CITY UNIVERSITY OF HONG KONG

Combined effects of bio-active components from Scutellaria barbata and Hedyotis diffusa on human leukemia cells
半枝蓮和白花蛇舌草內生物活性成份之組合對白血病細胞的作用

Submitted to
Department of Biology and Chemistry
生物及化學系
in Partial Fulfillment of the Requirements
for the Degree of Master of Philosophy
哲學碩士學位

by

Tse Kai Wing, Anfernee 謝啓榮

October 2002
二零零二年十月
ABSTRACT

*Scutellaria barbata* (半枝蓮, SB) and *Heydotis diffusa* (白花蛇舌草, HD) are two herbs commonly employed in the treatment of leukemia. In order to reveal the cytotoxic effects of cited bioactive components from these two herbs, tetrazolium colorimetric cytotoxic assay (MTT assay) was performed. Scutellarein (SC) from SB and ursolic acid (UA) and oleanolic acid (OA) from HD were identified as active principles for the death of human leukemia cell line, HL-60, while p-coumaric acid, which present abundantly in both herbs, exhibited very little inhibitory effect on the proliferation of the cells.

Drug combination experiments were performed to reveal the potential interactions between bioactive components in SB and HD. Synergistic cytotoxic effects were observed between SC (flavonoid) and p-coumaric acid (PCA, phenolic acid). On the other hand, upon exposure to PCA, the UA-induced cytotoxicity in HL-60 cells was significantly decreased.

Phenolic phytochemicals (flavonoids and phenolic acid) in the extracts of these two herbs were analyzed by capillary electrophoresis analysis. Combinations of p-coumaric acid and other two flavonoids, SC and apigenin, were found to have greater efficacy in the *in vitro* treatment of leukemia.

It was found that apoptosis of HL-60 cell was induced in the presence of UA through reactive oxygen species (ROS) mediated pathways. On the other hand, p-coumaric acid (PCA), a dietary antioxidant, was found in aqueous, ethanol and methanol extract of HD. Exposure of UA-treated HL-60 cells in PCA resulted in
decreased apoptosis percentage. This phenomenon was in association with the inhibitory effects of overall ROS and intracellular hydrogen peroxide (H₂O₂) production in UA-treated HL-60 cells by PCA. These results suggest that the combined uses of SB and HD were accomplished by the interactions between ROS-mediated active component and phenolic antioxidant.
CONTENT

ABSTRACT
CERTIFICATION OF APPROVAL BY THE PANEL OF EXAMINERS
ACKNOWLEDGEMENTS
CONTENT
BACKGROUND
AIM and OBJECTIVES of THIS STUDY
ABBREVIATIONS and SYMBOLS
LIST OF FIGURES and TABLES

1. INTRODUCTION

1.1 Problems of using Traditional Chinese Medicines (TCM) for cancer chemotherapy

1.1.1 Chinese herbs as cancer chemotherapeutic agents

1.1.2 Crude herbal extract for cancer chemotherapy

1.1.3 Purified bioactive components from herbs for cancer chemotherapy

1.1.4 Skeptical challenges of using TCM as cancer chemotherapeutic agents

1.1.5 Pragmatic problems of use of TCM

(a) Use of crude extract

(b) Use of isolated active compounds from herbs

1.1.6 Combined effects of active compounds in Chinese herbs

1.1.6.1 Chinese philosophy of herbal medication

1.1.6.2 Scientific base of combined effects of active compounds in Chinese herbs

1.2 Treatment of HL-60, acute promyelocytic leukemia

1.2.1 What is leukemia?

1.2.2 Background of HL-60 cells, an acute promyelocytic leukemia cell line

1.2.3 Strategies on treatment of acute promyelocytic leukemia

1.2.3.1 Cell Physiology level: Apoptosis or differentiation

(a) Apoptosis

(b) Differentiation
1.2.3.2 Molecular determinants of apoptosis and differentiation

1.2.3.2.1 Molecular determinants of apoptosis

- (i) Check point I: cell cycle arrest
- (ii) Checkpoint II: Genetic control by oncogenes an tumor suppressor genes
  (a) Tumor suppressor genes
  (b) Oncogenes
- (iii) Mitochondrial control of apoptosis
  (a) Capsases: executioners of apoptosis
  (b) Reactive oxygen species production

1.2.3.2.2 Molecular determinants of differentiation

(a) Mechanism of action of ATRA – interaction with RAR-α
(b) Cell cycle arrest and cell differentiation
(c) Role of protooncogene bcl-2 and c-myc in HL-60 cel differentiation
(d) Autocrine production
(e) Are reactive oxygen species involved in differentiatio of HL-60 cells?

