UNIVERSITI PUTRA MALAYSIA

STUDIES OF THE ANTI-CANCER EFFECTS OF FLAVOKAWIN B ON HUMAN BREAST CANCER CELL LINES, MCF7 AND MDA-MB-231

AJANTHA SINNIAH

FPSK(M) 2005 9
STUDIES OF THE ANTI-CANCER EFFECTS OF FLAVOKAWIN B ON HUMAN BREAST CANCER CELL LINES, MCF-7 AND MDA-MB-231

By

AJANThA SInNIAH

Thesis submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the requirements for the degree of Master’s of Science

MARCH 2005
This thesis is especially dedicated to:

Amma & Appa, who are infinitely precious to me

&

Anu, Aravind and Abirami, who have filled my life with joy and happiness

&

My friends, who were there for me!
Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Master’s of Science

STUDIES OF THE ANTI-TUMORIGENIC EFFECTS OF FLAVOKAWIN B ON HUMAN BREAST CANCER CELL LINES, MCF-7 AND MDA-MB-23

By

AJANTHA SINNIAH

MARCH 2005

Chairman: Ahmad Bustamam Abdul, PhD

Faculty: Medicine and Health Sciences

A natural compound, Flavokawin B, isolated and purified from extract of Alpinia zerumbet was investigated for its anti-cancer properties on breast cancer cell lines, estrogen dependant MCF-7 and estrogen non-dependant MDA-MB-23. Tamoxifen, a non-steroidal anti-estrogen, primarily exploited as a drug against hormone-dependent breast cancer, acts as the positive control for this study. MCF-10A, mammary epithelial cells serve as the negative control. The cytotoxicities of Flavokawin B and Tamoxifen on human breast cells were investigated using the MTT assay. The results showed that the IC$_{50}$ (± S.E.M) value of Flavokawin B on MCF-7 cell line was determined to be $11.5 \pm 0.015$ µM/ml whilst the IC$_{50}$ with Tamoxifen was at $10.2 \pm 0.012$ µM/ml. The IC$_{50}$ value of Flavokawin B on MDA-MB-231 cell line was determined to be $17.5 \pm 0.019$ µM/ml whilst the IC$_{50}$ value
of Tamoxifen was at 32.5 ± 4.2 µM/ml. The MTT assay results on normal epithelial cell line, MCF-10A treated with Flavokawin B demonstrated that the IC₅₀ value was 38.0 ± 0.032 µM/ml whereas MCF-10A treated with Tamoxifen had an IC₅₀ value of 28 ± 0.021 µM/ml. All values were statistically significant (p<0.05), as analysed using one sample T-test. The breast cancer cell lines treated at IC₅₀ concentration of both compounds before proceeding using confocal microscopy. There were no significant changes observed in the untreated cells. However, apoptotic features were that include membrane blebbing and nucleus condensation were evident at 24 hours. At 48 and 72 hours post treatment, convolution of nuclear membrane, destruction of nuclear membrane and fragmentation of the nucleus were observed. The TUNEL assay is designed to specifically detect and quantify apoptotic cells within a cell population, which primarily consists of both apoptotic and non-apoptotic cells. The TUNEL assay conducted showed that Flavokawin B induces more apoptosis on MCF-7 and MDA-MB-231 compared to Tamoxifen. In contrast, Flavokawin B has lesser lethal effects on MCF-10A as compared to Tamoxifen. The levels of IL-6 secretion in MDA-MB-231 cell line decreased significantly after treatment with Flavokawin B. Immunofluorescence studies demonstrated that the levels of IL-6 secretion commensurate with the presence of membrane bound IL-6r when proliferation of the breast cells was inhibited during treatment with both the compounds. The MCF-7 and MDA-MB-231 cell lines were arrested at G1 phase when treated with both Flavokawin B
