

Molecular Mechanisms of Modulatory effects of Naturally Occurring Compounds on the Efficacy of Cancer Therapy

Thesis Submitted for the Degree of PhD in Pharmaceutical Sciences
(Pharmacology & Toxicology)

By

El-Shaimaa Abdel Razek A. Arafa

M.Sc Pharmacology, Cairo University
Assistant Lecturer of Pharmacology & Toxicology
Faculty of Pharmacy, Beni-Suef University

Under the Supervision of

Prof. Dr. Hekma A. Abdel Latif

Professor and Head of Pharmacology &
Toxicology Department
Faculty of Pharmacy
Cairo University

Prof. Dr. Mostafa E. El-Sayed

Professor of Pharmacology & Toxicology
Faculty of Pharmacy
Cairo University

Prof. Dr. Helmy Moawad

Professor of Pharmacology & Toxicology
Faculty of Pharmacy
Cairo University

Prof. Dr. Altaf A. Wani

Professor of Biochemistry
Radiology Department
James Cancer Hospital and Research Institute
The Ohio State University, USA

Dr. Mohamed A. El-Mahdy

Assistant professor of Pharmacology & Toxicology
Faculty of Pharmacy, El-Azhar University
Radiobiology department, The Ohio State University, USA

Faculty of Pharmacy
Cairo University

2009

بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيمِ

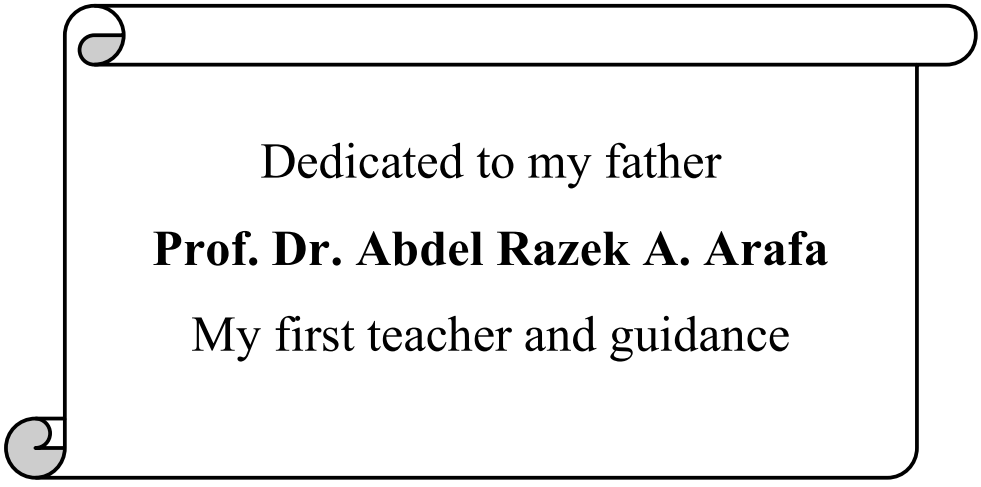
“قَالُوا سُبْحَانَكَ لَا عِلْمَ لَنَا إِلَّا

مَا عَلَّمْتَنَا إِنَّكَ أَنْتَ الْعَلِيمُ

الْحَكِيمُ”

صدق الله العظيم

(البقرة ٣٢)



Dedicated to my father

Prof. Dr. Abdel Razek A. Arafa

My first teacher and guidance

Acknowledgements

*I am sincerely grateful to **Prof. Dr. Hekma A. Abd El-Latif**, Professor and head of Pharmacology & Toxicology department, Faculty of Pharmacy, Cairo University for her keen supervision, deep experience, generous assistance and kind relation. I would also like to express to her my gratitude for being patient with me during all the stages of this work and for her critical reviews for the dissertation.*

*I wish to express my sincere gratitude and profound thanks to **Prof. Dr. Mostafa E. El-Sayed**, Professor of Pharmacology & Toxicology, Faculty of Pharmacy, Cairo University who was not only a supervisor but also a father to me I really wish to thank him for his kindness, indispensable remarks, generous assistance and continuous encouragement. You taught me how to do good science and how to be problem-solver.*

*I want to express my gratitude to **Prof. Dr. Helmy Moawad**, Professor of Pharmacology & Toxicology, Faculty of Pharmacy, Cairo University for his kindness, patience, creative suggestions, valuable discussions, generous assistance, sincere help and time that he sacrificed for me.*

*I wish to thank my advisor **Prof. Dr. Altaf A. Wani**, Professor of Biochemistry, The Ohio State University for the opportunity to partake in research that has been an excellent intellectual journey and for his guidance, support, kindness and always providing great opportunity for his students.*

