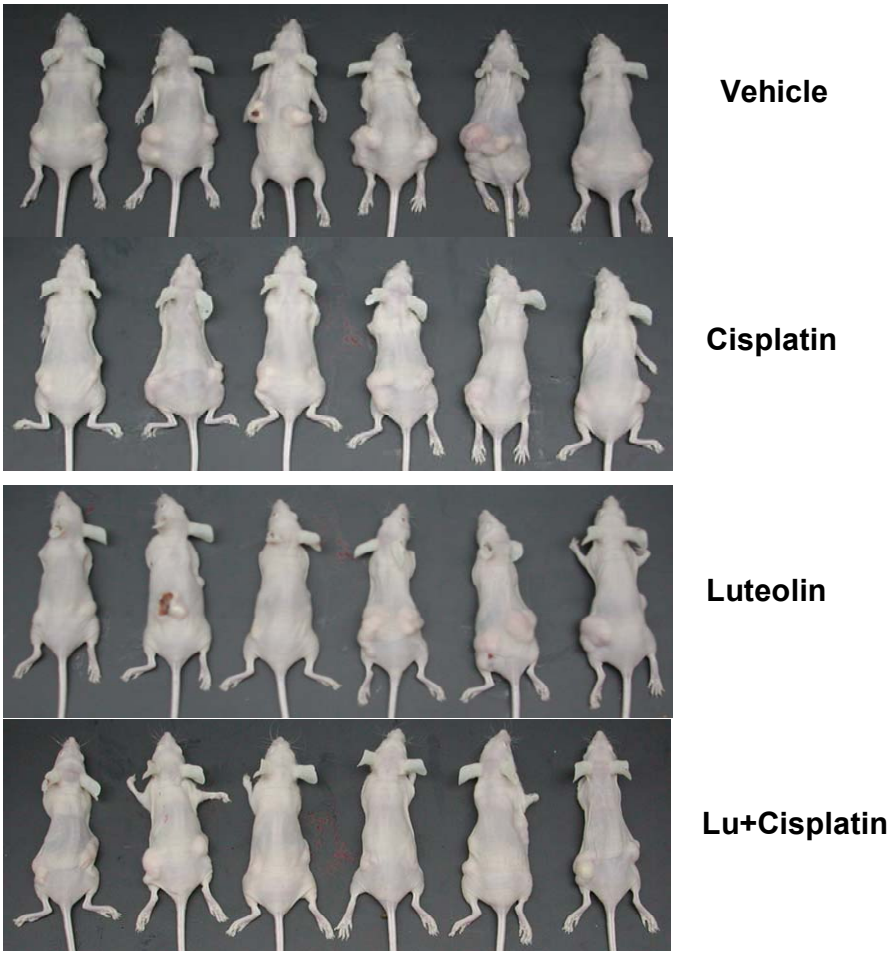


Figure 6.16 Luteolin enhances the anti-cancer effect of cisplatin *in vivo*

Six-eight weeks Balc/B nude mice (about 20 g) were inoculated with 10^7 HCT116 cells in 100 μl PBS. After one week, mice bearing tumor were treated with PBS as vehicle, or luteolin (40 mg/kg), or cisplatin (1.25 mg/kg), or luteolin (40 mg/kg) with cisplatin (1.25 mg/kg), through i.p. injection (3 times per week) for 3 weeks. At the end of treatment, mice were sacrificed by CO_2 inhalation and photographed.

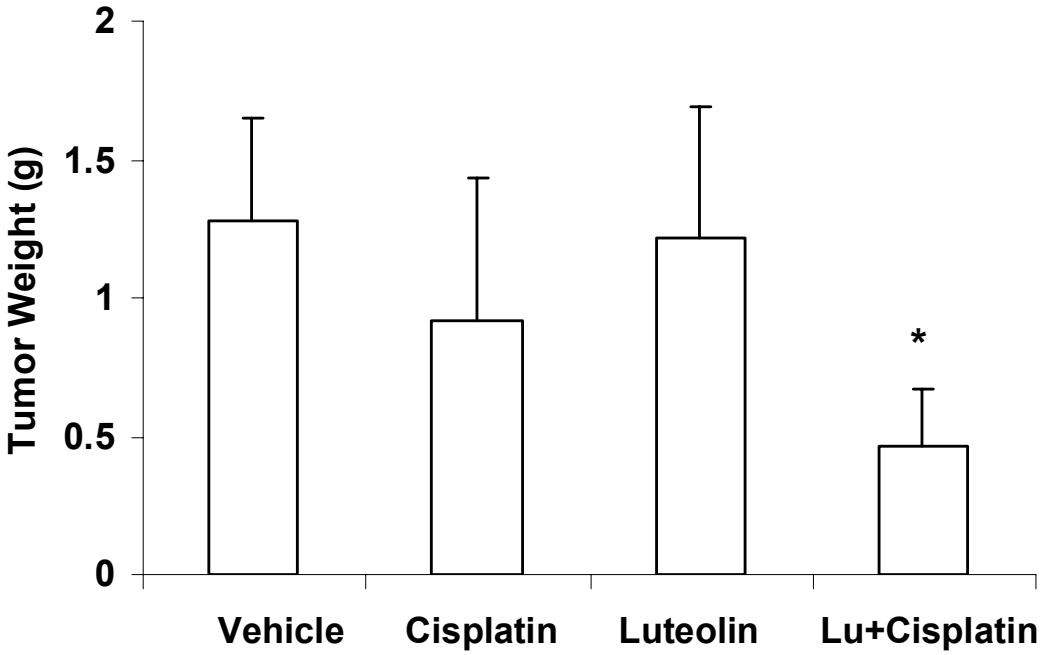


Figure 6.17 Luteolin enhances the anti-cancer effect of cisplatin *in vivo* (Quantification)

Tumor weights (g) of mice treated in Figure 6.16 were measured. *Column*, mean of tumor weight; *bar*, SD. Six mice in one group.

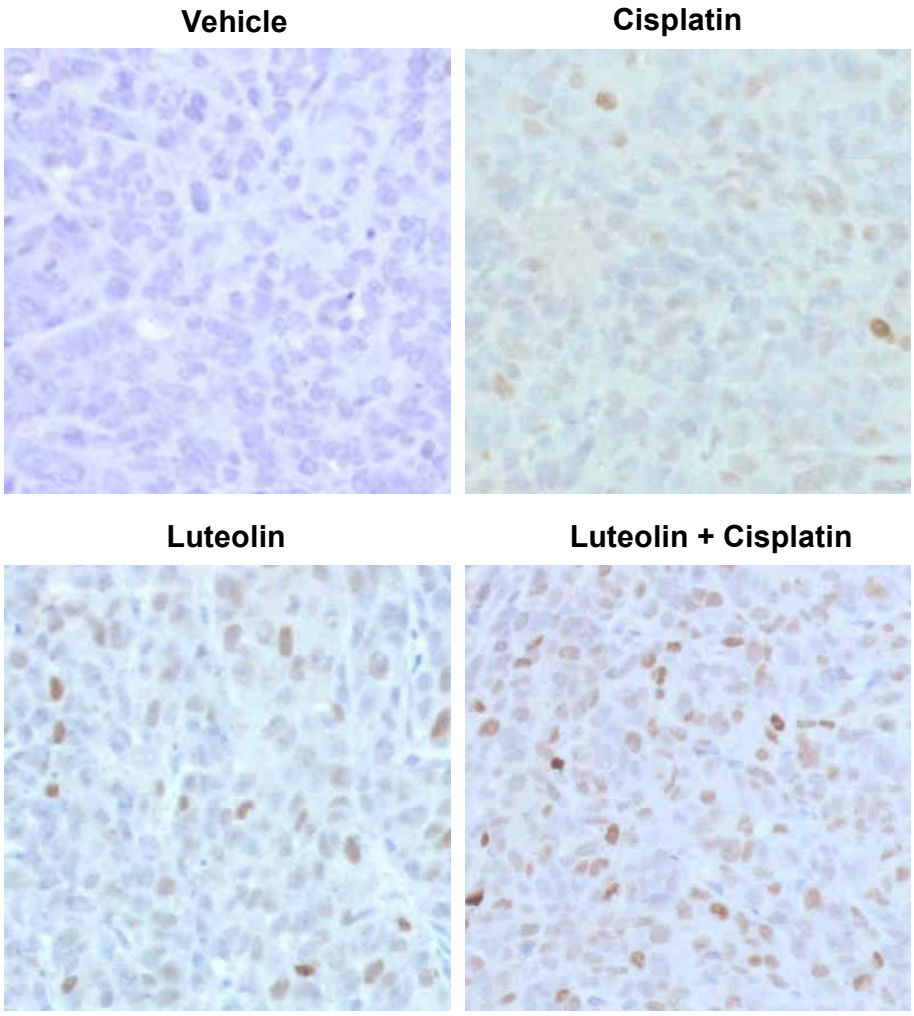


Figure 6.18 Luteolin and cisplatin elevate p53 protein level *in vivo*

Tumor samples from the mice treated in Figure 6.16 were fixed by buffered formalin for paraffin sectioning, p53 expression was evaluated by immunohistochemistry after counter staining. The representative field for p53 expression was photographed. The p53 positive cell stains brown and the negative cell stains blue.

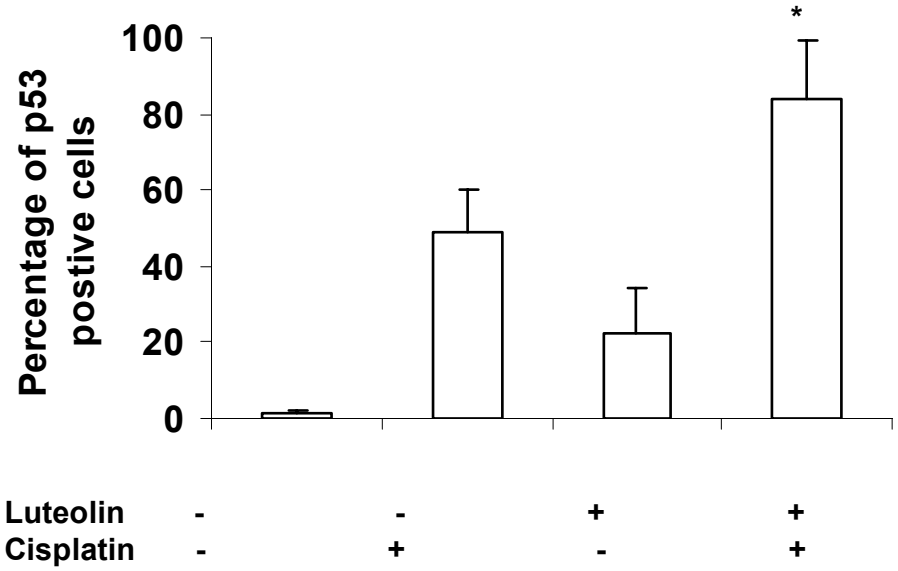


Figure 6.19 Luteolin and cisplatin elevate p53 protein level *in vivo* (Quantification)

The percentages of p53 positive cells in Figure 6.18 were counted in representative fields. *Column*, mean; *bar*, SD.

6.4 DISCUSSION

Cisplatin is widely used to treat a variety of tumors. However, resistance to this drug is a major limitation for its clinical use (Kartalou and Essigmann, 2001; Siddik, 2003). Therefore, treatment together with other agents is a strategy to enhance the anti-cancer potential of cisplatin (Duan *et al*., 2001; Iwase *et al*., 2003; Kim *et al*., 2003a; Fulda and Debatin, 2005; Mohanty *et al*., 2005). Here we reported that a natural flavonoid, luteolin can enhance the anticancer potential of cisplatin through a novel mechanism, stabilizing p53 protein and causing p53 and Bax mitochondrial translocation.

As shown in our early studies, luteolin could sensitize TNF and TRAIL-induced apoptosis in a variety of human cancer cells (Chapters 4 and 5). We thus further examine whether luteolin has a synergistic effect when combined with anti-cancer drugs, such as cisplatin.

As shown in Figure 6.1, several human cancer cells are resistant to cisplatin, while luteolin, at its non-toxic concentration, significantly enhanced cisplatin-induced apoptosis (Figure 6.3). Moreover, this sensitization effect was only observed in p53 wild type HCT116 and HepG2 cells, but not in p53 mutant cells such as Hep3B and HT29 (Figure 6.5). The critical role of p53 status was further supported by the following observations: (i) the significant elevation of p53 protein (Figure 6.4), (ii) knockdown of p53 by siRNA offered significant protection against apoptosis induced by a combined treatment of luteolin and cisplatin (Figures 6.6 and 6.7).

To be noted, the increase of p53 protein by luteolin is rapid, occurred as soon as 3 h after treatment (Figure 6.4). It has been previously reported that luteolin can activate p53 in p53 wild type cancer cells (Plaumann et al., 1996), but the mechanism

was not known. It is possible that luteolin can inhibit topoisomerase I (Mittra *et al.*, 2000; Chowdhury *et al.*, 2002) to cause DNA damage (Leung *et al*., 2005). However, this could not explain the significant and rapid elevation of p53 protein within 3 h, since cisplatin, a typical DNA-damaging agent, could only elevate p53 protein after 6 h in HCT116 (Figure 6.4). Further, we showed that the rapid p53 elevation is not dependent on p53 mRNA increase (Figure 6.8) but through stabilizing of this short-lived protein (Figure 6.9). p53 stability is mainly regulated by its interaction with MDM2 protein, which interacts with p53 directly and promotes its ubiquitination and proteasomal degradation. In this study, we demonstrated that the rapid p53 accumulation by luteolin is through decreasing MDM2 protein (Figure 6.11) and disrupting the MDM2-p53 interaction (Figure 6.10). In Chapter 5, we reported that luteolin, in the presence of TRAIL, promoted XIAP ubiquitination and proteasomal degradation without affecting its mRNA level. Here we tested whether luteolin also affected MDM2 protein via a similar mechanism. Very interestingly, we found that luteolin decreased MDM2 mRNA level (Figure 6.12) but did not promote MDM2 ubiquitination (data not shown). Since MDM2 is one of the target gene of the transcriptional factor p53, it was expected to see that luteolin caused elevation of MDM2 mRNA and protein level corresponding with the marked increase of total p53 protein level (Figure 6.4) induced by luteolin. Conversely, we found that MDM2 mRNA level decreased after luteolin treatment, suggesting that luteolin may either suppress the transcriptional activity of p53 or adversely affect MDM2 mRNA stability. The exact mechanism remains to be further investigated.

There were reports that the anti-cancer effect of cisplatin largely depends on the p53 status of the tumors (Song *et al.*, 1998; Kanata *et al*., 2000; Tang and Grimm, 2004; Beuvink *et al.,* 2005). It has been demonstrated that the resistance to cisplatin

could be overcome by enhancing or restoring the wild type p53 in p53 mutant cancer cells (Song *et al.*, 1997). The apoptosis induced by p53 is mainly mediated by the mitochondria pathway, a central regulator of apoptosis. In response to death stimuli including p53 activation, cytochrome C and SMAC are released from mitochondria to cytosal and facilitate the caspase activation and nuclear fragmentation (Green, 2000b). The cytochrome c release is not present in either luteolin or cisplatin treated cells (Figure 6.13). A combined treatment of luteolin and cisplatin led to the release of cytochrome c to cytosol prior to apoptosis, indicating the involvement of mitochondria in the apoptosis.