and Tamoxifen. This shows that both the treatment follows similar mechanism to induce cell phase arrest. In conclusion, it could be confirmed that the pure compound Flavokawin B induces apoptosis in MCF-7 and MDA-MB-231 breast cancer cell lines contributing to the discovery of new alternative treatment strategy for breast cancer.
Sebatian semulajadi Flavokawin B yang diasingkan dan ditulinkan daripada ekstrak Alpinia zerumbet telah dikaji bagi menentukan fungsi sebagai antikanser terhadap sel-sel selanjar payudara, samada bergantung kepada estrogen MCF-7 atau tidak bergantung kepada estrogen MDA-MB-231. Tamoxifen, anti-estrogen bukan steroid yang digunakan sebagai drug ke atas kanser payudara bergantung kepada estrogen digunakan sebagai kawalan positif bagi kajian ini. MCF-10A yang merupakan sel selanjar epithelial payudara digunakan sebagai kawalan negatif. Asai MTT digunakan untuk mengkaji kesan sitotoksik rawatan. Keputusan menunjukkan bahawa nilai IC₅₀ (±S.E.M) untuk rawatan Flavokawin B ke atas sel selanjar MCF-7 ditentukan sebagai 11.5 ± 0.015 μM/ml sementara nilai IC₅₀ bagi rawatan Tamoxifen ialah 10.2 ± 0.012 μM/ml. Nilai IC₅₀ bagi Flavokawin B ke atas sel selanjar MDA-MB-231 ditentukan sebagai 17.5 ± 0.019 μM/ml
sementara nilai IC₅₀ bagi rawatan Tamoxifen ialah 32.5 ± 4.2 μM/ml. Keputusan asai MTT ke atas sel selanjarg MCF-10A menunjukkan nilai IC₅₀ bagi Flavokawin B ialah 38.0 ± 0.032 μM/ml sementara nilai IC₅₀ bagi rawatan Tamoxifen ialah 28 ± 0.021 μM/ml. Semua nilai IC₅₀ adalah signifikan setelah dianalisis menggunakan satu sampel T-test (P <0.05). Kajian mikroskop konfokal diteruskan bagi semua sel-sel selanjarg dirawat dengan Flavokawin B dan Tamoxifen pada kepekatan IC₅₀ masing-masing. Tiada perubahan yang signifikan dilihat pada kumpulan kawalan. Walaubagaimanapun, ciri-ciri apoptosis telah dilihat seperti pengembungan membran dan kondensasi nukleus pada 24 jam. Pada 48 dan 72 jam selepas rawatan, konvolusi membran nucleus, pemusnahan dan fragmentasi membran nukleus telah dapat dilihat. Asai TUNEL telah direka untuk mengesan dan mengira sel-sel apoptotic dalam satu kumpulan sel yang terdiri daripada sel apoptotic dan bukan apoptotic. Berdasarkan keputusan, Flavokawin B merangsang lebih banyak apoptosis kepada MCF-7 dan MDA-MB-231 berbanding dengan rawatan Tamoxifen. Walaubagaimanapun, Flavokawin B kurang menghasilkan kesan kematian kepada sel selanjarg MCF-10A berbanding dengan rawatan Tamoxifen. Tahap IL-6 bagi sel selanjarg MDA-MB-231 berkurang selepas dirawat dengan Flavokawin B. Kajian immunofluorescence menunjukkan bahawa tahap rembesan IL-6 bergantung kepada kewujudan IL-6r yang terdapat pada membran sel apabila pertumbuhan sel kanser terbantut ketika dirawat dengan kedua-dua rawatan tersebut. Sel selanjarg MCF-7 dan MDA-MB-231
ACKNOWLEDGEMENT

I would like to take this opportunity to thank all those who gave great support to me while doing the thesis. First and foremost, I would like to extend my heartfelt gratitude to my supervisor, Dr. Ahmad Bustamam, for guiding me and giving endless support, advice and encouragement throughout the completion of this thesis. I am also deeply indebted to my co-supervisor, Assoc. Prof. Dr. Nazrul Hakim, for his invaluable advice and guidance.