*A simple thank you could never be enough for **Dr. Mohamed El-Mahdy**, assistant professor of Pharmacology & Toxicology, Faculty of Pharmacy, El-Azhar University and The Ohio State University, for all the support you have given. You taught me everything I know in the field of molecular biology, and shaped me into the scientist I am today.*

*I wish to thank all present and past lab members at The Ohio State University, especially **Bassant Barakat** and **Hany Arab** for their friendship, encouragement and continued support.*

My gratitude should be extended to include all my colleagues in the Department of Pharmacology & Toxicology, Faculty of Pharmacy, Beni-Suef University for their continuous cooperation and moral support.

*I owe a very special thank you to my husband **Hany Mostafa** and to my kids **Mohamed** and **Salma** for their daily encouragement and endless patience, especially throughout the last three years.*

*Finally, I would like to express my gratitude to my mother who, although separated by distance, has been with me through every step of the way. I would like to extend my gratitude to my brothers **Mohamed** and **Ahmed**, my sister **Aliaa** and their families for believing in me and supporting me.*

El-Shaimaa A. Arafa

Contents

Subject	Page
List of Tables	iii
List of Figures	v
List of Abbreviations	ix
Introduction	
- Cancer: An overview.....	1
- Cell cycle.....	3
- Apoptosis.....	8
- PI3K/PDK-1/Akt signaling pathway.....	20
- Cisplatin.....	27
- Mechanism of cisplatin resistance.....	31
- Ovarian cancer.....	36
- Flavonoids and cancer therapy.....	39
- Tangeretin.....	41
Aim of the Work	44
Materials and Methods	46
• Materials	46
• Experimental Design	51
• Methods	
I. Cytotoxicity assay.....	59
a. MTT-Based cytotoxicity assay.....	59
b. Clonogenic assay.....	60
II. PicoGreen dsDNA Quantitation.....	61
III. DNA fragmentation assay.....	62
IV. ELISA cell death assay.....	65
V. Protein quantitation.....	68

Subject	Page
VI. Gel electrophoresis and immuno-blot analysis of proteins (western blot).....	71
VII. Flow cytometric analysis.....	75
VIII. Transforming competent cells.....	76
IX. Plasmid purification.....	78
X. Restriction enzyme digestion for plasmid identification.....	82
XI. Plasmid DNA transfection.....	84
XII. Akt knockdown by siRNA transfection.....	85
XIII. Statistical Analysis.....	87
Results	88
Discussion	162
Summary and conclusions	176
References	182
Arabic summary	1

List of Tables

Table	Page
i. Summary of morphologic and biochemical characteristics of apoptosis.....	13
ii. Reagent preparation for DNA standard curve using PicoGreen assay.	62
iii. Reagent preparation for BSA standard curve using DC protein assay..	69
iv. Effective range of separation of SDS-polyacrylamide gels.....	74
v. Solutions for preparing resolving gels for Tris-glycine SDS-Polyacrylamide gel electrophoresis.....	74
vi. Solutions for preparing 5% stacking gels for Tris-glycine SDS-Polyacrylamide gel electrophoresis.....	74
vii. Composition of QIAGEN kit for plasmid purification.....	80
1. Effect of cisplatin on survival of different human cancer cells.....	89
2. Effect of tangeretin on survival of different human cancer cells.....	92
3. Effect of different tangeretin-cisplatin combination sequences on survival of human cisplatin-sensitive ovarian cancer cells.....	95
4. Effect of different tangeretin-cisplatin combination sequences on survival of human cisplatin-resistant ovarian cancer cells.....	98
5. Effect of different tangeretin-cisplatin combination sequences on survival of human hepatocellular carcinoma cells	101
6. Effect of different tangeretin-cisplatin combination sequences on survival of human breast adenocarcinoma cells	104
7. Effect of different tangeretin-cisplatin combination sequences on survival of human colon cancer cells	107