Generally there are two pathways in p53-mediated apoptosis: the transcription dependent and transcriptional activity-independent pathway. In the former, p53 could enhance the transcription of some pro-apoptotic proteins, such as Bax, Noxa and PUMA (Miyashita and Reed, 1995; Attardi *et al.*, 2000; Oda *et al.*, 2000b; Yu *et al.*, 2001). However, in this study we did not observe the elevation of Bax after the combined treatment. On the contrary, we found that the mRNA level of MDM2 and p21, two downstream genes regulated by p53, reduced, indicating that the p53 transcription-dependent pathway may not be involved in the apoptosis induced by luteolin and cisplatin. Currently there is accumulating evidence suggesting that p53-mediated apoptosis is independent of its transcriptional activity, a process mainly involving mitochondrial translocation of p53 and some pro-apoptotic Bcl-2 family members such as Bax (Chipuk *et al*., 2003; Erster et al., 2004; Arima *et al.*, 2005). In non-stimulated cells, Bax exists as a monomer either in the cytosol or loosely attached to the outer mitochondrial membrane. Although Bax total protein level was not affected in the present study (Figure 6.4), we observed that luteolin alone could induce translocation of p53 and Bax to mitochondria (Figure 6.14). In contrast,

cisplatin alone only induced marginal p53 and Bax mitochondrial translocation. The combined treatment of luteolin and cisplatin further enhanced p53 and Bax mitochondrial translocation as well as Bax transformation, suggesting the importance of such changes.

In this part of our study, the anti-cancer effect of combined treatment of luteolin and cisplatin was further examined using *in vivo* xenograft model. The dose of cisplatin used in this study was much lower than that in other studies (Hofmann *et al.*, 1990; Okamoto *et al.*, 2001; Goto *et al.*, 2004). In our study, cisplatin at 1.25 mg/ml only slightly suppressed the tumor growth (Figures 6.16 and 6.17). Immunohistochemisty showed that the p53 protein was elevated by cisplatin treatment, which is consistent with the *in vitro* data (Figure 6.4). The p53 protein level was also elevated by luteolin. Interestingly, luteolin plus cisplatin markedly elevated the p53 protein level and significantly inhibited the tumor growth (Figures 6.16, 6.17, 6.18 and 6.19). This *in vivo* finding suggests that luteolin can enhance the anti-tumor effect with a low dose of cisplatin by elevating p53.

In conclusion, we found that luteolin, a major flavonoid in chrysanthemum, can significantly enhance the anti-cancer effect of cisplatin. The enhancement is through decreasing MDM2 mRNA as well as protein level, disrupting the p53-MDM2 interaction, thus to stabilize p53 protein, leading to mitochondrial translocation of p53 and Bax, and eventually apoptotic cell death. Data from both the *in vivo* and *in vitro* experiments provide convincing evidence to support the potential application of luteolin as a chemosensitizer in cancer therapy.

CHAPTER SEVEN

DISCUSSION AND CONCLUSION

Chrysanthemum morifoium is a common traditional herbal medicine for treatment of fever, eye irritation and hypertension, etc in many Asian countries (Jiang, 2002). The main pharmacological activity of chrysanthemum includes its anti-inflammatory and anti-oxidant function (Yu and Xie, 1987; Chen *et al.*, 2003). Preliminary data from our laboratory have found that the water extract of chrysanthemum significantly inhibited the growth of xenografted tumor in nude mice (Shen *et al.,* unpublished data), suggesting the potential anti-cancer property of chrysanthemum.

Many types of active components identified in chrysanthemum have been found to own potential anti-cancer function. For instance, more than 50 terpenoids identified from chrysanthemum have been shown to possess anti-tumor effects in a number of cancer cell lines (Ukiya *et al*., 2001; Ukiya *et al*., 2002). However, terpenoids are unlikely the active components in the water extract due to their insolubility in water. On the other hand, flavonoids are a group of phytochemicals ubiquitiously present in plants, and their anti-tumor effects have been well documented (Ross and Kasum, 2002). A dozen of flavonoids have been identified from chrysanthemum (Hu *et al*., 1994; Liu *et al.,* 2001; Lee *et al*., 2003; Hu *et al.*, 2004). As flavonoids are mostly water soluble, thus are expected to be present in the water extract of chrysanthemum. Therefore, it was of interest to determine whether those flavonoids are responsible for the anti-cancer potential of chrysanthemum.

Apoptosis is a type of cell death characterized by specific morphological and biochemical changes (Hengartner, 2000). Insufficient apoptosis is believed to be one of the causes of tumor formation (Hanahan and Weinberg, 2000). Thus, induction of apoptosis in cancer cells has been an established strategy in cancer therapy. Apoptosis is mediated mainly through two signaling pathways, the extrinsic death receptor

pathway and intrinsic mitochondrial pathway. In the death receptor pathway, the death receptors are triggered by their ligands, such as TNFα and TRAIL, leading to the recruitment of adaptor molecules that activate the caspase cascade (Chen and Goeddel, 2002). In the mitochondrial pathway, cell death signals converge on mitochondria, causing the release of an array of apoptosis regulatory proteins such as cytochrome c and SMAC that activates the caspase cascade (Kroemer, 2000). The role of mitochondria in apoptosis is mainly mediated by the Bcl-2 family proteins in response to a variety of cell death stimuli, including DNA damaging agents or cancer therapeutics such as cisplatin.

One of the important issues in cancer therapy is resistance. Some cancer cells acquire the resistance to apoptosis through a variety of biochemical changes. For example, the rapid activation of NF-κB pathway by TNFα will trigger the expression of a series of anti-apoptotic proteins, including A20, XIAP and c-IAPs, etc (Krikos *et al*., 1992; Wang *et al.*, 1998; Micheau *et al.,* 2001). These anti-apoptotic proteins can then suppress the caspase cascade triggered by TNFα or TRAIL to block apoptosis. For example, XIAP can directly interact with caspase-3 to inhibit its activation and activity (Deveraux *et al*., 1997). It is known that cisplatin induces cancer cell apoptosis mainly through activating p53 (Siddik, 2003). Thus, high expression of MDM2 protein, which suppresses p53 protein level, may cause cancer cells to be resistant to cisplatin (Kondo et al., 1995). Therefore, it was important to seek effective strategies to overcome the resistance in cancer therapy.

As one of the major flavonoids of chrysanthemum (Chapter 2), luteolin has been demonstrated to possess a variety of activity, including antioxidant, anti-inflammation, inhibition of cell proliferation, induction of cell cycle arrest or apoptosis, inhibition of topoisomerases and protein kinases, and suppression of

metastasis and angiogenesis (see more detailed discussion in Section 1.2). To be noted, some of these effects are closely related to the ability of luteolin in modulating the activation and function of NF-κB and p53. Luteolin has been demonstrated to block LPS-induced NF-κB transcriptional activity in macrophages (Xagorari *et al.*, 2002), Rat-1 fibroblasts (Kim *et al.*, 2003b) and rat IEC-18 cells (Kim and Jobin, 2005). In addition, luteolin activates wild type p53 in several cells (Plaumann et al., 1996). It is thus postulated that luteolin may interfere with the cell death signaling pathway elicited by TNF, TRAIL and cisplatin to modulate the apoptotic cell death process.

The main objectives of this study are to investigate the anti-tumor property of chrysanthemum and its major flavonoids, luteolin. The whole study included the following investigation: 1) identification of the major anti-tumor components of the water extract of chrysanthemum, (presented in Chapter 2); 2) evaluation of the anti-tumor effects of the major active components, (presented in Chapter 3); 3) investigation of the combined effects of luteolin with cancer therapeutic agents, including TNF, TRAIL and cisplatin (presented in Chapters 4, 5 and 6).

7.1 Flavonoids are the major anti-tumor components of chrysanthemum water extract

To investigate the major anti-tumor components in the water extract of chrysanthemum, we obtained a crude water extract (Fraction A) and then partitioned it into three fractions according to their polarity (Figure 2.1). The bioassay showed that the EtOAc fraction were the most potent in inhibiting cancer cell growth (Figure 2.2). Its cytotoxicity was much higher than that of the crude chrysanthemum water extract and other two fractions, suggesting that EtOAc fraction contains the major cytotoxic components of the water extract of chrysanthemum.

Further chemical assays suggested that there are 13 flavonoids in the EtOAc fraction and all of them are conjugated with sugar substitutes. We found that the chrysanthemum flavonoids mixture, at 0.25 and 0.5 mg/ml, exerted significant cytotoxicity in several colorectal cancer cells (Figure 3.1). It was further showed that the cytotoxicity was through induction of apoptosis (Figures 3.2 and 3.3). This was in line with the effects of flavonoid extracts of many other plants, which have shown anti-tumor effects in the range of 0.05-1 mg/ml (Ye et al., 1999; Kim, 2004; Kim et al., 2005d).

As luteolin is the most abundant flavonoid in the EtOAc extract (Figure 3.4), it could have played an important role in the anti-tumor effects of chrysanthemum. However, the effects of other components in the chrysanthemum can not be excluded. For example, apigenin has also been demonstrated to be highly capable of killing cancer cells (Figure 3.4) (Way *et al.*, 2004; Shukla *et al.*, 2005; Torkin *et al.*, 2005; Zheng *et al.*, 2005). On the other hand, there may be synergistic effects between different flavonoids (Liu, 2004). There is evidence that combination of low doses of flavonoids may work cooperatively in blocking cell-cycle progression of cancer cells (Wang *et al.*, 2004).

7.2 Luteolin sensitizes TNFα-induced apoptosis in human cancer cells

Many cancer therapeutic agents are capable of eliminating cancer cells by inducing apoptotic cell death (Ferreira *et al.*, 2002). TNFα has been regarded as a cancer therapeutic cytokine due to its potential of inducing apoptosis in cancer cells (Tracey and Cerami, 1993). TNFα induces apoptosis through a typical death receptor pathway. It binds to TNFR1, a death receptor, and causes recruitment of a number of molecules which can subsequently trigger a caspase cascade (Chen and Goeddel, 2002). However, most cells are resistant to TNFα-induced apoptosis, mainly due to

the fact that TNFα simultaneously activates NF-κB, a cell survival signal transduction pathway. Activation of NF-κB induces expression of a number of anti-apoptotic proteins such as c-IAP-1, c-IAP-2, XIAP, FLIPs, survivin and A20 (Krikos *et al.*, 1992; Wang *et al.*, 1998; Micheau *et al.,* 2001). On the other hand, the activation of NF-κB also suppresses JNK activation, a signal generally regarded as pro-apoptotic in TNF signaling (De Smaele *et al.*, 2001; Tang *et al.,* 2001). Therefore, the therapeutic value of TNFα alone in cancer therapy is rather limited.

As one of the major flavonoids in chrysanthemum, luteolin can inhibit cancer cell growth to certain extent (Figure 3.4), and induce apoptosis in certain cancer cell lines (Figure 3.5). Interestingly, in the presence of nontoxic concentrations of luteolin, TNF could induce apoptosis rapidly in cancer cells (Figure 4.1). This striking synergistic effect suggests that luteolin can interfere with the cell survival mechanism elicited by TNFα.

Further studies showed that the sensitization is via an inhibition on NF-κB (Figure 4.9). TNFα-triggered NF-κB activation and over-expression of at least two anti-apoptotic proteins, c-IAP-1 and A20, were suppressed by luteolin (Figure 4.14). On the other hand, JNK activation was prolonged in the presence of luteolin due to removal of the blocking effect of NF-κB (Figure 4.15).

It has been reported that luteolin inhibits LPS-induced NF-κB activation in rat fibroblasts without affecting IκBα degradation, p65 nuclear translocation and p65-DNA binding (Xagorari *et al.*, 2001), which is in line with our findings (Figures 4.10 and 4.11). However, luteolin was found to inhibit LPS-induced NF-κB activation by suppressing IκBα degradation in macrophages (Dhanalakshmi *et al.*, 2002), indicating that the effect of luteolin on NF-κB may be cell type or stimulus-specific. Furthermore, we proved that luteolin inhibits NF-κB activation by disrupting the

interaction between p65 and its coactivator, CBP (Gerritsen *et al*., 1997), one of the critical step in p65 transcriptional activation (Kim *et al*., 2003b).

Taken together, data from this part of our study demonstrated a new anti-cancer function of luteolin: sensitization of human cancer cells to TNFα-induced apoptosis. Understanding such an effect of luteolin supports the potential application of luteolin as a chemotherapeutic agent against cancer together with TNFα.

7.3 Luteolin sensitizes TRAIL induced apoptosis in human cancer cells

In Chapter 4, we studied the synergistic effect between luteolin and TNFα. In our subsequent study, we then focused on the effect of luteolin on TRAIL-induced apoptosis. TRAIL is a newly identified member of the TNFR family. The unique property of TRAIL is its selectivity: it can kill cancerous or transformed cells but spare most of the normal cells (Wang and El Deiry, 2003), thus making TRAIL an ideal cancer therapeutic agent. However, one of the major obstacles in its clinical application is that many cancer cells are found to be resistant to TRAIL-induced apoptosis (Wang and El Deiry, 2003).

In this part of study, we observed that luteolin pretreatment greatly enhances TRAIL induced-apoptosis in human cancer cells, including those TRAIL-resistant cancer cells (Figures 5.2 and 5.3), indicating the potential of using luteolin as a chemosensitizer to overcome TRAIL resistance. In search of the molecular mechanisms involved in the sensitization, we first excluded the possibility of NF-κB inhibition or altered expression of DR4 and DR5, two death receptors for TRAIL (Figures 5.7, 5.8 and 5.9). Instead, we found significant reduction of XIAP protein level in cells treated with luteolin and TRAIL (Figure 5.11). XIAP is known to be the most important member of IAP family as it can directly bind to and inhibit both

caspase 9 and caspase activity (Deveraux *et al*., 1997; Deveraux and Reed, 1999; Riedl *et al.*, 2001).

The expression level of XIAP could be regulated at both transcriptional and post-transcriptional levels. At transcriptional level, XIAP is known to be one of the target genes of NF-κB (Deveraux and Reed, 1999). Since TRAIL fails to activate NF-κB in our system (Figure 5.9) and there is no change of its mRNA level (Figure 5.14). We then focused on the post-transcriptional regulatory mechanisms of XIAP. It is known that the RING finger domain of XIAP has ubiquitin protease ligase (E3) activity and is responsible for its autoubiquitination and proteasomal degradation (Yang *et al*., 2000b). It is also known that XIAP ubiquitination and degradation depends partly on its phosphorylation status as protein kinases such as AKT have been shown to block XIAP ubiquitination and degradation via phosphorylation (Dan *et al*., 2004). In this study, we demonstrated a novel PKC signaling mechanism: PKC activation contributes to XIAP protein stabilization via enhanced XIAP phosphorylation and reduced protein ubiquitination and degradation. More importantly, luteolin is probably acting as a PKC inhibitor to inhibit XIAP phosphorylation and to promote its ubiquitination and proteasomal degradation. Such a finding is important since many cancer cells contain elevated basal PKC level and many tumor promoters such as PMA are known to be potent PKC activators (Harper *et al*., 2003b).