I would also like to express my deepest appreciation and sincere gratitude to Prof. Nordin Lajis for providing us the compound used in this study. A special thanks is owed to Assoc. Prof. Dr. Rozita Rosli, for letting us use the tissue culture lab during our initial part of the study. I would also like to extend my gratitude to Assoc. Prof. Dr. Fauziah Othman, for allowing me to use the Confocal Microscopy at Infoport.

I would also like to acknowledge En. Nasir and Pn. Normawati, for their precious time in providing me with professional assistance during my study. A special thanks is owed to my colleague Zetty Nadia, without whom I would not have completed this research. I am also grateful to my friends Nazefah, Nirmala, Shaban and Kak Abdah who were willing to share their valuable knowledge and giving their full cooperation and encouragement to me. A special thanks is owed to my friend, Mr. Saravanan, for his continuous encouragement and support throughout my research.
I would like to extend my deepest and warmest thanks to my parents, sisters and brother for always being there for me, believing in me and supporting me in whatever that I indulge in.

Above all, I thank God for His mercy and blessings on me.
I certify that an Examination Committee met on 9th March 2005 to conduct the final examination of Ajantha Sinniah on her Master of Science thesis entitled "Anticancer Effects of Flavokawin B on Human Breast Cancer Cell Lines, MCF-7 and MDA-MB-231" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

Zarida Hambali, PhD
Associate Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Chairman)

Asmah Rahmat, PhD
Associate Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Internal Examiner)

Chong Pei Pei, PhD
Lecturer
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Internal Examiner)

Iekhsan Othman, PhD
Professor
Faculty of Medicine
University of Malaya
(External Examiner)

GULAM RUSUL RAHMAT ALI, PhD
Professor/Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 20 JUN 2005
This thesis submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the degree of Master’s of Science. The members of the Supervisory Committee are as follows:

Ahmad Bustamam Abdul, PhD
Lecturer
Department of BioMedical Sciences,
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Chairman)

Mohammad Nazrul Hakim Abdullah, D.V.M. PhD
Associate Professor
Department of BioMedical Sciences,
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Member)

AINI IDERIS, PhD
Professor/Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 15 JUL 2005
DECLARATION

I hereby declare that the thesis is based on my original work except for the quotations and citations, which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

AJANTHA A/P SINNIAH

Date: 17 JUNE 2005
TABLE OF CONTENTS

DEDICATION ii
ABSTRACT iii
ABSTRAK vi
ACKNOWLEDGEMENTS ix
APPROVAL xi
DECLARATION xii
TABLE OF CONTENT xi
LIST OF TABLES xv
LIST OF FIGURES xvi
LIST OF ABBREVIATIONS xx

CHAPTER

I INTRODUCTION 1
   Research Objectives 4

II. LITERATURE REVIEW 5
   Breast cancer 7
      Risk factors for breast cancer 7
      Breast cancer therapy 9
      Breast cancer cell lines 11
   Flavonoids and cancer 14
      Chalcone Flavokawin B 18
      Tamoxifen citrate 20
   Cell proliferation and cell viability 23
      Cell Viability 24
      Cell Proliferation 24
   Apoptosis 26
      Morphology of apoptosis 26
      Distinction of apoptosis and necrosis 28
   Cell cycle regulation 30
      Control of the Cell Cycle 30
   Overview of IL-6 32
      IL-6 and breast cancer 34

III. METHODOLOGY 35
   Materials and equipments 35
   Methodology 38
      Cell culture and maintenance 38
      Thawing cryopreserved cells 39
      Cryopreservation of cell lines 40

xiii
IV. RESULTS

MTT ASSAY

Morphological studies of cell lines using inverted microscope

Confocal microscopy

MCF-7 cell lines

MDA-MB-231 cell lines

Tunel micrographs

Flowcytometry studies

ELISA IL-6

Immunofluorescence technique to detect IL-6r.

V. DISCUSSION

MTT assay and apoptosis induction.