Table	Page
8. Determination of the half maximal inhibitory concentration of cisplatin in cisplatin-sensitive and –resistant ovarian cancer cells.....	110
9. Determination of the half maximal inhibitory concentration of tangeretin in cisplatin –resistant ovarian cancer cells.....	113
10. Combination index values for tangeretin-cisplatin combination in cisplatin –resistant ovarian cancer cells.....	117
11. Combination index values for cisplatin-tangeretin combination in cisplatin –resistant ovarian cancer cells.....	119
12. Effect of different tangeretin-cisplatin combination sequences on survival of cisplatin-resistant ovarian cancer cells.....	122
13. Effect of tangeretin-cisplatin combination on cell cycle distribution of cisplatin-resistant ovarian cancer cells.....	127
14. Effect of tangeretin-cisplatin combination on DNA fragmentation in cisplatin-resistant ovarian cancer cells in the presence and absence of z-VAD using ELISA kit	133
15. Effect of tangeretin-cisplatin combination on DNA fragmentation in cisplatin-resistant ovarian cancer cells in presence and absence of SB216763 using ELISA kit.....	146
16. Effect of tangeretin-cisplatin combination on DNA fragmentation in constitutively active Akt plasmid transfected and non-transfected cisplatin-resistant ovarian cancer cells using ELISA kit.....	151
17. Effect of tangeretin-cisplatin combination on DNA fragmentation in cisplatin-resistant ovarian cancer cells in presence and absence of LY294002 using ELISA kit.....	155
18. Effect of tangeretin-cisplatin combination on DNA fragmentation in cisplatin-resistant ovarian cancer cells after silencing Akt using siRNA.....	159

List of Figures

Figure	Page
i. Checkpoints and the cell cycle.....	4
ii. Schematic representation of the four major periods in the cell cycle and the cyclin/Cdk complexes that regulate progression through each period.....	7
iii. The morphological features of apoptosis and necrosis.....	12
iv. Death receptor-dependent and –independent pathways in anticancer drug-induced apoptosis.....	18
v. Regulation of apoptosis by the Bcl-2 family.....	19
vi. Regulation of intracellular Akt activity.....	22
vii. PI3K/PDK-1/Akt pathway.....	26
viii. Overview of pathways involved in mediating cisplatin-induced cellular effects.....	30
ix. Molecular mechanisms of resistance to platinum drugs.....	33
x. Standard curve of double stranded DNA.....	63
xi. Scheme of the principle of the Cell Death Detection ELISA ^{PLUS}	66
xii. Standard curve of bovine serum albumin.....	70
xiii. Map of HA PKB T308D S473D pcDNA3 plasmid containing Akt insert.....	77
xiv. Schematic representation for the plasmid DNA purification.....	81
xv. Akt plasmid digestion.....	83
xvi. Mechanism of plasmid transfection.....	84

Figure	Page
1. Effect of cisplatin on survival of different human cancer cells.....	90
2. Effect of tangeretin on survival of different human cancer cells.....	93
3. Effect of different tangeretin-cisplatin combination sequences on survival of human cisplatin-sensitive ovarian cancer cells.....	96
4. Effect of different tangeretin-cisplatin combination sequences on survival of cisplatin –resistant ovarian cancer cells.....	99
5. Effect of different tangeretin-cisplatin combination sequences on survival of human hepatocellular carcinoma cells.....	102
6. Effect of different tangeretin-cisplatin combination sequences on survival of human breast adenocarcinoma cells.....	105
7. Effect of different tangeretin-cisplatin combination sequences on survival of human colon cancer cells.....	108
8. Determination of the half maximal inhibitory concentration of cisplatin in cisplatin-sensitive and -resistant ovarian cancer cells.....	111
9. Determination of the half maximal inhibitory concentration of tangeretin in cisplatin-resistant ovarian cancer cells.....	114
10. Combination index values for tangeretin-cisplatin combination in cisplatin-resistant ovarian cancer cells.....	118
11. Combination index values for cisplatin-tangeretin combination in cisplatin-resistant ovarian cancer cells.....	120
12. Effect of different tangeretin-cisplatin combination sequences on survival of cisplatin-resistant ovarian cancer cells.....	123
13. Effect of tangeretin-cisplatin combination on colony formation of cisplatin-resistant ovarian cancer cells.....	125
14. a. Effect of tangeretin-cisplatin combination on cell cycle distribution of cisplatin-resistant ovarian cancer cells.....	128