Combination of TRAIL with other anti-cancer agents has been a promising strategy to enhance the therapeutic efficiency of TRAIL and to overcome TRAIL resistance (Bagli *et al.*, 2004; Huerta-Yepez *et al*., 2004; Rosato *et al*., 2004; von Haefen *et al.*, 2004). Our data provide convincing evidence for the potential therapeutic application of luteolin in overcoming TRAIL resistance.

7.4 Luteolin enhances the anticancer effect of cisplatin *in vitro* and *in vivo*

Cisplatin has been used successfully as an anti-cancer drug in variety of cancers. It has been well established that cisplatin kills the cancer cells via induction of DNA damage and p53 activation (Siddik, 2003). However, changes in p53 signaling pathway, for example, elevation of MDM2 protein level, confer cancer cells to resistant to cisplatin (Kondo *et al.,* 1995).

In this part of our study, we focused on effect of luteolin on the anti-cancer efficacy of cisplatin using both *in vitro* cell culture and an *in vivo* animal model. One significant finding is that a functional p53 is required for cell death induced by combined treatment of luteolin and cisplatin. Moreover, luteolin alone is capable of markedly increasing p53 protein level. It has been reported that luteolin activates wild type p53 in several cells (Plaumann *et al.*, 1996), without knowing the mechanism involved. Here we provided clear evidence that luteolin is capable of stabilizing p53 protein through suppression of MDM2 gene transcription. Such a finding is indeed consistent with a previous report that apigenin could activate p53 through decreasing MDM2 protein level, indicating that there might exist a common mechanism by which flavonoids activate p53.

The chemosensitization effect of luteolin was further tested in a nude mice xenograft model. While a relatively low dose of luteolin or cisplatin only marginally suppressed the tumor cell growth, the combined treatment of luteolin and cisplatin led to significant reduction of tumor size (Figures 6.16 and 6.17). Importantly, higher level of p53 protein was also observed in tumor tissues in mice receiving combined treatment of luteolin and cisplatin, suggesting that luteolin acts via a similar mechanism as observed *in vitro* to enhance the therapeutic efficacy of cisplatin *in vivo*. Although the combined treatment of luteolin and cisplatin will only be workable in

cancers with wild-type p53, luteolin is certainly valuable as a chemosensitizer to improve the efficacy of cisplatin or other DNA damaging agents in cancer therapy.

7.5 Luteolin as a chemosensitizer in cancer therapy

One of the focuses of this study is to examine the synergistic effect of luteolin with other cancer therapeutic agents, although luteolin alone at a relatively high concentration, is capable of inducing apoptotic cell death in cancer cells (Chapter 3). Systematic studies were conducted to demonstrate the sensitization activity of luteolin on cancer cell apoptosis induced by TNFα (Chapter 4), TRAIL (Chapter 5) and cisplatin (Chapter 6).

In the summary of the sensitization activity of luteolin, one important point emerges: luteolin is capable of utilizing distinct mechanisms depending on the nature of the cell death stimuli. For TNFα-induced apoptosis, luteolin acts as a NF-κB inhibitor (Chapter 4). In the presence of TRAIL, it promotes XIAP ubiquitination and proteasomal degradation by inhibiting PKC (Chapter 5). In cisplatin-treated cells, luteolin is able to stabilize p53 protein via inhibition of MDM2 expression (Chapter 6). Although distinct mechanisms are involved in different parts of our study, it is worth mentioning that some of the above mechanisms are functionally interlinked. For instance, XIAP is one of the target genes of NF-κB (Deveraux et al., 1997) and constitutively active NF-κB activation found in some cancers would render resistance to cancer therapy (Baldwin, 2001). Therefore, treatment with luteolin would then offer multiple impacts on XIAP: suppression of XIAP expression via reduced NF-κB and promotion of XIAP ubiquitination and degradation. On the other hand, it is known that NF-κB activation will lead to decreased p53 stabilization (Tergaonkar *et al.*, 2002). NF-κB activation is also implicated in resistance to cisplatin-induced apoptosis (Chuang *et al.*, 2002). Therefore, treatment with luteolin may increase p53

protein stability via multiple mechanisms, including reduced MDM2 expression and suppressed NF-κB signaling pathway. Taken together, such a unique property of luteolin makes this compound desirable as a chemosensitizer in cancer therapy. The significant synergistic effect of luteolin and cisplatin observed in the *in vivo* animal model tends to support the above notion.

Based on the literature and our earlier observations that luteolin could have multiple functions and effects in cancer cells, possibly with multiple targets and affecting different anti-cancer pathways. It was reported that luteolin can directly inhibit the activities of several kinases (Ferriola et al., 1989; Huang, 1996; Conseil, 1998). However, the linkage of the inhibition with the biological effects has not been elucidated. Furthermore, it is not clear whether other molecular targets are also involved. Sporadic reports have also shown that many flavonoids showed similar biological effects as luteolin (Gerritsen et al., 1997; Plaumann *et al.*, 1996; Farah *et al.*, 2003; Kim *et al*., 2003b). Thus, it is worthwhile to explore the structure-activity relationship systematically. The identification of the molecular targets of some of the flavonoids as well as the structure-activity relationship study will also help to optimize its pharmacokinetics and pave a way to its clinical application.

7.6 Conclusions

In this study, we carried out systematic investigation on the anti-tumor properties of chrysanthemum. We first confirmed that flavonoids are the main active components responsible of the anti-cancer effect of chrysanthemum. Subsequently we focused on the anti-tumor activity of luteolin by examining its sensitization effect on cancer therapeutic agents, including TNFα, TRAIL and cisplatin.

The major findings are:

1) Flavonoids are the major anti-tumor components of the chrysanthemum water extract;

2) Chrysanthemum flavonoids exert their anti-tumor activity by inducing caspase-dependent apoptosis;

3) Luteolin is the major flavonoid in chrysanthemum, and induces caspase-dependent apoptosis in human cancer cells;

4) Luteolin sensitizes TNFα-induced apoptosis in cancer cells by suppressing NF-κB activation and augmenting JNK activation;

5) Luteolin sensitizes TRAIL-induced apoptosis in cancer cell by promoting XIAP ubiquitination and proteasomal degradation via its inhibitory effect on PKC;

6) Luteolin enhances the anti-cancer activity of cisplatin by stabilizing p53 protein via suppression of MDM2 gene expression;

7) Luteolin enhances the anti-cancer activity of cisplatin in a nude mice xenograft model.

In summary, data from this study clearly demonstrate the anti-tumor activity of luteolin, a major flavonoid from chrysanthemum. More importantly, this study provides evidence showing that luteolin is highly capable of sensitizing TNFα, TRAIL and cisplatin-induced cancer cell apoptosis. Such findings support the potential application of luteolin as a chemosensitizer in cancer therapy.

CHAPTER EIGHT

REFERENCE

Reference:

Abdollahi, T., Robertson, N. M., Abdollahi, A., and Litwack, G. (2003). Identification of Interleukin 8 as an Inhibitor of Tumor Necrosis Factor-related Apoptosis-inducing Ligand-induced Apoptosis in the Ovarian Carcinoma Cell Line OVCAR3. Cancer Res *63*, 4521-4526.

Adams, J. M., and Cory, S. (1998). The Bcl-2 Protein Family: Arbiters of Cell Survival. Science *281*, 1322-1326.

Agullo, G., Gamet-Payrastre, L., Manenti, S., Viala, C., Remesy, C., Chap, H., and Payrastre, B. (1997). Relationship between flavonoid structure and inhibition of phosphatidylinositol 3-kinase: a comparison with tyrosine kinase and protein kinase C inhibition. *53*, 1649.

Akihisa, T., Yasukawa, K., Oinuma, H., Kasahara, Y., Yamanouchi, S., Takido, M., Kumaki, K., and Tamura, T. (1996). Triterpene alcohols from the flowers of compositae and their anti-inflammatory effects. *43*, 1255.

Akira, S., Taga, T., and Kishimoto, T. (1993). Interleukin-6 in biology and medicine. Adv Immunol *54*, 1-78.

Ardizzoni, A., Antonelli, G., Grossi, F., Tixi, L., Cafferata, M., and Rosso, R. (1999). The combination of etoposide and cisplatin in non-small-cell lung cancer (NSCLC). Ann Oncol *10 Suppl 5*, S13-17.

Arima, Y., Nitta, M., Kuninaka, S., Zhang, D., Fujiwara, T., Taya, Y., Nakao, M., and Saya, H. (2005). Transcriptional blockade induces p53-dependent apoptosis associated with translocation of p53 to mitochondria. J Biol Chem *280*, 19166-19176.

Ashcroft, M., and Vousden, K. H. (1999). Regulation of p53 stability. Oncogene *18*, 7637-7643.

Ashkenazi, A. (2002). Targeting death and decoy receptors of the tumour-necrosis factor superfamily. *2*, 420.

Asselin, E., Mills, G. B., and Tsang, B. K. (2001). XIAP Regulates Akt Activity and Caspase-3-dependent Cleavage during Cisplatin-induced Apoptosis in Human Ovarian Epithelial Cancer Cells. Cancer Research *61*, 1862.

Attardi, L. D., Reczek, E. E., Cosmas, C., Demicco, E. G., McCurrach, M. E., Lowe, S. W., and Jacks, T. (2000). PERP, an apoptosis-associated target of p53, is a novel member of the PMP-22/gas3 family. Genes Dev *14*, 704-718.

Baeuerle, P. A., and Baltimore, D. (1996). NF-kappa B: ten years after. Cell *87*, 13-20.

Baeuerle, P. A., and Henkel, T. (1994). Function and activation of NF-kappa B in the immune system. Annu Rev Immunol *12*, 141-179.

Bagli, E., Stefaniotou, M., Morbidelli, L., Ziche, M., Psillas, K., Murphy, C., and Fotsis, T. (2004). Luteolin inhibits vascular endothelial growth factor-induced angiogenesis; inhibition of endothelial cell survival and proliferation by targeting phosphatidylinositol 3'-kinase activity. Cancer Res *64*, 7936-7946.

Baldwin, A. S. (2001). Control of oncogenesis and cancer therapy resistance by the transcription factor NF-kappaB. J Clin Invest *107*, 241-246.

Bartholomeusz, C., Itamochi, H., Yuan, L. X. H., Esteva, F. J., Wood, C. G., Terakawa, N., Hung, M.-C., and Ueno, N. T. (2005). Bcl-2 Antisense Oligonucleotide Overcomes Resistance to E1A Gene Therapy in a Low HER2-Expressing Ovarian Cancer Xenograft Model. Cancer Res *65*, 8406-8413.

Behnia, M., Foster, R., Einhorn, L. H., Donohue, J., and Nichols, C. R. (2000). Adjuvant bleomycin, etoposide and cisplatin in pathological stage II non-seminomatous testicular cancer: the Indiana University experience. European Journal of Cancer *36*, 472.

Benassayag, C., Perrot-Applanat, M., and Ferre, F. (2002). Phytoestrogens as modulators of steroid action in target cells. Journal of Chromatography B *777*, 233.

Bennett, B. L., Sasaki, D. T., Murray, B. W., O'Leary, E. C., Sakata, S. T., Xu, W., Leisten, J. C., Motiwala, A., Pierce, S., Satoh, Y.*, et al.* (2001). SP600125, an anthrapyrazolone inhibitor of Jun N-terminal kinase. Proc Natl Acad Sci U S A *98*, 13681-13686.

Bennett, M., Macdonald, K., Chan, S. W., Luzio, J. P., Simari, R., and Weissberg, P. (1998). Cell surface trafficking of Fas: a rapid mechanism of p53-mediated apoptosis. Science *282*, 290.

Beuvink, I., Boulay, A., Fumagalli, S., Zilbermann, F., Ruetz, S., O'Reilly, T., Natt, F., Hall, J., Lane, H. A., and Thomas, G. (2005). The mTOR Inhibitor RAD001 Sensitizes Tumor Cells to DNA-Damaged Induced Apoptosis through Inhibition of p21 Translation. Cell *120*, 747.

Birt, D. F., Hendrich, S., and Wang, W. (2001). Dietary agents in cancer prevention: flavonoids and isoflavonoids. Pharmacol Ther *90*, 157-177.

Boulares, A. H., Yakovlev, A. G., Ivanova, V., Stoica, B. A., Wang, G., Iyer, S., and Smulson, M. (1999). Role of Poly(ADP-ribose) Polymerase (PARP) Cleavage in Apoptosis. J Biol Chem *274*, 22932-22940.

Boyer, J., McLean, E. G., Aroori, S., Wilson, P., McCulla, A., Carey, P. D., Longley, D. B., and Johnston, P. G. (2004). Characterization of p53 Wild-Type and Null Isogenic Colorectal Cancer Cell Lines Resistant to 5-Fluorouracil, Oxaliplatin, and Irinotecan. Clin Cancer Res *10*, 2158-2167.

Brattain, M. G., Fine, W. D., Khaled, F. M., Thompson, J., and Brattain, D. E. (1981). Heterogeneity of malignant cells from a human colonic carcinoma. Cancer Res *41*, 1751-1756.

Brinckerhoff, C. E., and Matrisian, L. M. (2002). MATRIX METALLOPROTEINASES: A TAIL OF A FROG THAT BECAME A PRINCE. Nature Reviews Molecular Cell Biology *3*, 207.

Brown, J. E., and Rice-Evans, C. A. (1998). Luteolin-rich artichoke extract protects low density lipoprotein from oxidation in vitro. Free Radic Res *29*, 247-255.

Brown, M. D. (1999). Green tea (Camellia sinensis) extract and its possible role in the prevention of cancer. Altern Med Rev *4*, 360-370.

Bubici, C., Papa, S., Pham, C., Zazzeroni, F., and Franzoso, G. (2006). The NF-kappaB-mediated control of ROS and JNK signaling. Histol Histopathol *21*, 69-80.

Buening, M. K., Chang, R.L., Huang, M.T., Fortner, J.G., Wood, A.W., and Conney, A.H. (1981). Activation and inhibition of benzo(a)pyrene and aflatoxin B1 metabolism in human liver microsomes by naturally occurring flavonoids. Cancer Research *41*, 67-72.

Buschmann, T., Potapova, O., Bar-Shira, A., Ivanov, V. N., Fuchs, S. Y., Henderson, S., Fried, V. A., Minamoto, T., Alarcon-Vargas, D., Pincus, M. R.*, et al.* (2001). Jun NH2-Terminal Kinase Phosphorylation of p53 on Thr-81 Is Important for p53 Stabilization and Transcriptional Activities in Response to Stress. Mol Cell Biol *21*, 2743-2754.

Cande, C., Cecconi, F., Dessen, P., and Kroemer, G. (2002). Apoptosis-inducing factor (AIF): key to the conserved caspase-independent pathways of cell death? J Cell Sci *115*, 4727-4734.

Cao, G., Sofic, E., and Prior, R. L. (1997). Antioxidant and Prooxidant Behavior of Flavonoids: Structure-Activity Relationships. Free Radical Biology and Medicine *22*, 749.

Casagrande, F., and Darbon, J. M. (2001). Effects of structurally related flavonoids on cell cycle progression of human melanoma cells: regulation of cyclin-dependent kinases CDK2 and CDK1. Biochem Pharmacol *61*, 1205-1215.

