

**MOLECULAR STUDIES ON
GROWTH ARREST AND
APOPTOSIS INDUCED BY
ANDROGRAPHOLIDE IN HUMAN
HEPATOMA HEPG2 CELLS**

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**Molecular Studies on Growth Arrest and
Apoptosis Induced by Andrographolide in
Human Hepatoma HepG2 Cells**

穿心莲内酯诱导人肝癌细胞 HepG2 的生长抑制和
凋亡的分子学研究

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ABSTRACT

Cell cycle regulation and apoptosis are two important control mechanisms for the proliferation of cells. These two processes are cooperatively interlinked so that tissue homeostasis and normal development of organisms could be effectively maintained. Loss of cell cycle control or apoptosis will result in many diseases, and cancer is somewhat one of the most jeopardous examples. Hepatocellular carcinoma (HCC) is a liver cancer prevalent in Asia, especially in the Mainland China and in Hong Kong. The major modalities of treatment for HCC include liver transplantation, chemotherapy and radiation, all of which have some limitations. Nowadays, chemotherapy involving the use of Traditional Chinese Medicines (TCMs), however, deserves exploration for the little side effects.

Andrographolide (Andro) is the main bioactive component of *Andrographis paniculata* (AP; Traditional Chinese: 穿心蓮; pinyin: Chuan Xin Lian) and has been reported to inhibit the proliferation of some tumor cell lines. However, there is no report about the anticancer effect of Andro to HCC cells although it has been claimed to be hepatoprotective and immunostimulative, both of which are supposed to be beneficial to normal liver cells.

This study reports for the first time that Andro is the most potent cytotoxic component among five analogues to two HCC cell lines. The IC₅₀ to human hepatoma HepG2 cells was $40.2 \pm 9.6 \mu\text{M}$ and to rat hepatoma H4IIE cells was $66.2 \pm 14.6 \mu\text{M}$ after 48 h of treatment. Compared to its cytotoxicity to normal liver L02 cells (IC₅₀ at 48 h was $135.1 \pm 30.7 \mu\text{M}$), Andro was found preferential targeting to tumor cells. When cell cycle and apoptotic study was monitored, it was found HepG2 cells was arrested at gap 2/mitosis (G2/M) phase of the cell cycle that subsequently

led to a late apoptosis. Fragmentation of chromosomal DNA was observed if the cells were treated with 200 μ M Andro for 48 h or with 50 μ M Andro for a prolonged period of 96 h. Analysis of apoptotic signal indicated that caspase-3 was activated during the process. Flow cytometric analysis of the treated cell after fluorescein isothiocyanate (FITC)-phospho-histone H3 plus propidium iodide (PI) staining revealed that the mitotic index increased from 5.5 to 49.1%, which was about 10 times of the control. As the G2/M phase cells were increased from 17% to 62.7%, these results mean that the ratio of cells at interphase G2 changed little upon Andro exposure while the arrest took place mainly at mitosis.

Immunoblotting analysis showed that upon Andro treatment, the tumor suppressor p53 (also known as a nuclear transcription factor), was up-regulated to almost 2-fold of the control. The activation of p53 subsequently turns on the transcription of the target Bax, responsible for causing apoptosis. Western blot analysis also showed that phosphorylated form of p34^{cdc2} at Thr161 was up-regulated to 3-fold of the control. This activation was confirmed by the enhanced expression of Cdk7, a cyclin-dependent kinase which is in control of the Thr161 phosphorylation after combined with Cyclin H. Because Thr161-phosphorylated p34^{cdc2} can form a complex named M-phase promoting factor (MPF) with Cyclin B and trigger cells pass through the G2/M checkpoint, this means that cells could enter into mitosis after Andro treatment. During mitosis, there is another cell cycle checkpoint, the metaphase-to-anaphase transition. At this transition, the MPF activates another complex - the anaphase-promoting complex (APC), which destroys Cyclin B reciprocally. From our result, the Cyclin B level was determined stable after Andro treatment, indicative of inactivation of APC thus cells could not pass through this transition and were arrested at metaphase. Studies with synchronized cells revealed

that the cells arrest at G2 phase were more sensitive to Andro treatment, followed by M phase cells. These results confirmed our hypothesis that upon Andro treatment, cells pass through the G2/M checkpoint and accumulate at M phase, during which apoptosis occurs.

Many previous studies indicated that the hepatoprotective effect of Andro was mainly due to the antioxidant effects. But in this study, we found that Andro caused the overproduction of hydrogen peroxide, a main form of reactive oxygen species (ROS) in HepG2 hepatoma cells. On the contrary, the level of superoxide radicals was decreased upon Andro treatment in both time- and concentration-dependent manners. When some well-known antioxidants were added prior to Andro treatment, it was found that N-acetylcysteine (NAC, a non-toxic precursor of GSH), catalase (an enzyme that degrading hydrogen peroxide) or reduced glutathione (GSH) inhibited the cytotoxic effect of Andro. Through an in vitro test, we observed that Andro could rapidly scavenge superoxide radicals generated by a KO_2 system. Cellular SOD activity was enhanced after Andro treatment, indicating that Andro may has some SOD-like activity. Because the GSH system contains the glutathione peroxidase which is one of the most important hydrogen peroxide-removing enzymes in mammalian mitochondria, the elevated level of hydrogen peroxide implies that GSH depletion might take place by Andro in the cells. Therefore, the intracellular GSH level was determined and it was found that the GSH system was destroyed by glutathione depletion after the addition of Andro. This depletion was confirmed by that Andro could react directly with GSH under certain conditions. When a GSH peroxidase (Gpx) mimicking antioxidant named ebselen was added prior to Andro, the cytotoxicity was also greatly attenuated.