Figure	Page
b. Effect of tangeretin-cisplatin combination on G2/M phase distribution of cisplatin-resistant ovarian cancer cells.....	129
c. The effect of tangeretin-cisplatin combination on Sub-G1 phase distribution of cisplatin-resistant ovarian cancer cells.....	130
15. Effect of tangeretin-cisplatin combination on DNA fragmentation in cisplatin-resistant ovarian cancer cells in the presence and absence of z-VAD using ELISA kit	134
16. Effect of tangeretin-cisplatin combination on DNA fragmentation in cisplatin-resistant ovarian cancer cells in the presence and absence of z-VAD using agarose gel electrophoresis.....	135
17. Effect of tangeretin-cisplatin combination on caspase-7 in cisplatin-resistant ovarian cancer cells using western blot analysis.....	138
18. Effect of tangeretin-cisplatin combination on PARP cleavage in cisplatin-resistant ovarian cancer cells using western blot analysis.....	139
19. Effect of tangeretin-cisplatin combination on the expression of phospho-Akt in cisplatin-resistant ovarian cancer cells using western blot analysis.....	141
20. Effect of tangeretin-cisplatin combination on the expression of NF- κ B in cisplatin-resistant ovarian cancer cells using western blot analysis....	142
21. Effect of tangeretin-cisplatin combination on the expression of phospho-GSK3 β in cisplatin-resistant ovarian cancer cells using western blot analysis.....	143
22. Effect of tangeretin-cisplatin combination on the expression of phospho-Bad in cisplatin-resistant ovarian cancer cells using western blot analysis.....	144
23. Effect of tangeretin-cisplatin combination on DNA fragmentation in cisplatin-resistant ovarian cancer cells in presence and absence of SB216763 using ELISA kit.....	147

Figure	Page
24. Effect of tangeretin-cisplatin combination on PARP cleavage in cisplatin-resistant ovarian cancer cells in presence and absence of SB216763 using western blot analysis.....	148
25. Akt and p-Akt expression 24 hours after transfection of cisplatin-resistant ovarian cancer cells with constitutively active Akt plasmid....	150
26. Effect of tangeretin-cisplatin combination on DNA fragmentation in constitutively active Akt plasmid in transfected and non-transfected cisplatin-resistant ovarian cancer cells using ELISA kit.....	152
27. Effect of tangeretin-cisplatin combination on PARP cleavage in constitutively active Akt plasmid in transfected and non-transfected cisplatin-resistant ovarian cancer cells using western blot analysis.....	153
28. Effect of tangeretin-cisplatin combination on DNA fragmentation in cisplatin-resistant ovarian cancer cells in presence and absence of LY294002 using ELISA kit.....	156
29. Effect of tangeretin-cisplatin combination on DNA fragmentation in cisplatin-resistant ovarian cancer cells in presence and absence of LY294002 using western blot analysis.....	157
30. Effect of tangeretin-cisplatin combination on DNA fragmentation in cisplatin-resistant ovarian cancer cells after silencing Akt using siRNA.....	160
31. Effect of tangeretin-cisplatin combination on PARP cleavage in cisplatin-resistant ovarian cancer cells after silencing Akt using siRNA.....	161

List of Abbreviations

Apaf-1	Apoptotic-protease-activating-factor-1
BAD	Bcl-2-associated death protein
CA-125	Cancer antigen 125
CAD	caspase-activated DNase
Caspases	cysteine-aspartic acid proteases
Cdk	Cyclin-dependent kinase
Cis	Cisplatin
CKIs	Cyclin-dependent kinase inhibitors
COX-2	Cyclooxygenase 2
dATP	Deoxyadenosine triphosphate
DDP	diamminedichloroplatinum
ELISA	Enzyme-linked immunosorbent assay
ERK1/2	Extracellular signal-regulated kinases
FADD	Fas- associated death domain adapter protein
Fas L	Fas-Ligand
HER2/neu	Human epidermal growth factor 2
FKHRL	forkhead family transcription factor
GSK3β	Glycogen synthase kinase 3 β
HCT-116	Human colon cancer cells
ICAD	Inhibitor of caspase-activated DNase
IKBs	Inhibitors of NF- κ B
IKK	Inhibitor of NF- κ B kinase
JNK	c-Jun N-terminal kinases
MAPK	Mitogen-activated protein kinase

MDM2	The murine double minute
MOPS	3-morpholinopropanesulfonic acid
mTOR	mammalian target of rapamycin
MTT	(3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide
NER	Nucleotide excision repair
NF-κB	Nuclear factor κ B
P70S6K	Ribosomal protein S6 kinase
PDK	Pyruvate dehydrogenase kinase
PI3K	Phosphatidylinositol-3-kinase
PIP2	Phosphatidylinositol-4,5- diphosphate
PIP3	Phosphatidylinositol-3, 4, 5-triphosphate
PKB	Protein kinase B
Plks	Polo-Like Kinases
PTEN	Phosphatase and tensin homolog
tBid	Truncated Bid
TBST	Tris-buffered saline-tween
TG	Tangeretin
TNF	Tumor necrosis factor
TRADD	TNF receptor-associated death domain adapter protein
VDAC	Voltage-dependent anion channel
z-VAD-FMK	N-Benzyloxycarbonyl-Val-Ala-Asp(O-Me) fluoromethyl ketone