Chang, Y. C., Lee, Y.S., Tejima, T., Tanaka, K., Omura, S., Heintz, N.H., Mitsui, Y., and Magae, J. (1998). mdm2 and bax, downstream mediators of the p53 response, are degraded by the ubiquitin-proteasome pathway. Cell Growth Differ 1998 Jan;9(1):79-84 *9*, 79-84.

Chen, C.-C., Chow, M.-P., Huang, W.-C., Lin, Y.-C., and Chang, Y.-J. (2004). Flavonoids Inhibit Tumor Necrosis Factor-{alpha}-Induced Up-Regulation of Intercellular Adhesion Molecule-1 (ICAM-1) in Respiratory Epithelial Cells through Activator Protein-1 and Nuclear Factor-{kappa}B: Structure-Activity Relationships. Mol Pharmacol *66*, 683-693.

Chen, G., and Goeddel, D. V. (2002). TNF-R1 signaling: a beautiful pathway. Science *296*, 1634-1635.

Chen, W., Weng, Y. M., and Tseng, C. Y. (2003). Antioxidative and antimutagenic activities of healthy herbal drinks from Chinese medicinal herbs. *31*, 523.

Chen, X., Thakkar, H., Tyan, F., Gim, S., Robinson, H., Lee, C., Pandey, S. K., Nwokorie, C., Onwudiwe, N., and Srivastava, R. K. (2001). Constitutively active Akt is an important regulator of TRAIL sensitivity in prostate cancer. *%20;20*, 6073.

Cheng, A.-C., Huang, T.-C., Lai, C.-S., and Pan, M.-H. (2005a). Induction of apoptosis by luteolin through cleavage of Bcl-2 family in human leukemia HL-60 cells. European Journal of Pharmacology *509*, 1.

Cheng, W., Li, J., You, T., and Hu, C. (2005b). Anti-inflammatory and immunomodulatory activities of the extracts from the inflorescence of Chrysanthemum indicum Linne. J Ethnopharmacol *101*, 334-337.

Cheng, W., Li, J., You, T., and Hu, C. (2005). Anti-inflammatory and immunomodulatory activities of the extracts from the inflorescence of Chrysanthemum indicum Linne. J Ethnopharmacol *101*, 334-337.

Chi, S.-W., Lee, S.-H., Kim, D.-H., Ahn, M.-J., Kim, J.-S., Woo, J.-Y., Torizawa, T., Kainosho, M., and Han, K.-H. (2005). Structural details on MDM2-P53 interaction. J Biol Chem, M508578200.

Chinnaiyan, A. M., O'Rourke, K., Tewari, M., and Dixit, V. M. (1995). FADD, a novel death domain-containing protein, interacts with the death domain of Fas and initiates apoptosis. Cell *81*, 505-512.

Chipuk, J. E., Bouchier-Hayes, L., Kuwana, T., Newmeyer, D. D., and Green, D. R. (2005). PUMA Couples the Nuclear and Cytoplasmic Proapoptotic Function of p53. Science *309*, 1732-1735.

Chipuk, J. E., Maurer, U., Green, D. R., and Schuler, M. (2003). Pharmacologic activation of p53 elicits Bax-dependent apoptosis in the absence of transcription. Cancer Cell *4*, 371.

Cholbi, M. R., Paya, M., and Alcaraz, M.J. (1991). Inhibitory effects of phenolic compounds on CCl4-induced microsomal lipid peroxidation. Experientia *47*, 195-199.

Chowdhury, A. R., Sharma, S., Mandal, S., Goswami, A., Mukhopadhyay, S., and Majumder, H. K. (2002). Luteolin, an emerging anti-cancer flavonoid, poisons eukaryotic DNA topoisomerase I. Biochem J *366*, 653-661.

Chuang, S. E., Yeh, P. Y., Lu, Y. S., Lai, G. M., Liao, C. M., Gao, M., and Cheng, A. L. (2002). Basal levels and patterns of anticancer drug-induced activation of nuclear factor-kappaB (NF-kappaB), and its attenuation by tamoxifen, dexamethasone, and curcumin in carcinoma cells. Biochem Pharmacol *63*, 1709-1716.

Claesson-Welsh, L. (1994). Platelet-derived growth factor receptor signals. J Biol Chem *269*, 32023-32026.

Colditz, G. A. (2005). Estrogen, Estrogen Plus Progestin Therapy, and Risk of Breast Cancer. Clin Cancer Res *11*, 909s-917.

Collins-Burow, B. M., Burow, M.E., Duong, B.N., and McLachlan, J.A. (2000). Estrogenic and antiestrogenic activities of flavonoid phytochemicals through estrogen receptor binding-dependent and -independent mechanisms. Nutr Cancer *38*, 229-244.

Conseil, G., Baubichon-Cortay, H., Dayan, G., Jault, J.M., Barron, D., and Di Pietro, A. (1998). Flavonoids: a class of modulators with bifunctional interactions at vicinal ATP- and steroid-binding sites on mouse P-glycoprotein. Proc Natl Acad Sci *95*, 9831-9836.

Constantinou, A., Mehta, R., Runyan, C., Rao, K., Vaughan, A., and Moon, R. (1995). Flavonoids as DNA topoisomerase antagonists and poisons: structure-activity relationships. J Nat Prod *58*, 217-225.

Corbett, K. D., and Berger, J. M. (2004). Structure, molecular mechanisms, and evolutionary relationships in DNA topoisomerases. Annu Rev Biophys Biomol Struct *33*, 95-118.

Dan, H. C., Sun, M., Kaneko, S., Feldman, R. I., Nicosia, S. V., Wang, H. G., Tsang, B. K., and Cheng, J. Q. (2004). Akt phosphorylation and stabilization of X-linked inhibitor of apoptosis protein (XIAP). *279*, 5405.

Davis, R. J. (2000). Signal transduction by the JNK group of MAP kinases. Cell *103*, 239-252.

De Smaele, E., Zazzeroni, F., Papa, S., Nguyen, D. U., Jin, R., Jones, J., Cong, R., and Franzoso, G. (2001). Induction of gadd45beta by NF-kappaB downregulates pro-apoptotic JNK signalling. Nature *414*, 308-313.

Denecker, G., Vercammen, D., Steemans, M., Vanden Berghe, T., Brouckaert, G., Van Loo, G., Zhivotovsky, B., Fiers, W., Grooten, J., Declercq, W., and Vandenabeele, P. (2001). Death receptor-induced apoptotic and necrotic cell death: differential role of caspases and mitochondria. Cell Death Differ *8*, 829-840.

Deng, Y., Ren, X., Yang, L., Lin, Y., and Wu, X. (2003). A JNK-dependent pathway is required for TNFalpha-induced apoptosis. Cell *115*, 61-70.

Desagher, S., Osen-Sand, A., Nichols, A., Eskes, R., Montessuit, S., Lauper, S., Maundrell, K., Antonsson, B., and Martinou, J. C. (1999). Bid-induced conformational change of Bax is responsible for mitochondrial cytochrome c release during apoptosis. The Journal Of Cell Biology *144*, 891.

Devasagayam, T. P., Subramanian, M., Singh, B. B., Ramanathan, R., and Das, N. P. (1995). Protection of plasmid pBR322 DNA by flavonoids against single-stranded breaks induced by singlet molecular oxygen. J Photochem Photobiol B *30*, 97-103.

Deveraux, Q. L., and Reed, J. C. (1999). IAP family proteins--suppressors of apoptosis. *13*, 239.

Deveraux, Q. L., Takahashi, R., Salvesen, G. S., and Reed, J. C. (1997). X-linked IAP is a direct inhibitor of cell-death proteases. *388*, 300.

Dhanalakshmi, S., Singh, R. P., Agarwal, C., and Agarwal, R. (2002). Silibinin inhibits constitutive and TNFalpha-induced activation of NF-kappaB and sensitizes human prostate carcinoma DU145 cells to TNFalpha-induced apoptosis. Oncogene *21*, 1759-1767.

Dijt, F. J., Fichtinger-Schepman, A.M., Berends, F., and Reedijk, J. (1988). Formation and repair of cisplatin-induced adducts to DNA in cultured normal and repair-deficient human fibroblasts. Cancer Res *48*, 6058-6062.

Ding, H. F., Lin, Y. L., McGill, G., Juo, P., Zhu, H., Blenis, J., Yuan, J., and Fisher, D. E. (2000). Essential role for caspase-8 in transcription-independent apoptosis triggered by p53. The Journal Of Biological Chemistry *275*, 38905.

Donjerkovic, D., and Scott, D. W. (2000). Regulation of the G1 phase of the mammalian cell cycle. Cell Res *10*, 1-16.

Duan, L., Aoyagi, M., Tamaki, M., Nakagawa, K., Nagashima, G., Nagasaka, Y., Ohno, K., Yamamoto, K., and Hirakawa, K. (2001). Sensitization of human malignant glioma cell lines to tumor necrosis factor-induced apoptosis by cisplatin. J Neurooncol *52*, 23-36.

Duh, P. D. (1999). Antioxidant activity of water extract of Harng Jyur (Chrysanthemum morifolium Ramat), Y. Y. Y. G. C. Tu, ed., pp. 269.

Duthie, S. J., and Dobson, V. L. (1999). Dietary flavonoids protect human colonocyte DNA from oxidative attack in vitro. *38*, 28.

Ekholm, S. V., and Reed, S. I. (2000). Regulation of G1 cyclin-dependent kinases in the mammalian cell cycle. Current Opinion in Cell Biology *12*, 676.

Elangovan, V., Sekar, N., and Govindasamy, S. (1994). Chemopreventive potential of dietary bioflavonoids against 20-methylcholanthrene-induced tumorigenesis. Cancer Letters *87*, 107.

Ende, C., and Gebhardt, R. (2004). Inhibition of matrix metalloproteinase-2 and -9 activities by selected flavonoids. Planta Med *70*, 1006-1008.

Erster, S., Mihara, M., Kim, R. H., Petrenko, O., and Moll, U. M. (2004). In Vivo Mitochondrial p53 Translocation Triggers a Rapid First Wave of Cell Death in

Response to DNA Damage That Can Precede p53 Target Gene Activation. Mol Cell Biol *24*, 6728-6741.

Fang, J., Xia, C., Cao, Z., Zheng, J. Z., Reed, E., and Jiang, B.-H. (2005). Apigenin inhibits VEGF and HIF-1 expression via PI3K/AKT/p70S6K1 and HDM2/p53 pathways. FASEB J *19*, 342-353.

Farah, M., Parhar, K., Moussavi, M., Eivemark, S., and Salh, B. (2003). 5,6-Dichloro-ribifuranosylbenzimidazole- and apigenin-induced sensitization of colon cancer cells to TNF-alpha-mediated apoptosis. Am J Physiol Gastrointest Liver Physiol *285*, G919-928.

Ferrara, N., and Gerber, H.P. (2001). The role of vascular endothelial growth factor in angiogenesis. Acta Haematol *106*, 148-156.

Ferreira, C. G., Epping, M., Kruyt, F. A. E., and Giaccone, G. (2002). Apoptosis: Target of Cancer Therapy. Clin Cancer Res *8*, 2024-2034.

Ferriola, P. C., Cody, V., and Middleton, E., Jr. (1989). Protein kinase C inhibition by plant flavonoids. Kinetic mechanisms and structure-activity relationships. *38*, 1617.

Fischer, U., Janicke, R. U., and Schulze-Osthoff, K. (2003). Many cuts to ruin: a comprehensive update of caspase substrates. Cell Death Differ *10*, 76-100.

Folkman, J. (1995). Angiogenesis in cancer, vascular, rheumatoid and other disease. Nat Med *1*, 27-31.

Fotsis, T., Pepper, M.S., Aktas, E., Breit, S., Rasku, S., Adlercreutz, H., Wahala, K., Montesano, R., and Schweigerer, L. (1997). Flavonoids, dietary-derived inhibitors of cell proliferation and in vitro angiogenesis. Cancer Research *57*, 2916-2921.

Fuentes, L., Perez, R., Nieto, M. L., Balsinde, J., and Balboa, M. A. (2003). Bromoenol lactone promotes cell death by a mechanism involving phosphatidate phosphohydrolase-1 rather than calcium-independent phospholipase A2. *278*, 44683.

Fulda, S., and Debatin, K. M. (2005). Sensitization for anticancer drug-induced apoptosis by betulinic Acid. Neoplasia *7*, 162-170.

Galati, G., and O'Brien, P. J. (2004). Potential toxicity of flavonoids and other dietary phenolics: significance for their chemopreventive and anticancer properties. Free Radical Biology and Medicine *37*, 287.

Galati, G., Sabzevari, O., Wilson, J. X., and O'Brien, P. J. (2002). Prooxidant activity and cellular effects of the phenoxyl radicals of dietary flavonoids and other polyphenolics. Toxicology *177*, 91.

Gerritsen, M. E., Williams, A. J., Neish, A. S., Moore, S., Shi, Y., and Collins, T. (1997). CREB-binding protein/p300 are transcriptional coactivators of p65. Proc Natl Acad Sci U S A *94*, 2927-2932.

Ghobrial, I. M., Witzig, T. E., and Adjei, A. A. (2005). Targeting Apoptosis Pathways in Cancer Therapy. CA Cancer J Clin *55*, 178-194.

Gibson, S. B., Oyer, R., Spalding, A. C., Anderson, S. M., and Johnson, G. L. (2000). Increased expression of death receptors 4 and 5 synergizes the apoptosis response to combined treatment with etoposide and TRAIL. *20*, 205.

Gonzalez, V. M., Fuertes, M. A., Alonso, C., and Perez, J. M. (2001). Is Cisplatin-Induced Cell Death Always Produced by Apoptosis? Mol Pharmacol *59*, 657-663.

Goping, I. S., Gross, A., Lavoie, J. N., Nguyen, M., Jemmerson, R., Roth, K., Korsmeyer, S. J., and Shore, G. C. (1998). Regulated targeting of BAX to mitochondria. The Journal Of Cell Biology *143*, 207.

Goto, H., Yano, S., Matsumori, Y., Ogawa, H., Blakey, D. C., and Sone, S. (2004). Sensitization of tumor-associated endothelial cell apoptosis by the novel vascular-targeting agent ZD6126 in combination with cisplatin. Clin Cancer Res *10*, 7671-7676.

Green, D. R. (2000a). Apoptotic pathways: paper wraps stone blunts scissors. *102*, 1.

Green, D. R. (2000b). Apoptotic Pathways: Paper Wraps Stone Blunts Scissors. Cell *102*, 1.

Griffiths, L. A., and Barrow, A. (1972). Metabolism of flavonoid compounds in germ-free rats. Biochem J *130*, 1161-1162.

Hah, J. O., Lee, K. A., Choi, Y. J., Kim, W. D., Park, J. W., and Kwon, T. K. (2003). Inactive caspase 3 activates Akt in human leukemia cells susceptible or resistant to apoptosis induced by phorbol ester. *22*, 1111.

Han, D. H., Denison, M.S., Tachibana, H., and Yamada, K. (2002). Relationship between estrogen receptor-binding and estrogenic activities of environmental estrogens and suppression by flavonoids. Biosci Biotechnol Biochem *66*, 1479-1487.

Hanahan, D., and Weinberg, R. A. (2000). The Hallmarks of Cancer. Cell *100*, 57.

Hanasaki, Y., Ogawa, S., and Fukui, S. (1994). The correlation between active oxygens scavenging and antioxidative effects of flavonoids. Free Radical Biology and Medicine *16*, 845.