Changes in the redox status after the addition of Andro also coupled with the

collapse of the mitochondrial membrane potential (MMP). This MMP loss, however, could be eliminated by NAC. On the other hand, the overproduction of hydrogen peroxide could not be attenuated by cyclosporine A (CsA), a well-known mitochondrial permeability transition pore (MPTP) inhibitor. Taken all the data present, it indicates that Andro treatment triggered changes in a redox signaling pathway via mitochondrial depolarization. Induction of cell cycle arrest at M phase was the main cause of growth inhibition and eventually led cells into apoptosis.

Finally, the redox variations of hepatoma HepG2 cells and normal liver L02 cells after Andro treatment was investigated. Less redox change was noted in L02 cells. Our results thus suggest that a redox signaling pathway played an important role in the cytotoxic effect of Andro to tumor cell and provide some valuable knowledge in the understanding of cell-type specific response to this drug.

TABLE OF CONTENTS

	Page
ABSTRACT	i-iv
TABLE OF CONTENTS	v-xi
ACKNOWLEDGEMENTS	xii
DECLARATION	xiii
ABBREVIATIONS	xiv-xvi
LIST OF TABLES	xvii
LIST OF FIGURES	xviii-xx
LIST OF PUBLICATIONS	xxi
SECTION A GENERAL INTRODUCTION	1
CHAPTER 1 OVERVIEW OF CELL CYCLE	2
1.1 Phases of Cell Cycle	2
1.2 Cell Cycle Control Systems	3
1.2.1 Regulation of the cell cycle	4
1.2.2 Checkpoints of cell cycle	5
1.2.3 Cyclins, Cyclin-dependent protein kinases (Cdks) and Cyclin-dependent kinase inhibitors (CKIs)	6
1.3 Cell Cycle and Cancer	12
CHAPTER 2 OVERVIEW OF APOPTOSIS	14
2.1 Apoptosis and Necrosis	14
2.2 Molecular Mechanisms of Apoptosis Signaling Pathways	16
2.2.1 Death receptor pathway (extrinsic pathway)	17
2.2.2 Intrinsic apoptosis pathway (mitochondria pathway)	20
2.2.3 Caspases: central initiators and executioners of apoptosis	21

2.3 Regulation of Apoptosis	22
2.3.1 The Bcl-2 family	23
2.3.2 IAPs (inhibitor of apoptosis proteins)	25
2.3.3 Tumor protein p53	27
2.4 Apoptosis, Cell Cycle Regulation and Cancer Therapy	29
CHAPTER 3 INTRODUCTION OF ANDROGRAPHOLIDE	31
DITERPENES AND THE PHARMACOLOGICAL EFFECTS	
3.1 <i>Andrographis paniculata</i> (AP)	31
3.2 Chemical Ingredients of AP	31
3.2.1 Diterpene lactones and diterpenoids	32
3.2.2 Flavones and flavonoids	33
3.2.3 Other constituents	33
3.3 Pharmacological Effects	34
3.3.1 Hepatoprotective activity	36
3.3.2 Immune system stimulator	38
3.3.3 Anti-cancer effect	40
CHAPTER 4 AIMS AND STRATEGIES OF THIS STUDY	43
4.1 Hepatocellular Carcinoma	43
4.2 Previous Research of AP in Our Laboratory	44
4.3 Aims and Strategies of This Study	45
SECTION B EXPERIMENTAL	48
CHAPTER 5 MATERIALS AND EQUIPMENTS	49
5.1 Cell Lines	49
5.1.1 Human hepatocellular carcinoma cell, HepG2	49
5.1.2 Human leukemia cell, HL 60	49

5.1.3 Rat hepatoma cell, H4IIE	49
5.1.4 Human normal liver cell, L02	49
5.2 Culture Media	49
5.3 Drugs and Chemicals	50
5.3.1 Drugs	50
5.3.2 Chemicals	50
5.4 Glasswares and Equipments	52
CHAPTER 6 BASIC EXPERIMENTAL TECHNIQUES	
6.1 Recovery of Cells from Storage	53
6.2 Subculture of Cell Lines	53
6.2.1 Subculture of adherent cells	53
6.2.2 Subculture of suspension cells	55
6.3 Cell Counting and Assessment of Cell Viability	55
6.4 Freezing Cells for Storage	57
6.5 Drug Preparation	57
6.6 Determination of Growth Curve of Cell Lines	58
6.7 Results of Growth Curves and Population Doubling Time (PDT)	59
CHAPTER 7 CYTOTOXICITY STUDY OF ANDRO AND ITS ANALOGUES	61
7.1 Introduction	61
7.2 Experimental	62
7.2.1 MTT assay	62
7.2.2 Trypan blue exclusive assay	63
7.3 Determination of the Median Dose (IC ₅₀)	63
7.4 Results	64