1.2.3.3 Drug combination in chemotherapy of leukemia

1.2.3.4 Leukemia treatment using traditional Chinese medicines

1.3 Anti-leukemia Chinese medical plants: Scutellaria barbata and Hedyotis diffusa

1.3.1 General information of Scutellaria barbata and Hedyotis diffusa

1.3.2 Pharmacology and applications of SB and HD in Chinese medical point of view

1.3.3 Pharmacology and applications of SB and HD in Western medical point of view

1.3.4 Major known bioactive components from SB and HD

1.3.4.1 Bioactive components from SB

1.3.4.2 Bioactive components from HD

2 MATERIALS AND METHODS

2.1 Materials

2.1.1 Cell line

2.1.2 Drugs
2.2 Methods
2.2.1 Preparation of crude herbal extracts 61
2.2.2 Capillary electrophoresis (CE) analysis 61-62
2.2.3 MTT assay 62
2.2.4 Morphological evaluation of apoptosis, differentiation and necrosis by laser confocal microscopy 63
2.2.5 Combination effect analysis 63-68
2.2.6 Visualization of DNA fragmentation 68-69
2.2.7 Flow cytometry analysis 69
2.2.8 Quantitation of apoptosis by flow cytometry 69
2.2.9 NBT reduction assay 70
2.2.10 Analysis of cell surface markers expression, CD11b and CD14 70
2.2.11 Enzyme-linked immunosorbent assay (ELISA) of IL-6 and TNF-α production 71
2.2.12 Measurement of Caspase activity 71
2.2.13 Western blot analysis 71-72
2.2.14 Measurement of reactive oxygen species 72-73
2.2.15 Measurement of mitochondrial membrane potential 73
2.2.16 Data analysis 73

3 EXPERIMENTAL

3.1 CHAPTER 1 - Cytotoxic study of various bioactive components from Scutellaria barbata and Hedyotis diffusa on human acute promyelocytic leukemia HL-60 cells 74-99

3.2 CHAPTER 2 - Combined Cytotoxic effects of biological active phenolic phytochemicals from Scutellaria barbata and Hedyotis diffusa on HL-60 cells 100-118

3.3 CHAPTER 3 - Activation of cellular differentiation and apoptosis of human promyelocytic leukemia, HL-60 cells by ursolic acid and oleanolic acid is concentration dependent 119-136

3.4 CHAPTER 4 - Apoptosis of HL-60 cells induced by Ursolic acid is related to the over production of reactive oxygen species (ROS) 137-150
<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Pages</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.5</td>
<td>CHAPTER 5 - Antagonistic cytotoxic and apoptotic effect between bioactive components from traditional Chinese her <em>Hedyotis diffusa</em>: p-coumaric acid scavenges ursolic acid-induce reactive oxygen species production in HL-60 cells</td>
<td>151-163</td>
</tr>
<tr>
<td>4</td>
<td>SUMMARY OF THESIS</td>
<td>164-167</td>
</tr>
<tr>
<td>5</td>
<td>REFERENCES</td>
<td>168-199</td>
</tr>
</tbody>
</table>
LIST OF FIGURES AND TABLES

Figures:

Introduction:
Figure 1a: Monthly variations of biologically active compounds in herbs
Figure 1b: Yearly variations of biologically active compounds in herbs
Figure 2: The overall production of ROS in cell

Chapter 1:
Figure 1.1: Dose response curves for biological active compounds alone
Figure 1.2: DNA fragmentation after treatment of HL-60 cells with ethanol extracts of SB or HD
Figure 1.3a-c: Assessment of herbal extracts-induced morphological changes in HL-60 cells by laser confocal microscopy
Figure 1.4a-c: Fluorescence images of HL-60 cells after drug treatments
Figure 1.5a-c: Combination effects of p-coumaric acid in combined with (a) scutellarein, (b) ursolic acid and (c) oleanolic acid
Figure 1.6a-c: Combination effects of stearic acid in combined with (a) scutellarein, (b) ursolic acid and (c) oleanolic acid
Figure 1.7a-b: Combination effects of scutellarein combined with (a) ursolic acid and (b) oleanolic acid
Figure 1.8a-c: IC$_{50}$ isobolograms of p-coumaric acid in combination with (a) scutellarein, (b) ursolic acid and (c) oleanolic acid
Figure 1.9a-c: IC$_{50}$ isobolograms of stearic acid in combination with (a) scutellarein, (b) ursolic acid and (c) oleanolic acid
Figure 1.10a-c: IC$_{50}$ isobolograms of scutellarein in combination with (a) ursolic acid and (b) oleanolic acid
Figure 1.11a-c: Zero interaction response surface for a 4 days drug exposure of p-coumaric acid in combined with (a) scutellarein, (b) ursolic acid and (c) oleanolic acid
Figure 1.12a-c: Zero interaction response surface for a 4 days drug exposure of stearic acid in combined with (a) scutellarein, (b) ursolic acid and (c) oleanolic acid
Figure 1.13a-c: Zero interaction response surface for a 4 days drug exposure of scutellarein in combined with (a) ursolic acid and (b) oleanolic acid