Hanson, J. R. (2001). The development of strategies for terpenoid structure determination. Nat Prod Rep *18*, 607-617.

Harborne, J. B., and Williams, C. A. (2000). Advances in flavonoid research since 1992. Phytochemistry *55*, 481.

Harper, N., Hughes, M., MacFarlane, M., and Cohen, G. M. (2003a). Fas-associated Death Domain Protein and Caspase-8 Are Not Recruited to the Tumor Necrosis

Factor Receptor 1 Signaling Complex during Tumor Necrosis Factor-induced Apoptosis. J Biol Chem *278*, 25534-25541.

Harper, N., Hughes, M. A., Farrow, S. N., Cohen, G. M., and MacFarlane, M. (2003b). Protein kinase C modulates tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis by targeting the apical events of death receptor signaling. *278*, 44338.

Hasebe, Y., Egawa, K., Yamazaki, Y., Kunimoto, S., Hirai, Y., Ida, Y., and Nose, K. (2003). Specific inhibition of hypoxia-inducible factor (HIF)-1 alpha activation and of vascular endothelial growth factor (VEGF) production by flavonoids. Biol Pharm Bull *26*, 1379-1383.

He, K. L., and Ting, A. T. (2002). A20 inhibits tumor necrosis factor (TNF) alpha-induced apoptosis by disrupting recruitment of TRADD and RIP to the TNF receptor 1 complex in Jurkat T cells. Mol Cell Biol *22*, 6034-6045.

Hehner, S. P., Hofmann, T. G., Droge, W., and Schmitz, M. L. (1999). The antiinflammatory sesquiterpene lactone parthenolide inhibits NF-kappa B by targeting the I kappa B kinase complex. J Immunol *163*, 5617-5623.

Hehner, S. P., Hofmann, T. G., Ratter, F., Dumont, A., Droge, W., and Schmitz, M. L. (1998). Tumor necrosis factor-alpha-induced cell killing and activation of transcription factor NF-kappaB are uncoupled in L929 cells. J Biol Chem *273*, 18117-18121.

Hengartner, M. O. (2000). The biochemistry of apoptosis. Nature *407*, 770.

Herrant, M., Jacquel, A., Marchetti, S., Belhacene, N., Colosetti, P., Luciano, F., and Auberger, P. (2004). Cleavage of Mcl-1 by caspases impaired its ability to counteract Bim-induced apoptosis. *23*, 7863.

Hibasami, H., Jin, Z.X., Hasegawa, M., Urakawa, K., Nakagawa, M., Ishii, Y., adn Yoshioka, K. (2000). Oolong tea polyphenol extract induces apoptosis in human stomach cancer cells. Anticancer Res *20*, 4403-4406.

Hofmann, J., Fiebig, H. H., Winterhalter, B. R., Berger, D. P., and Grunicke, H. (1990). Enhancement of the antiproliferative activity of cis-diamminedichloroplatinum(II) by quercetin. Int J Cancer *45*, 536-539.

Holland, M. B., and Roy, D. (1995). Estrone-induced cell proliferation and differentiation in the mammary gland of the female Noble rat. Carcinogenesis *16*, 1955-1961.

Hollman, P. C., de Vries, J. H., van Leeuwen, S. D., Mengelers, M. J., and Katan, M. B. (1995). Absorption of dietary quercetin glycosides and quercetin in healthy ileostomy volunteers. Am J Clin Nutr *62*, 1276-1282.

Hollman, P. C. H., and Katan, M. B. (1999). Dietary Flavonoids: Intake, Health Effects and Bioavailability. Food and Chemical Toxicology *37*, 937.

Hollstein, M., Sidransky, D., Vogelstein, B., and Harris, C.C. (1991). p53 mutations in human cancers. Science 1991 Jul 5;253(5015):49-53 *253*, 49-53.

Horinaka, M., Yoshida, T., Shiraishi, T., Nakata, S., Wakada, M., Nakanishi, R., Nishino, H., Matsui, H., and Sakai, T. (2005). Luteolin induces apoptosis via death receptor 5 upregulation in human malignant tumor cells. *24*, 7180.

Hu, B. B., Jiang, H. D., Yang, J., and Zeng, S. (2004). [Determination of luteolin and luteolin-7-beta-D-glucoside in Chrysanthemum morfolium Ramat. from different collection time by RP-HPLC]. Zhejiang Da Xue Xue Bao Yi Xue Ban *33*, 29-32.

Hu, C., and Kitts, D. D. (2004). Luteolin and luteolin-7-O-glucoside from dandelion flower suppress iNOS and COX-2 in RAW264.7 cells. Mol Cell Biochem *265*, 107-113.

Hu, C. Q., Chen, K., Shi, Q., Kilkuskie, R. E., Cheng, Y. C., and Lee, K. H. (1994). Anti-AIDS agents, 10. Acacetin-7-O-beta-D-galactopyranoside, an anti-HIV principle from Chrysanthemum morifolium and a structure-activity correlation with some related flavonoids. J Nat Prod *57*, 42-51.

Hu, S., Vincenz, C., Ni, J., Gentz, R., and Dixit, V. M. (1997). I-FLICE, a Novel Inhibitor of Tumor Necrosis Factor Receptor-1- and CD-95-induced Apoptosis. J Biol Chem *272*, 17255-17257.

Huang, M. T., Wood, A.W., Newmark, H.L., Sayer, J.M., Yagi, H., Jerina, D.M., and Conney, A.H. (1983). Inhibition of the mutagenicity of bay-region diol-epoxides of polycyclic aromatic hydrocarbons by phenolic plant flavonoids. Carcinogenesis *4*, 1631-1637.

Huang, Y. T., Hwang, J. J., Lee, P. P., Ke, F. C., Huang, J. H., Huang, C. J., Kandaswami, C., Middleton, E., Jr., and Lee, M. T. (1999a). Effects of luteolin and quercetin, inhibitors of tyrosine kinase, on cell growth and metastasis-associated properties in A431 cells overexpressing epidermal growth factor receptor. Br J Pharmacol *128*, 999-1010.

Huang, Y. T., Hwang, J. J., Lee, P. P., Ke, F. C., Huang, J. H., Huang, C. J., Kandaswami, C., Middleton, E., Jr., and Lee, M. T. (1999b). Effects of luteolin and quercetin, inhibitors of tyrosine kinase, on cell growth and metastasis-associated properties in A431 cells overexpressing epidermal growth factor receptor. Br J Pharmacol *128*, 999.

Huang, Y. T., Kuo, M.L., Liu, J.Y., Huang, S.Y., and Lin, J.K. (1996). Inhibitions of protein kinase C and proto-oncogene expressions in NIH 3T3 cells by apigenin. Eur J Cancer *32A*, 146-151.

Huang, Y. T., Lee, L.T., Lee, P.P., Lin, Y.S., and Lee, M.T. (2005). Targeting of focal adhesion kinase by flavonoids and small-interfering RNAs reduces tumor cell migration ability. Anticancer Res *25*, 2017-2025.

Hubbard, A. K., and Rothlein, R. (2000). Intercellular adhesion molecule-1 (ICAM-1) expression and cell signaling cascades. Free Radic Biol Med *28*, 1379-1386.

Huerta-Yepez, S., Vega, M., Jazirehi, A., Garban, H., Hongo, F., Cheng, G., and Bonavida, B. (2004). Nitric oxide sensitizes prostate carcinoma cell lines to TRAIL-mediated apoptosis via inactivation of NF-kappa B and inhibition of Bcl-xl expression. *23*, 4993.

Hyer, M. L., Croxton, R., Krajewska, M., Krajewski, S., Kress, C. L., Lu, M., Suh, N., Sporn, M. B., Cryns, V. L., Zapata, J. M., and Reed, J. C. (2005). Synthetic Triterpenoids Cooperate with Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand to Induce Apoptosis of Breast Cancer Cells. Cancer Res *65*, 4799-4808.

Irmler, M., Thome, M., Hahne, M., Schneider, P., Hofmann, K., Steiner, V., Bodmer, J.-L., Schroter, M., Burns, K., Mattmann, C.*, et al.* (1997). Inhibition of death receptor signals by cellular FLIP. Nature *388*, 190.

Itoh, N., and Nagata, S. (1993). A novel protein domain required for apoptosis. Mutational analysis of human Fas antigen. J Biol Chem *268*, 10932-10937.

Iwase, M., Watanabe, H., Kondo, G., Ohashi, M., and Nagumo, M. (2003). Enhanced susceptibility of oral squamous cell carcinoma cell lines to FAS-mediated apoptosis by cisplatin and 5-fluorouracil. Int J Cancer *106*, 619-625.

Iwashita, K., Kobori, M., Yamaki, K., and Tsushida, T. (2000). Flavonoids inhibit cell growth and induce apoptosis in B16 melanoma 4A5 cells. Biosci Biotechnol Biochem *64*, 1813-1820.

Izeradjene, K., Douglas, L., Tillman, D. M., Delaney, A. B., and Houghton, J. A. (2005). Reactive oxygen species regulate caspase activation in tumor necrosis factor-related apoptosis-inducing ligand-resistant human colon carcinoma cell lines. Cancer Res *65*, 7436-7445.

Jiang, H. (2002). Studies on cardic effects of Chrysanthemum morifolium Ramat and their underlying mechanism, In Modernillization of Traditional Chinese Medicine, Q. Xia, W. Xu, and C. Cao, eds., pp. 31.

Jiang, H., Xia, Q., Xu, W., and Zheng, M. (2004). Chrysanthemum morifolium attenuated the reduction of contraction of isolated rat heart and cardiomyocytes induced by ischemia/reperfusion. Die Pharmazie *59*, 565.

Jiang, X., and Wang, X. (2004). Cytochrome C-mediated apoptosis. Annu Rev Biochem *73*, 87-106.

Joussen, A. M., Rohrschneider, K., Reichling, J., Kirchhof, B., and Kruse, F. E. (2000). Treatment of Corneal Neovascularization with Dietary Isoflavonoids and Flavonoids. Experimental Eye Research *71*, 483.

Kanata, H., Yane, K., Ota, I., Miyahara, H., Matsunaga, T., Takahashi, A., Ohnishi, K., Ohnishi, T., and Hosoi, H. (2000). CDDP induces p53-dependent apoptosis in tongue cancer cells. Int J Oncol *17*, 513-517.

Karin, M., and Ben-Neriah, Y. (2000). Phosphorylation meets ubiquitination: the control of NF-[kappa]B activity. Annu Rev Immunol *18*, 621-663.

Karin, M., and Delhase, M. (2000). The I kappa B kinase (IKK) and NF-kappa B: key elements of proinflammatory signalling. Semin Immunol *12*, 85-98.

Karin, M., and Lin, A. (2002). NF-kappaB at the crossroads of life and death. Nat Immunol *3*, 221-227.

Kartalou, M., and Essigmann, J. M. (2001). Mechanisms of resistance to cisplatin. *478*, 23.

Kaufmann, S. H., and Hengartner, M. O. (2001). Programmed cell death: alive and well in the new millennium. Trends in Cell Biology *11*, 526.

Kelekar, A., and Thompson, C. B. (1998). Bcl-2-family proteins: the role of the BH3 domain in apoptosis. Trends in Cell Biology *8*, 324.

Kelliher, M. A., Grimm, S., Ishida, Y., Kuo, F., Stanger, B. Z., and Leder, P. (1998). The death domain kinase RIP mediates the TNF-induced NF-kappaB signal. Immunity *8*, 297-303.

Kim, E. H., Kim, S. U., Shin, D. Y., and Choi, K. S. (2004a). Roscovitine sensitizes glioma cells to TRAIL-mediated apoptosis by downregulation of survivin and XIAP. *23*, 446.

Kim, H. J., Lee, S.B., Park, S.K., Kim, H.M., Park, Y.I., and Dong, M.S. (2005). Effects of hydroxyl group numbers on the B-ring of 5,7-dihydroxyflavones on the differential inhibition of human CYP 1A and CYP1B1 enzymes. Archives of Pharmacy Research *28*, 1114-1121.

Kim, H. K., Cheon, B. S., Kim, Y. H., Kim, S. Y., and Kim, H. P. (1999). Effects of naturally occurring flavonoids on nitric oxide production in the macrophage cell line RAW 264.7 and their structure-activity relationships. Biochemical Pharmacology *58*, 759.

Kim, H. S., Chang, I., Kim, J. Y., Choi, K.-H., and Lee, M.-S. (2005a). Caspase-Mediated p65 Cleavage Promotes TRAIL-Induced Apoptosis. Cancer Res *65*, 6111-6119.

Kim, H. Y., Yu, R., Kim, J. S., Kim, Y. K., and Sung, M. K. (2004b). Antiproliferative crude soy saponin extract modulates the expression of IkappaBalpha, protein kinase C, and cyclooxygenase-2 in human colon cancer cells. Cancer Lett *210*, 1-6.

Kim, J.-A., Kim, D.-K., Kang, O.-H., Choi, Y.-A., Park, H.-J., Choi, S.-C., Kim, T.-H., Yun, K.-J., Nah, Y.-H., and Lee, Y.-M. (2005b). Inhibitory effect of luteolin on TNF-[alpha]-induced IL-8 production in human colon epithelial cells. International Immunopharmacology *5*, 209.

Kim, J.-H., Jin, Y.-R., Park, B.-S., Kim, T.-J., Kim, S.-Y., Lim, Y., Hong, J.-T., Yoo, H.-S., and Yun, Y.-P. (2005c). Luteolin prevents PDGF-BB-induced proliferation of vascular smooth muscle cells by inhibition of PDGF [beta]-receptor phosphorylation. Biochemical Pharmacology *69*, 1715.

Kim, J. H., Cho, Y.H., Park, S.M., Lee, K.E., Lee, J.J., Lee, B.C., Pyo, H.B., Song, K.S., Park, H.D., and Yun, Y.P. (2004). Antioxidants and inhibitor of matrix metalloproteinase-1 expression from leaves of Zostera marina L. Arch Pharm Res *27*, 177-183.

Kim, J. S., and Jobin, C. (2005). The flavonoid luteolin prevents lipopolysaccharide-induced NF- B signalling and gene expression by blocking I B kinase activity in intestinal epithelial cells and bone-marrow derived dendritic cells. Immunology *115*, 375-387.

Kim, K. S., Rhee, K. H., Yoon, J. H., Lee, J. G., Lee, J. H., and Yoo, J. B. (2005d). Ginkgo biloba extract (EGb 761) induces apoptosis by the activation of caspase-3 in oral cavity cancer cells. Oral Oncol *41*, 383-389.

Kim, M. S., Blake, M., Baek, J. H., Kohlhagen, G., Pommier, Y., and Carrier, F. (2003a). Inhibition of histone deacetylase increases cytotoxicity to anticancer drugs targeting DNA. Cancer Res *63*, 7291-7300.

Kim, S. H., Shin, K. J., Kim, D., Kim, Y. H., Han, M. S., Lee, T. G., Kim, E., Ryu, S. H., and Suh, P. G. (2003b). Luteolin inhibits the nuclear factor-kappa B transcriptional activity in Rat-1 fibroblasts. Biochem Pharmacol *66*, 955-963.