7.4.1 Growth inhibition of Andro and analogues to HepG2 cells	64
7.4.2 Growth inhibition of Andro to different cell lines	65
7.4.3 Cell viability	67
7.5 Chapter Summary	67
CHAPTER 8 CELL CYCLE ARREST AND APOPTOSIS OF ANDRO-TREATED HEPG2 CELLS	70
8.1 Introduction	70
8.2 Experimental	72
8.2.1 Cell morphological analysis	72
8.2.1.1 Hoechst 33342 staining	72
8.2.1.2 Acridine orange/ethidium bromide (AO/EB) staining	73
8.2.2 Cell cycle analysis	73
8.2.3 Identification of G2 arrest cells and mitotic cells	74
8.2.4 DNA fragmentation assay	74
8.2.5 Quantification of apoptosis by Annexin V-PI staining	75
8.2.6 Caspase-3 activity	76
8.3 Results	76
8.3.1 Morphological analysis	76
8.3.1.1 Apoptotic body formation	76
8.3.1.2 Nuclear condensation	77
8.3.1.3 Late apoptosis and necrosis	78
8.3.2 Cell cycle arrest at G2/M	81
8.3.3 Mitotic accumulation	86
8.3.4 DNA laddering	86
8.3.5 Apoptosis quantification	88

8.3.6 Caspase-3 activation	91
8.4 Chapter Summary	91
CHAPTER 9 MOLECULAR STUDY OF THE ANDRO-INDUCED	93
CELL CYCLE ARREST AND APOPTOSIS IN HEPG2 CELLS	
9.1 Introduction	93
9.2 Experimental	95
9.2.1 Effect of Andro on synchronized HepG2 cells	95
9.2.1.1 Effect of Andro in G2 phase synchronized cells	95
9.2.1.2 Effect of Andro in mitosis arrest cells	95
9.2.2 Protein extraction	96
9.2.2.1 Total cell extracts	96
9.2.2.2 Nuclear extracts	96
9.2.3 Western blotting analysis	97
9.2.4 NF- κ B transcriptional assay	98
9.3 Results	99
9.3.1 Effect of Andro on synchronized HepG2 cells	99
9.3.2 Expression of Bcl-2 family proteins	103
9.3.3 Cell cycle regulators	103
9.3.4 Up-regulation of tumor suppressor p53	104
9.3.5 Independent of NF- κ B activation	106
9.4 Chapter Summary	106
CHAPTER 10 STUDY OF REDOX SIGNALING	108
10.1 Introduction of Redox Signaling	108
10.1.1 Reactive oxygen species (ROS)	108
10.1.2 Biological effects of ROS and redox signaling	108

10.1.3 Cellular antioxidants	110
10.1.4 Mitochondrial membrane potential (MMP)	110
10.2 Experimental	112
10.2.1 Detection of hydrogen peroxide	112
10.2.2 Detection of superoxide radicals	113
10.2.3 Intracellular glutathione	114
10.2.4 Mitochondrial membrane potential (MMP) assay	115
10.2.5 In vitro scavenge of superoxide by Andro	115
10.2.5 Pretreatments prior to Andro	116
10.3 Results	116
10.3.1 Preserved cell viability by certain antioxidants and CsA	117
10.3.2 Pretreatment effect on cell cycle regulation	118
10.3.3 Elevation of H ₂ O ₂ level in cytosol	119
10.3.4 Change of O ₂ ^{·-} level in cytosol	120
10.3.5 Effect of Andro on intracellular level of GSH	123
10.3.6 Ability of Andro to scavenge O ₂ ^{·-}	125
10.3.7 MMP collapse	125
10.3.8 Pretreatments effect on certain protein expression	128
10.3.9 Effect of pretreatments on NF-κB transcriptional level	128
SECTION C DISCUSSIONS	131
CHAPTER 11 CELL CYCLE ARREST AND APOPTOSIS	132
INDUCED BY ANDRO	
11.1 Effect on Mitotic Division	132
11.2 p53-mediated Apoptosis	134
CHAPTER 12 MITOCHONDRIA-MEDIATED REDOX SIGNALING	139

12.1 ROS Alteration	139
12.2 GSH Depletion	140
12.3 Independent of NF- κ B	144
12.4 Mitochondrial Signaling	145
12.5 Basal ROS Level in L02 and HepG2 cells	148
SECTION D CONCLUSION AND SUGGESTIONS	151
CHAPTER 13 CONCLUSION AND SUGGESTIONS FOR	152
FUTURE WORK	
13.1 Summary Conclusion	152
13.2 Suggestions for Future Work	154
SECTION E REFERENCES AND APPENDIX	156
REFERENCES	157
APPENDIX I	190
APPENDIX II	192