Chapter 2:
Figure 2.1a-b: Dose response curves for (a) flavonoids and (b) phenolic acids
Figure 2.2a-c: Separation of (a) phenolic phytochemicals standard, (b) ethanol extract of SB and (c) ethanol extract of HD by micellar electrokinetic capillary chromatography

Figure 2.3a-d: Medain effects analysis: combination effects of p-coumaric acid (PCA) in combined with (a and b) scutellarein and (c and d) apigenin

Figure 2.4a-d: IC$_{50}$ Isobolgram of p-coumaric acid in combined with (a and b) scutellarein and (c and d) apigenin.

Figure 2.5a-d: Zero interaction response surface for a 2 or 4 days drug exposure of p-coumaric acid in combined with (a and b) scutellarein and (c and d) apigenin.

Figure 2.6a-f: Zero interaction response surface for a 2 or 4 days drug exposure of p-coumaric acid in combined with (a and b) biochanin A, (c and d) flavone and (e and f) genistein.

Chapter 3:
Figure 3.1a-b: Cytotoxicities of (a) ursolic acid and (b) oleanolic acid for HL-60 cells at indicated time

Figure 3.2a-b: Effects of (a) ursolic acid and (b) oleanolic acid on the induction of apoptosis of leukemia cells

Figure 3.3: DNA fragmentation after treatment of HL-60 cells with ursolic acid and oleanolic acid

Figure 3.4: Effects of ursolic acid, oleanolic acid and crude extract of *Hedyotis diffusa* on the induction of differentiation of HL-60 cells (1x10$^6$) as measured by NBT reduction assay

Figure 3.5a-b: Flow cytometric analysis of (a) CD11b and (b) CD14 expression on HL-60 cells after treatment

Figure 3.6a-c: Fluorescence images of HL-60 cells after drug treatments

Figure 3.7a-b: Cytokines production in HL-60 cell cultures

Figure 3.8: Induction of caspase activities by various drugs

Figure 3.9: Concentration-dependent suppression of bcl-2 protein.

Chapter 4:
Figure 4.1: The time dependency of ROS production in HL-60 cells treated with 10 µg/ml UA or OA

Figure 4.2: The dose dependency of ROS production in HL-60 cells treated with UA or OA

Figure 4.3a-b: Inhibition of UA-induced apoptosis by BHA

Figure 4.4: The dose dependency of H$_2$O$_2$ production in HL-60 cells treated with
LIST OF FIGURES AND TABLES

Figure 4.5: Changes of mitochondrial membrane potential in drug-treated HL-60 cells
Figure 4.6: The inhibition of UA-mediated overall ROS and H$_2$O$_2$ production by calcium inhibitor or chelator

Chapter 5:
Figure 5.1a-c: Combined cytotoxic effects between ursolic acid (UA) and p-coumaric acid (PCA) on HL-60 cells
Figure 5.2: Inhibition of UA-induced HL-60 cells apoptosis by p-coumaric acid
Figure 5.3: The time dependency of inhibition of ROS production in UA-treated HL-60 cells with PCA
Figure 5.4: The inhibition of ROS production in HL-60 cells treated with various concentrations of UA and PCA at 90th minutes
Figure 5.5: The time dependency of inhibition of H$_2$O$_2$ production in HL-60 cells treated with various concentrations of UA and PCA
Figure 5.6: Inhibition of UA-induced caspase activities by PCA

Tables:

Introduction:
Table 1: Characteristics of HL-60 cell line
Table 2: Some typical drugs used in the treatment of acute myelogenous leukemia
Table 3: Assays for the detection of reactive oxygen species
Table 4: General information of SB and HD
Table 5: Pharmacological properties of SB and HD
Table 6: Chinese anti-leukemia herbal formulas using HD and/or SB

Chapter 2:
Table 2.1: Capillary electrophoresis profile of phenolic phytochemicals from SB and HD

Chapter 3:
Table 3.1: Cell cycle analysis of HL-60 cells after 96h exposure to ursolic acid (UA), oleanolic acid (OA) and crude extract of *Hedyotis diffusa* (HD)

Chapter 4:
Table 4.1: Effects of antioxidants on UA-induced apoptosis in HL-60 cells