Kimata, Shichijo, Miura, Serizawa, Inagaki, and Nagai (2000). Effects of luteolin, quercetin and baicalein on immunoglobulin E-mediated mediator release from human cultured mast cells. Clinical & Experimental Allergy *30*, 501-508.

Kinjo, J., Nagao, T., Tanaka, T., Nonaka, G., Okawa, M., Nohara, T., and Okabe, H. (2002). Activity-guided fractionation of green tea extract with antiproliferative activity against human stomach cancer cells. Biol Pharm Bull *25*, 1238-1240.

Knekt, P., Jarvinen, R., Seppanen, R., Hellovaara, M., Teppo, L., Pukkala, E., and Aromaa, A. (1997). Dietary flavonoids and the risk of lung cancer and other malignant neoplasms. Am J Epidemiol *146*, 223-230.

Knowles, L. M., Zigrossi, D.A., Tauber, R.A., Hightower, C., and Milner, J.A. (2000). Flavonoids suppress androgen-independent human prostate tumor proliferation. Nutr Cancer *38*, 116-120.

Ko, L. J., and Prives, C. (1996). p53: puzzle and paradigm. Genes Dev *10*, 1054-1072.

Ko, W. G., Kang, T. H., Lee, S. J., Kim, Y. C., and Lee, B. H. (2002). Effects of luteolin on the inhibition of proliferation and induction of apoptosis in human myeloid leukaemia cells. *16*, 295.

Kobayashi, T., Nakata, T., and Kuzumaki, T. (2002). Effect of flavonoids on cell cycle progression in prostate cancer cells. Cancer Lett *176*, 17-23.

Koechli, O., Schaer, G.N., Seifert, B., Hornung, R., Haller, U., Eppenberger, U., and Mueller, H. (1994). Mutant p53 protein associated with chemosensitivity in breast cancer specimens. Lancet *344*, 1647-1648.

Kondo, S., Barnett, G. H., Hara, H., Morimura, T., and Takeuchi, J. (1995). MDM2 protein confers the resistance of a human glioblastoma cell line to cisplatin-induced apoptosis. Oncogene *10*, 2001-2006.

Kostyuk, V. A., Kraemer, T., Sies, H., and Schewe, T. (2003). Myeloperoxidase/nitrite-mediated lipid peroxidation of low-density lipoprotein as modulated by flavonoids. FEBS Letters *537*, 146.

Kotanidou, A., Xagorari, A., Bagli, E., Kitsanta, P., Fotsis, T., Papapetropoulos, A., and Roussos, C. (2002). Luteolin Reduces Lipopolysaccharide-induced Lethal Toxicity and Expression of Proinflammatory Molecules in Mice. Am J Respir Crit Care Med *165*, 818-823.

Kovnar, E. H., Kellie, S. J., Horowitz, M. E., Sanford, R. A., Langston, J. W., Mulhern, R. K., Jenkins, J. J., Douglass, E. C., Etcubanas, E. E., and Fairclough, D. L. (1990). Preirradiation cisplatin and etoposide in the treatment of high-risk medulloblastoma and other malignant embryonal tumors of the central nervous system: a phase II study. J Clin Oncol *8*, 330-336.

Krikos, A., Laherty, C. D., and Dixit, V. M. (1992). Transcriptional activation of the tumor necrosis factor alpha-inducible zinc finger protein, A20, is mediated by kappa B elements. J Biol Chem *267*, 17971-17976.

Kroemer, G., and Reed, J.C. (2000). Mitochondrial control of cell death. Nat Med *6*, 513-519.

Kroemer, G., and Martin, S. J. (2005). Caspase-independent cell death. Nat Med *11*, 725.

Kubbutat, M. H., Jones, S. N., and Vousden, K. H. (1997). Regulation of p53 stability by Mdm2. Nature *387*, 299-303.

Kubbutat, M. H., Jones, S.N., and Vousden, K.H. (1997). Regulation of p53 stability by Mdm2. Nature *387*, 299-303.

Kucharczak, J., Simmons, M. J., Fan, Y., and Gelinas, C. (2003). To be, or not to be: NF-kappaB is the answer--role of Rel/NF-kappaB in the regulation of apoptosis. Oncogene *22*, 8961-8982.

Kuppusamy, U. R., Khoo, H.E., and Das, N.P. (1990). Structure-activity studies of flavonoids as inhibitors of hyaluronidase. Biochem Pharmacol *40*, 397-401.

Lademann, U., Kallunki, T., and Jaattela, M. (2001). A20 zinc finger protein inhibits TNF-induced apoptosis and stress response early in the signaling cascades and independently of binding to TRAF2 or 14-3-3 proteins. Cell Death Differ *8*, 265-272.

Lansky, E. P., Harrison, G., Froom, P., and Jiang, W.G. (2005). Pomegranate (Punica granatum) pure chemicals show possible synergistic inhibition of human PC-3 prostate cancer cell invasion across Matrigel. Invest New Drugs *23*, 121-122.

Lapidot, T., Walker, M. D., and Kanner, J. (2002). Antioxidant and Prooxidant Effects of Phenolics on Pancreatic Cells in Vitro. J Agric Food Chem *50*, 7220-7225.

Lee, E. G., Boone, D. L., Chai, S., Libby, S. L., Chien, M., Lodolce, J. P., and Ma, A. (2000). Failure to regulate TNF-induced NF-kappaB and cell death responses in A20-deficient mice. Science *289*, 2350-2354.

Lee, H. J., Wang, C. J., Kuo, H. C., Chou, F. P., Jean, L. F., and Tseng, T. H. (2005). Induction apoptosis of luteolin in human hepatoma HepG2 cells involving mitochondria translocation of Bax/Bak and activation of JNK. Toxicol Appl Pharmacol *203*, 124-131.

Lee, J. S., Kim, H. J., and Lee, Y. S. (2003). A new anti-HIV flavonoid glucuronide from Chrysanthemum morifolium. Planta Med *69*, 859-861.

Lee, L.-T., Huang, Y.-T., Hwang, J.-J., Lee, A. Y. L., Ke, F.-C., Huang, C.-J., Kandaswami, C., Lee, P.-P. H., and Lee, M.-T. (2004). Transinactivation of the epidermal growth factor receptor tyrosine kinase and focal adhesion kinase phosphorylation by dietary flavonoids: effect on invasive potential of human carcinoma cells. Biochemical Pharmacology *67*, 2103.

Lee, L. T., Huang, Y. T., Hwang, J. J., Lee, P. P., Ke, F. C., Nair, M. P., Kanadaswam, C., and Lee, M. T. (2002). Blockade of the epidermal growth factor receptor tyrosine kinase activity by quercetin and luteolin leads to growth inhibition and apoptosis of pancreatic tumor cells. Anticancer Res *22*, 1615-1627.

Leung, H. W., Wu, C. H., Lin, C. H., and Lee, H. Z. (2005). Luteolin induced DNA damage leading to human lung squamous carcinoma CH27 cell apoptosis. Eur J Pharmacol *508*, 77-83.

Leverkus, M., Sprick, M. R., Wachter, T., Mengling, T., Baumann, B., Serfling, E., Brocker, E. B., Goebeler, M., Neumann, M., and Walczak, H. (2003). Proteasome inhibition results in TRAIL sensitization of primary keratinocytes by removing the resistance-mediating block of effector caspase maturation. *23*, 777.

Levine, A. J. (1997). p53, the cellular gatekeeper for growth and division. Cell *88*, 323-331.

Levine, A. J., Momand, J., and Finlay, C. A. (1991). The p53 tumour suppressor gene. Nature *351*, 453.

Li, H., Zhu, H., Xu, C.J., and Yuan, J. (1998). Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. Cell *94*, 491-501.

Li, W. X., Cui, C. B., Cai, B., Wang, H. Y., and Yao, X. S. (2005). Flavonoids from Vitex trifolia L. inhibit cell cycle progression at G2/M phase and induce apoptosis in mammalian cancer cells. J Asian Nat Prod Res *7*, 615-626.

Li, X., Yang, Y., and Ashwell, J. D. (2002). TNF-RII and c-IAP1 mediate ubiquitination and degradation of TRAF2. Nature *416*, 345-347.

Lin, Y., Devin, A., Rodriguez, Y., and Liu, Z. G. (1999). Cleavage of the death domain kinase RIP by caspase-8 prompts TNF-induced apoptosis. Genes Dev *13*, 2514-2526.

Linden, J. C., Haigh, J. R., Mirjalili, N., and Phisaphalong, M. (2001). Gas concentration effects on secondary metabolite production by plant cell cultures. Adv Biochem Eng Biotechnol *72*, 27-62.

Lindenmeyer, F., Li, H., Menashi, S., Soria, C., and Lu, H. (2001). Apigenin acts on the tumor cell invasion process and regulates protease production. Nutr Cancer *39*, 139-147.

Liu, J. Q., Shen, Q. Q., Liu, J. S., Wu, D. L., and Wang, J. T. (2001). [Studies on the chemical constituents from Chrysanthemum morifolium Ramat]. Zhongguo Zhong Yao Za Zhi *26*, 547-548.

Liu, L.-Z., Fang, J., Zhou, Q., Hu, X., Shi, X., and Jiang, B.-H. (2005). Apigenin Inhibits Expression of Vascular Endothelial Growth Factor and Angiogenesis in Human Lung Cancer Cells: Implication of Chemoprevention of Lung Cancer. Mol Pharmacol *68*, 635-643.

Liu, R. H. (2004). Potential Synergy of Phytochemicals in Cancer Prevention: Mechanism of Action. J Nutr *134*, 3479S-3485.

Liu, T., Zhao, L., Wang, N., Lu, Z. and Sun Z. (1998). Effects of Ju Hua Jian in ulcerative colitis and on the contents of CD44 and CD62p, In Chinese J of Covalescent Medicine. ed., pp. 38.

Liu, X., Yue, P., Khuri, F. R., and Sun, S.-Y. (2004). p53 Upregulates Death Receptor 4 Expression through an Intronic p53 Binding Site. Cancer Res *64*, 5078-5083.

Liu, Z. G., Hsu, H., Goeddel, D. V., and Karin, M. (1996). Dissection of TNF receptor 1 effector functions: JNK activation is not linked to apoptosis while NF-kappaB activation prevents cell death. Cell *87*, 565-576.

Loehrer, P. J., and Einhorn, L.H. (1984). Drugs five years later. Cisplatin. Ann Intern Med *100*, 704-713.

Lucas, M., and Sanchez-Margalet, V. (1995). Protein kinase C involvement in apoptosis. Gen Pharmacol *26*, 881-887.

Luo, X., Budihardjo, I., Zou, H., Slaughter, C., and Wang, X. (1998). Bid, a Bcl2 Interacting Protein, Mediates Cytochrome c Release from Mitochondria in Response to Activation of Cell Surface Death Receptors. Cell *94*, 481.

Mafune, K., Tanaka, Y., Mimori, K., Mori, M., Takubo, K., and Makuuchi, M. (1999). Increased expression of ornithine decarboxylase messenger RNA in human esophageal carcinoma. *5*, 4073.

Makino, T., Ito, M., Kiuchiu, F., Ono, T., Muso, E., and Honda, G. (2001). Inhibitory effect of decoction of Perilla frutescens on cultured murine mesangial cell proliferation and quantitative analysis of its active constituents. Planta Med 2001 Feb;67(1):24-8 *67*, 24-28.

Mangan, J. K., Rane, S. G., Kang, A. D., Amanullah, A., Wong, B. C., and Reddy, E. P. (2004). Mechanisms associated with IL-6-induced up-regulation of Jak3 and its role in monocytic differentiation. Blood *103*, 4093-4101.

Markaverich, B. M., Roberts, R.R., Alejandro, M.A., Johnson, G.A., Middleditch, B.S., and Clark, J.H. (1988). Bioflavonoid interaction with rat uterine type II binding sites and cell growth inhibition. J Steroid Biochem *30*, 71-78.

Massague, J. (2004). G1 cell-cycle control and cancer. Nature *432*, 298.

Matsuda, H., Morikawa, T., Toguchida, I., Harima, S., and Yoshikawa, M. (2002). Medicinal flowers. VI. Absolute stereostructures of two new flavanone glycosides and a phenylbutanoid glycoside from the flowers of Chrysanthemum indicum L.: their inhibitory activities for rat lens aldose reductase. *50*, 972.

Matsukawa, Y., Marui, N., Sakai, T., Satomi, Y., Yoshida, M., Matsumoto, K., Nishino, H., and Aoike, A. (1993). Genistein arrests cell cycle progression at G2-M. Cancer Res *53*, 1328-1331.

McGahon, A. J., Martin, S. J., Bissonnette, R. P., Mahboubi, A., Shi, Y., Mogil, R. J., Nishioka, W. K., and Green, D. R. (1995). The end of the (cell) line: methods for the study of apoptosis in vitro. Methods Cell Biol *46*, 153-185.

McManus, D. C., Lefebvre, C.A., Cherton-Horvat, G., St-Jean, M., Kandimalla, E.R., Agrawal, S., Morris, S.J., Durkin, J.P., and Lacasse, E.C. (2004). Loss of XIAP protein expression by RNAi and antisense approaches sensitizes cancer cells to functionally diverse chemotherapeutics. Oncogene *23*, 8105-8117.

Meng, X. W., Heldebrant, M. P., and Kaufmann, S. H. (2002). Phorbol 12-myristate 13-acetate inhibits death receptor-mediated apoptosis in Jurkat cells by disrupting recruitment of Fas-associated polypeptide with death domain. *277*, 3776.

Micheau, O., Lens, S., Gaide, O., Alevizopoulos, K., and Tschopp, J. (2001). NF-kappaB signals induce the expression of c-FLIP. *21*, 5299.

Micheau, O., and Tschopp, J. (2003). Induction of TNF Receptor I-Mediated Apoptosis via Two Sequential Signaling Complexes. Cell *114*, 181.

Mihara, M., Erster, S., Zaika, A., Petrenko, O., Chittenden, T., Pancoska, P., and Moll, U. M. (2003). p53 Has a Direct Apoptogenic Role at the Mitochondria. Molecular Cell *11*, 577.

Mittra, B., Saha, A., Chowdhury, A. R., Pal, C., Mandal, S., Mukhopadhyay, S., Bandyopadhyay, S., and Majumder, H. K. (2000). Luteolin, an abundant dietary component is a potent anti-leishmanial agent that acts by inducing topoisomerase II-mediated kinetoplast DNA cleavage leading to apoptosis. Mol Med *6*, 527-541.

Miyashita, T., and Reed, J. C. (1995). Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. Cell *80*, 293-299.

Mohanty, S., Huang, J., and Basu, A. (2005). Enhancement of cisplatin sensitivity of cisplatin-resistant human cervical carcinoma cells by bryostatin 1. Clin Cancer Res *11*, 6730-6737.

Monasterio, A., Urdaci, M. C., Pinchuk, I. V., Lopez-Moratalla, N., and Martinez-Irujo, J. J. (2004). Flavonoids induce apoptosis in human leukemia U937 cells through caspase- and caspase-calpain-dependent pathways. Nutr Cancer *50*, 90-100.

Mora, A., Paya, M., Rios, J.L., and Alcaraz, M.J. (1990). Structure-activity relationships of polymethoxyflavones and other flavonoids as inhibitors of non-enzymic lipid peroxidation. Biochemical Pharmacology *40*, 793-797.

Morin, D., Barthelemy, S., Zini, R., Labidalle, S., and Tillement, J.-P. (2001). Curcumin induces the mitochondrial permeability transition pore mediated by membrane protein thiol oxidation. FEBS Letters *495*, 131.

Mouria, M., Gukovskaya, A. S., Jung, Y., Buechler, P., Hines, O. J., Reber, H. A., and Pandol, S. J. (2002). Food-derived polyphenols inhibit pancreatic cancer growth through mitochondrial cytochrome C release and apoptosis. International Journal Of Cancer Journal International Du Cancer *98*, 761.

Muller, M., Wilder, S., Bannasch, D., Israeli, D., Lehlbach, K., Li-Weber, M., Friedman, S. L., Galle, P. R., Stremmel, W., and Oren et, a. (1998). p53 activates the CD95 (APO-1/Fas) gene in response to DNA damage by anticancer drugs. The Journal Of Experimental Medicine *188*, 2033.

Murkies, A. L., Wilcox, G., and Davis, S. R. (1998). Phytoestrogens. J Clin Endocrinol Metab *83*, 297-303.

Muzio, M., Salvesen, G. S., and Dixit, V. M. (1997). FLICE induced apoptosis in a cell-free system. Cleavage of caspase zymogens. J Biol Chem *272*, 2952-2956.

Nagane, M., Pan, G., Weddle, J. J., Dixit, V. M., Cavenee, W. K., and Huang, H. J. (2000). Increased death receptor 5 expression by chemotherapeutic agents in human gliomas causes synergistic cytotoxicity with tumor necrosis factor-related apoptosis-inducing ligand in vitro and in vivo. *60*, 847.

Nagao, A., Seki, M., and Kobayashi, H. (1999). Inhibition of xanthine oxidase by flavonoids. Biosci Biotechnol Biochem *63*, 1787-1790.

Nakano, K., and Vousden, K. H. (2001). PUMA, a Novel Proapoptotic Gene, Is Induced by p53. Molecular Cell *7*, 683.

Nathan, C., and Xie, Q. W. (1994). Nitric oxide synthases: roles, tolls, and controls. Cell *78*, 915-918.

Natoli, C., Scognamiglio, M. T., Martino, M. T., Irtelli, L., De Tursi, M., Cianchetti, E., Mascitelli, E., Tinari, N., and Iacobelli, S. (2000). Chronomodulated infusion of cisplatin, 5-fluorouracil and folinic acid: lack of activity in advanced colorectal cancer. Anticancer Res *20*, 1253-1256.

Natoli, G., Costanzo, A., Ianni, A., Templeton, D. J., Woodgett, J. R., Balsano, C., and Levrero, M. (1997). Activation of SAPK/JNK by TNF receptor 1 through a noncytotoxic TRAF2-dependent pathway. Science *275*, 200-203.

Nechushtan, A., Smith, C. L., Hsu, Y. T., and Youle, R. J. (1999). Conformation of the Bax C-terminus regulates subcellular location and cell death. The EMBO Journal *18*, 2330.

Nesterov, A., Lu, X., Johnson, M., Miller, G. J., Ivashchenko, Y., and Kraft, A. S. (2001). Elevated AKT activity protects the prostate cancer cell line LNCaP from TRAIL-induced apoptosis. *276*, 10767.

Nicholson, D. W., and Thornberry, N. A. (1997). Caspases: killer proteases. Trends in Biochemical Sciences *22*, 299.

Noroozi, M., Angerson, W. J., and Lean, M. E. (1998). Effects of flavonoids and vitamin C on oxidative DNA damage to human lymphocytes. Am J Clin Nutr *67*, 1210-1218.

Oda, E., Ohki, R., Murasawa, H., Nemoto, J., Shibue, T., Yamashita, T., Tokino, T., Taniguchi, T., and Tanaka, N. (2000a). Noxa, a BH3-only member of the Bcl-2 family and candidate mediator of p53-induced apoptosis. Science *288*, 1053.

Oda, K., Arakawa, H., Tanaka, T., Matsuda, K., Tanikawa, C., Mori, T., Nishimori, H., Tamai, K., Tokino, T., Nakamura, Y., and Taya, Y. (2000b). p53AIP1, a potential mediator of p53-dependent apoptosis, and its regulation by Ser-46-phosphorylated p53. Cell *102*, 849-862.

Oguri, A., Suda, M., Totsuka, Y., Sugimura, T., and Wakabayashi, K. (1998). Inhibitory effects of antioxidants on formation of heterocyclic amines. Mutation Research *402*, 237-245.

Okamoto, M., Hara, I., Miyake, H., Hara, S., Gotoh, A., Arakawa, S., and Kamidono, S. (2001). Synergistic antitumor effect of ionomycin and cisplatin against renal cell carcinoma in vitro and in vivo. Urology *57*, 188-192.

Papa, S., Zazzeroni, F., Pham, C. G., Bubici, C., and Franzoso, G. (2004). Linking JNK signaling to NF-{kappa}B: a key to survival. J Cell Sci *117*, 5197-5208.

Park, M. S., De Leon, M., and Devarajan, P. (2002). Cisplatin Induces Apoptosis in LLC-PK1 Cells via Activation of Mitochondrial Pathways. J Am Soc Nephrol *13*, 858-865.

Petak, I., Douglas, L., Tillman, D. M., Vernes, R., and Houghton, J. A. (2000). Pediatric rhabdomyosarcoma cell lines are resistant to Fas-induced apoptosis and highly sensitive to TRAIL-induced apoptosis. Clin Cancer Res *6*, 4119-4127.

Pitot, H. C. (1993). The molecular biology of carcinogenesis. Cancer *72*, 962-970.

Plaumann, B., Fritsche, M., Rimpler, H., Brandner, G., and Hess, R. D. (1996). Flavonoids activate wild-type p53. Oncogene *13*, 1605-1614.

Quist, S. R., Wang-Gohrke, S., Kohler, T., Kreienberg, R., and Runnebaum, I.B. (2004). Cooperative effect of adenoviral p53 gene therapy and standard chemotherapy in ovarian cancer cells independent of the endogenous p53 status. Cancer Gene Ther *11*, 547-554.

Ramanathan, R., Das, N.P., and Tan, C.H. (1994). Effects of gamma-linolenic acid, flavonoids, and vitamins on cytotoxicity and lipid peroxidation. Free Radic Biol Med *16*, 43-48.

Reinhard, C., Shamoon, B., Shyamala, V., and Williams, L. T. (1997). Tumor necrosis factor alpha-induced activation of c-jun N-terminal kinase is mediated by TRAF2. Embo J *16*, 1080-1092.

Riedl, S. J., Renatus, M., Schwarzenbacher, R., Zhou, Q., Sun, C., Fesik, S. W., Liddington, R. C., and Salvesen, G. S. (2001). Structural basis for the inhibition of caspase-3 by XIAP. *104*, 791.

Robak, J., Shridi, F., Wolbis, M., and Krolikowska, M. (1988). Screening of the influence of flavonoids on lipoxygenase and cyclooxygenase activity, as well as on nonenzymic lipid oxidation. Pol J Pharmacol Pharm *40*, 451-458.

Rosato, R. R., Dai, Y., Almenara, J. A., Maggio, S. C., and Grant, S. (2004). Potent antileukemic interactions between flavopiridol and TRAIL/Apo2L involve flavopiridol-mediated XIAP downregulation.

Ross, J. A., and Kasum, C. M. (2002). Dietary flavonoids: bioavailability, metabolic effects, and safety. Annu Rev Nutr *22:19-34.*, 19-34.

Ryan, K. M., Phillips, A. C., and Vousden, K. H. (2001). Regulation and function of the p53 tumor suppressor protein. Current Opinion in Cell Biology *13*, 332.

Sadik, C. D., Sies, H., and Schewe, T. (2003). Inhibition of 15-lipoxygenases by flavonoids: structure-activity relations and mode of action. Biochemical Pharmacology *65*, 773.

Sakihama, Y., Cohen, M. F., Grace, S. C., and Yamasaki, H. (2002). Plant phenolic antioxidant and prooxidant activities: phenolics-induced oxidative damage mediated by metals in plants. Toxicology *177*, 67.

Salghetti, S. E., Kim, S. Y., and Tansey, W. P. (1999). Destruction of Myc by ubiquitin-mediated proteolysis: cancer-associated and transforming mutations stabilize Myc. Embo J *18*, 717-726.

Salvi, M., Brunati, A. M., Clari, G., and Toninello, A. (2002). Interaction of genistein with the mitochondrial electron transport chain results in opening of the membrane transition pore. Biochimica et Biophysica Acta (BBA) - Bioenergetics *1556*, 187.

Sandler, A. (2002). Irinotecan plus cisplatin in small-cell lung cancer. Oncology (Williston Park) *16*, 39-43.

Sarker, M., Ruiz-Ruiz, C., Robledo, G., and Lopez-Rivas, A. (2002). Stimulation of the mitogen-activated protein kinase pathway antagonizes TRAIL-induced apoptosis downstream of BID cleavage in human breast cancer MCF-7 cells. *%20;21*, 4323.

Savill, J., and Fadok, V. (2000). Corpse clearance defines the meaning of cell death. Nature *407*, 784.

Sax, J. K., Fei, P., Murphy, M. E., Bernhard, E., Korsmeyer, S. J., and El-Deiry, W. S. (2002). BID regulation by p53 contributes to chemosensitivity. Nature Cell Biology *4*, 842.

Scaffidi, C., Fulda, S., Srinivasan, A., Friesen, C., Li, F., Tomaselli, K. J., Debatin, K. M., Krammer, P. H., and Peter, M. E. (1998). Two CD95 (APO-1/Fas) signaling pathways. Embo J *17*, 1675-1687.

Scaffidi, C., Schmitz, I., Zha, J., Korsmeyer, S. J., Krammer, P. H., and Peter, M. E. (1999). Differential Modulation of Apoptosis Sensitivity in CD95 Type I and Type II Cells. J Biol Chem *274*, 22532-22538.

Schuler, M., and Green, D. R. (2001). Mechanisms of p53-dependent apoptosis. Biochemical Society Transactions *29*, 684.

Semenza, G. L. (2001). HIF-1 and mechanisms of hypoxia sensing. Current Opinion in Cell Biology *13*, 167.

Sergediene, E., Jonsson, K., Szymusiak, H., Tyrakowska, B., Rietjens, I. M. C. M., and Cenas, N. (1999). Prooxidant toxicity of polyphenolic antioxidants to HL-60 cells: description of quantitative structure-activity relationships. FEBS Letters *462*, 392.

Sesink, A. L. A., O'Leary, K. A., and Hollman, P. C. H. (2001). Quercetin Glucuronides but Not Glucosides Are Present in Human Plasma after Consumption of Quercetin-3-Glucoside or Quercetin-4'-Glucoside. J Nutr *131*, 1938-1941.

Shao, H., Sun, S. L., Kaplan, H. J., and Sun, D. (2004). Characterization of rat CD8+ uveitogenic T cells specific for interphotoreceptor retinal-binding protein 1177-1191. J Immunol *173*, 2849-2854.

Shen, S.-C., Ko, C. H., Tseng, S.-W., Tsai, S.-H., and Chen, Y.-C. (2004). Structurally related antitumor effects of flavanones in vitro and in vivo: involvement of caspase 3 activation, p21 gene expression, and reactive oxygen species production. Toxicology and Applied Pharmacology *197*, 84.

Sheridan, J. P., Marsters, S. A., Pitti, R. M., Gurney, A., Skubatch, M., Baldwin, D., Ramakrishnan, L., Gray, C. L., Baker, K., Wood, W. I.*, et al.* (1997). Control of TRAIL-Induced Apoptosis by a Family of Signaling and Decoy Receptors. Science *277*, 818-821.

Shi, Y. (2002). Mechanisms of caspase activation and inhibition during apoptosis. *9*, 459.

Shieh, S. Y., Ikeda, M., Taya, Y., and Prives, C. (1997). DNA damage-induced phosphorylation of p53 alleviates inhibition by MDM2. Cell 1997 Oct 31;91(3):325-34 *91*, 325-334.

Shieh, S. Y., Taya, Y., and Prives, C. (1999). DNA damage-inducible phosphorylation of p53 at N-terminal sites including a novel site, Ser20, requires tetramerization. EMBO J *18*, 1815-1823.

Shukla, S., Mishra, A., Fu, P., MacLennan, G. T., Resnick, M. I., and Gupta, S. (2005). Up-regulation of insulin-like growth factor binding protein-3 by apigenin leads to growth inhibition and apoptosis of 22Rv1 xenograft in athymic nude mice. FASEB J, 05-3740fje.

Siddik, Z. H. (2003). Cisplatin: mode of cytotoxic action and molecular basis of resistance. Oncogene *22*, 7265-7279.

Soengas, M. S., Capodieci, P., Polsky, D., Mora, J., Esteller, M., Opitz-Araya, X., McCombie, R., Herman, J. G., Gerald, W. L., and Lazebnik et, a. (2001). Inactivation of the apoptosis effector Apaf-1 in malignant melanoma. Nature *409*, 207.

Song, H. Y., Rothe, M., and Goeddel, D. V. (1996). The tumor necrosis factor-inducible zinc finger protein A20 interacts with TRAF1/TRAF2 and inhibits NF-kappaB activation. Proc Natl Acad Sci U S A *93*, 6721-6725.

Song, K., Fukushima, P., Seth, P., and Sinha, B. K. (1998). Role of p53 and apoptosis in sensitization of cis-diamminedichloroplatinum antitumor activity by interleukin-1 in ovarian carcinoma cells. Int J Oncol *12*, 299-304.

Song, K., Li, Z., Seth, P., Cowan, K. H., and Sinha, B. K. (1997). Sensitization of cis-platinum by a recombinant adenovirus vector expressing wild-type p53 gene in human ovarian carcinomas. Oncol Res *9*, 603-609.

Sonoda, Y., Matsumoto, Y., Funakoshi, M., Yamamoto, D., Hanks, S. K., and Kasahara, T. (2000). Anti-apoptotic role of focal adhesion kinase (FAK). Induction of inhibitor-of-apoptosis proteins and apoptosis suppression by the overexpression of FAK in a human leukemic cell line, HL-60. J Biol Chem *275*, 16309-16315.

Steegenga, W. T., van der Eb, A. J., and Jochemsen, A. G. (1996). How Phosphorylation Regulates the Activity of p53. Journal of Molecular Biology *263*, 103.

Stobiecki, M. (2000). Application of mass spectrometry for identification and structural studies of flavonoid glycosides. Phytochemistry *54*, 237.

Strasser, A., and Newton, K. (1999). FADD/MORT1, a signal transducer that can promote cell death or cell growth. Int J Biochem Cell Biol *31*, 533-537.

Strasser, A., O'Connor, L., and Dixit, V. M. (2000). APOPTOSIS SIGNALING. Annual Review of Biochemistry *69*, 217-245.

Tanaka, Y., Gavrielides, M. V., Mitsuuchi, Y., Fujii, T., and Kazanietz, M. G. (2003). Protein kinase C promotes apoptosis in LNCaP prostate cancer cells through activation of p38 MAPK and inhibition of the Akt survival pathway. *278*, 33753.

Tang, C.-H., and Grimm, E. A. (2004). Depletion of Endogenous Nitric Oxide Enhances Cisplatin-induced Apoptosis in a p53-dependent Manner in Melanoma Cell Lines. J Biol Chem *279*, 288-298.

Tang, G., Minemoto, Y., Dibling, B., Purcell, N. H., Li, Z., Karin, M., and Lin, A. (2001). Inhibition of JNK activation through NF-kappaB target genes. Nature *414*, 313-317.

Tergaonkar, V., Pando, M., Vafa, O., Wahl, G., and Verma, I. (2002). p53 stabilization is decreased upon NF-kappa B activation: A role for NF-kappa B in acquisition of resistance to chemotherapy. Cancer Cell *1*, 493-503.

Thakkar, H., Chen, X., Tyan, F., Gim, S., Robinson, H., Lee, C., Pandey, S. K., Nwokorie, C., Onwudiwe, N., and Srivastava, R. K. (2001). Pro-survival function of Akt/protein kinase B in prostate cancer cells. Relationship with TRAIL resistance. *%19;276*, 38361.

The Institute of Chinese Materia Medica, C. A. o. T. C. M. (1989). Medicinal plants in China: a selection of 150 commonly used species (Manila: World Health Organization Regional Office for the Western Pacific).

Ting, A. T., Pimentel-Muinos, F. X., and Seed, B. (1996). RIP mediates tumor necrosis factor receptor 1 activation of NF-kappaB but not Fas/APO-1-initiated apoptosis. Embo J *15*, 6189-6196.

Torkin, R., Lavoie, J.-F., Kaplan, D. R., and Yeger, H. (2005). Induction of caspase-dependent, p53-mediated apoptosis by apigenin in human neuroblastoma. Mol Cancer Ther *4*, 1-11.

Tormakangas, L., Vuorela, P., Saario, E., Leinonen, M., Saikku, P., and Vuorela, H. (2005). In vivo treatment of acute Chlamydia pneumoniae infection with the flavonoids quercetin and luteolin and an alkyl gallate, octyl gallate, in a mouse model. Biochemical Pharmacology *70*, 1222.

Tournier, C., Hess, P., Yang, D. D., Xu, J., Turner, T. K., Nimnual, A., Bar-Sagi, D., Jones, S. N., Flavell, R. A., and Davis, R. J. (2000). Requirement of JNK for stress-induced activation of the cytochrome c-mediated death pathway. Science *288*, 870-874.

Tracey, K. J., and Cerami, A. (1993). Tumor necrosis factor, other cytokines and disease. Annu Rev Cell Biol *9*, 317-343.

Tracey, M. D. K. J., and Cerami, P. D. A. (1994). TUMOR NECROSIS FACTOR: A Pleiotropic Cytokine and Therapuetic Target. Annual Review of Medicine *45*, 491-503.

Trauzold, A., Wermann, H., Arlt, A., Schutze, S., Schafer, H., Oestern, S., Roder, C., Ungefroren, H., Lampe, E., Heinrich, M.*, et al.* (2001). CD95 and TRAIL receptor-mediated activation of protein kinase C and NF-kappaB contributes to apoptosis resistance in ductal pancreatic adenocarcinoma cells. *20*, 4258.

Trochon, V., Mabilat-Pragnon, C., Bertrand, P., Legrand, Y., Soria, J., Soria, C., Delpech, B., and Lu, H. (1997). Hyaluronectin blocks the stimulatory effect of hyaluronan-derived fragments on endothelial cells during angiogenesis in vitro. FEBS Letters *418*, 6.

Ueda, H., Yamazaki, C., and Yamazaki, M. (2003). Inhibitory effect of Perilla leaf extract and luteolin on mouse skin tumor promotion. Biol Pharm Bull *26*, 560-563.

Ueda, S., Nakamura, H., Masutani, H., Sasada, T., Takabayashi, A., Yamaoka, Y., and Yodoi, J. (2002). Baicalin induces apoptosis via mitochondrial pathway as prooxidant. Molecular Immunology *38*, 781.

Ukiya, M., Akihisa, T., Tokuda, H., Suzuki, H., Mukainaka, T., Ichiishi, E., Yasukawa, K., Kasahara, Y., and Nishino, H. (2002). Constituents of Compositae plants III. Anti-tumor promoting effects and cytotoxic activity against human cancer cell lines of triterpene diols and triols from edible chrysanthemum flowers. *177*, 7.

Ukiya, M., Akihisa, T., Yasukawa, K., Kasahara, Y., Kimura, Y., Koike, K., Nikaido, T., and Takido, M. (2001). Constituents of compositae plants. 2. Triterpene diols, triols, and their 3-o-fatty acid esters from edible chrysanthemum flower extract and their anti-inflammatory effects. *49*, 3187.

Vassilev, L. T., Vu, B. T., Graves, B., Carvajal, D., Podlaski, F., Filipovic, Z., Kong, N., Kammlott, U., Lukacs, C., Klein, C., *et al.* (2004). In Vivo Activation of the p53 Pathway by Small-Molecule Antagonists of MDM2. Science *303*, 844-848.

von Haefen, C., Gillissen, B., Hemmati, P. G., Wendt, J., Guner, D., Mrozek, A., Belka, C., Dorken, B., and Daniel, P. T. (2004). Multidomain Bcl-2 homolog Bax but not Bak mediates synergistic induction of apoptosis by TRAIL and 5-FU through the mitochondrial apoptosis pathway.

Wallach, D., Varfolomeev, E. E., Malinin, N. L., Goltsev, Y. V., Kovalenko, A. V., and Boldin, M. P. (1999). TUMOR NECROSIS FACTOR RECEPTOR AND Fas SIGNALING MECHANISMS. Annual Review of Immunology *17*, 331-367.

Walle, T., Browning, A. M., Steed, L. L., Reed, S. G., and Walle, U. K. (2005). Flavonoid Glucosides Are Hydrolyzed and Thus Activated in the Oral Cavity in Humans. J Nutr *135*, 48-52.

Wang, C., and Kurzer, M.S. (1997). Phytoestrogen concentration determines effects on DNA synthesis in human breast cancer cells. Nutr Cancer 1997;28(3):236-47 *28*, 236-247.

Wang, C., and Kurzer, M.S. (1998). Effects of phytoestrogens on DNA synthesis in MCF-7 cells in the presence of estradiol or growth factors. Nutr Cancer *31*, 90-100.

Wang, C. Y., Mayo, M. W., Korneluk, R. G., Goeddel, D. V., and Baldwin, A. S., Jr. (1998). NF-kappaB antiapoptosis: induction of TRAF1 and TRAF2 and c-IAP1 and c-IAP2 to suppress caspase-8 activation. *281*, 1680.

Wang, S., and El Deiry, W. S. (2003). TRAIL and apoptosis induction by TNF-family death receptors. *22*, 8628.

Wang, T. T., Sathyamoorthy, N., and Phang, J.M. (1996). Molecular effects of genistein on estrogen receptor mediated pathways. Carcinogenesis *17*, 271-275.

Wang, W., VanAlstyne, P. C., Irons, K. A., Chen, S., Stewart, J. W., and Birt, D. F. (2004). Individual and interactive effects of apigenin analogs on G2/M cell-cycle arrest in human colon carcinoma cell lines. Nutr Cancer *48*, 106-114.

Way, T.-D., Kao, M.-C., and Lin, J.-K. (2004). Apigenin Induces Apoptosis through Proteasomal Degradation of HER2/neu in HER2/neu-overexpressing Breast Cancer Cells via the Phosphatidylinositol 3-Kinase/Akt-dependent Pathway. J Biol Chem *279*, 4479-4489.

Weinstein, I. B., Kahn, S. M., O'Driscoll, K., Borner, C., Bang, D., Jiang, W., Blackwood, A., and Nomoto, K. (1997). The role of protein kinase C in signal transduction, growth control and lipid metabolism. Adv Exp Med Biol *400A*, 313-321.

Wu, G. S., Burns, T.F., McDonald, E.R., Jiang, W., Meng, R., Krantz, I.D., Kao, G., Gan, D.D., Zhou, J.Y., Muschel, R., Hamilton, S.R., Spinner, N.B., Markowitz, S.,

Wu, G., and el-Deiry, W.S. (1997). KILLER/DR5 is a DNA damage-inducible p53-regulated death receptor gene. Nat Genet *17*, 141-143.

Wu, H. C., Lu, T. Y., Lee, J. J., Hwang, J. K., Lin, Y. J., Wang, C. K., and Lin, C. T. (2004a). MDM2 expression in EBV-infected nasopharyngeal carcinoma cells. Lab Invest *84*, 1547-1556.

Wu, M., Xu, L. G., Li, X., Zhai, Z., and Shu, H. B. (2002). AMID, an apoptosis-inducing factor-homologous mitochondrion-associated protein, induces caspase-independent apoptosis. J Biol Chem *277*, 25617-25623.

Wu, M. J., Wang, L., Ding, H. Y., Weng, C. Y., and Yen, J. H. (2004b). Glossogyne tenuifolia acts to inhibit inflammatory mediator production in a macrophage cell line by downregulating LPS-induced NF-kappa B. J Biomed Sci *11*, 186-199.

Xagorari, A., Papapetropoulos, A., Mauromatis, A., Economou, M., Fotsis, T., and Roussos, C. (2001). Luteolin inhibits an endotoxin-stimulated phosphorylation cascade and proinflammatory cytokine production in macrophages. J Pharmacol Exp Ther *296*, 181-187.

Xagorari, A., Roussos, C., and Papapetropoulos, A. (2002). Inhibition of LPS-stimulated pathways in macrophages by the flavonoid luteolin. Br J Pharmacol *136*, 1058-1064.

Yamamoto, Y., and Gaynor, R. B. (2001). Therapeutic potential of inhibition of the NF-kappaB pathway in the treatment of inflammation and cancer. *107*, 135.

Yamashita, N., and Kawanishi, S. (2000). Distinct mechanisms of DNA damage in apoptosis induced by quercetin and luteolin. Free Radic Res *33*, 623-633.

Yang, C.-F., Shen, H.-M., and Ong, C.-N. (1999). Protective effect of ebselen against hydrogen peroxide-induced cytotoxicity and DNA damage in HepG2 cells. Biochemical Pharmacology *57*, 273.

Yang, C. F., Shen, H. M., and Ong, C. N. (2000a). Intracellular thiol depletion causes mitochondrial permeability transition in ebselen-induced apoptosis. Arch Biochem Biophys *380*, 319-330.

Yang, Y., Fang, S., Jensen, J. P., Weissman, A. M., and Ashwell, J. D. (2000b). Ubiquitin protein ligase activity of IAPs and their degradation in proteasomes in response to apoptotic stimuli. *288*, 874.

Ye, X., Krohn, R. L., Liu, W., Joshi, S. S., Kuszynski, C. A., McGinn, T. R., Bagchi, M., Preuss, H. G., Stohs, S. J., and Bagchi, D. (1999). The cytotoxic effects of a novel IH636 grape seed proanthocyanidin extract on cultured human cancer cells. Mol Cell Biochem *196*, 99-108.

Yeh, W. C., Pompa, J. L., McCurrach, M. E., Shu, H. B., Elia, A. J., Shahinian, A., Ng, M., Wakeham, A., Khoo, W., Mitchell, K.*, et al.* (1998). FADD: essential for

embryo development and signaling from some, but not all, inducers of apoptosis. Science *279*, 1954-1958.

Yin, F., Giuliano, A.E., Van Herle, A.J. (1999). Growth inhibitory effects of flavonoids in human thyroid cancer cell lines. Thyroid *9*, 369-376.

Yoon, H. S., Moon, S. C., Kim, N. D., Park, B. S., Jeong, M. H., and Yoo, Y. H. (2000). Genistein Induces Apoptosis of RPE-J Cells by Opening Mitochondrial PTP. Biochemical and Biophysical Research Communications *276*, 151.

Yu, D. Q., and Xie, F. Z. (1987). [Studies on the chemical constituents of Chrysanthemum indicum L.]. Yao Xue Xue Bao *22*, 837-840.

Yu, J., Zhang, L., Hwang, P. M., Kinzler, K. W., and Vogelstein, B. (2001). PUMA induces the rapid apoptosis of colorectal cancer cells. Mol Cell *7*, 673-682.

Yu, Y., Sun, P., Sun, L.-c., Liu, G.-y., Chen, G.-h., Shang, L.-h., Wu, H.-b., Hu, J., Li, Y., and Mao, Y.-l. (2006). Downregulation of MDM2 expression by RNAi inhibits LoVo human colorectal adenocarcinoma cells growth and the treatment of LoVo cells with mdm2siRNA3 enhances the sensitivity to cisplatin. Biochemical and Biophysical Research Communications *339*, 71.

Zand, R. S., Jenkins, D.J., and Diamandis, E.P. (2000). Steroid hormone activity of flavonoids and related compounds. Breast Cancer Res Treat *62*, 35-49.

Zauli, G., Sancilio, S., Cataldi, A., Sabatini, N., Bosco, D., and Di Pietro, R. (2004). PI-3K/Akt and NF-kappaB/IkappaBalpha pathways are activated in Jurkat T cells in response to TRAIL treatment.

Zhang, H. G., Wang, J., Yang, X., Hsu, H. C., and Mountz, J. D. (2004). Regulation of apoptosis proteins in cancer cells by ubiquitin. *23*, 2009.

Zhang, L., Lau, Y.K., Xi, L., Hong, R.L., Kim, D.S., Chen, C.F., Hortobagyi, G.N., Chang, C., and Hung, M.C. (1998). Tyrosine kinase inhibitors, emodin and its derivative repress HER-2/neu-induced cellular transformation and metastasis-associated properties. Oncogene *16*, 2855-2863.

Zhao, W., Liang, C., Chen, Z., Pang, R., Zhao, B., and Chen, Z. (2002). Luteolin inhibits proliferation and collagen synthesis of hepatic stellate cells. Zhonghua Gan Zang Bing Za Zhi *10*, 204-206.

Zheng, P.-W., Chiang, L.-C., and Lin, C.-C. (2005). Apigenin induced apoptosis through p53-dependent pathway in human cervical carcinoma cells. Life Sciences *76*, 1367.

Zhou, B. P., Hu, M. C., Miller, S. A., Yu, Z., Xia, W., Lin, S. Y., and Hung, M. C. (2000). HER-2/neu blocks tumor necrosis factor-induced apoptosis via the Akt/NF-kappaB pathway. J Biol Chem *275*, 8027-8031.

Zhou, Y. D., Kim, Y. P., Li, X. C., Baerson, S. R., Agarwal, A. K., Hodges, T. W., Ferreira, D., and Nagle, D. G. (2004). Hypoxia-Inducible Factor-1 Activation by (-)-Epicatechin Gallate: Potential Adverse Effects of Cancer Chemoprevention with High-Dose Green Tea Extracts. J Nat Prod *67*, 2063-2069.

Zhou, Y. L. (1987). Chrysanthemum morifolium in the treatment of hypertension. Zhong Xi Yi Jie He Za Zhi *7*, 18-20, 14.

Zi, X., Feyes, D. K., and Agarwal, R. (1998). Anticarcinogenic effect of a flavonoid antioxidant, silymarin, in human breast cancer cells MDA-MB 468: induction of G1 arrest through an increase in Cip1/p21 concomitant with a decrease in kinase activity of cyclin-dependent kinases and associated cyclins. Clin Cancer Res *4*, 1055-1064.