Cell cycle phase arrest

Detection of IL-6 and IL-6r in breast cancer cell lines

VI. CONCLUSION

Future work and Recommendation

REFERENCES

APPENDICES

BIODATA OF THE AUTHOR
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1</td>
<td>Diagram depicting an example of the arrangement of blanks, standards and samples in the microwell strips.</td>
<td>49</td>
</tr>
<tr>
<td>4.1</td>
<td>The IC$_{50}$ value of MCF-7, MDA-MB-231 and MCF-10A</td>
<td>55</td>
</tr>
</tbody>
</table>
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>: <em>Alpinia zerumbet</em> and fruits of <em>Alpinia zerumbet</em></td>
<td>19</td>
</tr>
<tr>
<td>2.2</td>
<td>: Molecular structure of Flavokawin B</td>
<td>19</td>
</tr>
<tr>
<td>2.4</td>
<td>: Molecular structure of Tamoxifen citrate</td>
<td>21</td>
</tr>
<tr>
<td>2.5</td>
<td>: Mechanism of action of Tamoxifen citrate</td>
<td>22</td>
</tr>
<tr>
<td>4.1</td>
<td>: A representative of dose response curve of MCF-7 cell line.</td>
<td>56</td>
</tr>
<tr>
<td>4.2</td>
<td>: A representative of dose response curve of MDA-MB-231 cell line.</td>
<td>57</td>
</tr>
<tr>
<td>4.3</td>
<td>: A representative of dose response curve of MCF-10A cell line.</td>
<td>58</td>
</tr>
<tr>
<td>4.4</td>
<td>: Morphology changes of MCF-7 cell line after treatment 24 hours with Flavokawin B and Tamoxifen</td>
<td>61</td>
</tr>
<tr>
<td>4.5</td>
<td>: Morphology changes of MCF-7 cell line after treatment 48 hours with Flavokawin B and Tamoxifen</td>
<td>62</td>
</tr>
<tr>
<td>4.6</td>
<td>: Morphology changes of MCF-7 cell line after treatment 72 hours with Flavokawin B and Tamoxifen</td>
<td>63</td>
</tr>
<tr>
<td>4.7</td>
<td>: Morphology changes of MDA-MB-231 cell line after treatment 24 hours with Flavokawin B and Tamoxifen</td>
<td>64</td>
</tr>
<tr>
<td>4.8</td>
<td>: Morphology changes of MDA-MB-231 cell line after treatment 48 hours with Flavokawin B and Tamoxifen</td>
<td>65</td>
</tr>
<tr>
<td>4.9</td>
<td>: Morphology changes of MDA-MB-231 cell line after treatment 72 hours with Flavokawin B and Tamoxifen</td>
<td>66</td>
</tr>
<tr>
<td>4.10</td>
<td>: Confocal micrographs of MCF-7 cell line treated with Flavokawin B and Tamoxifen at their IC₅₀ concentrations at 24 hours post-treatment.</td>
<td>68</td>
</tr>
</tbody>
</table>
4.11: Confocal micrographs of MCF-7 cell line treated with Flavokawin B and Tamoxifen at their IC$_{50}$ concentrations at 48 hours post-treatment.

4.12: Confocal micrographs of MCF-7 cell line treated with Flavokawin B and Tamoxifen at their IC$_{50}$ concentrations at 72 hours post-treatment.

4.13: Confocal micrographs of MDA-MB-231 cell line treated with Flavokawin B and Tamoxifen at their IC$_{50}$ concentrations at 24 hours post-treatment.

4.14: Confocal micrographs of MDA-MB-231 cell line treated with Flavokawin B and Tamoxifen at their IC$_{50}$ concentrations at 48 hours post-treatment.

4.15: Confocal micrographs of MDA-MB-231 cell line treated with Flavokawin B and Tamoxifen at their IC$_{50}$ concentrations at 72 hours post-treatment.

4.16: Mean number of apoptotic cells in control and treated MCF-10A cell line.

4.17: TUNEL micrographs of MCF-10A cell line treated with Flavokawin B and Tamoxifen at 24 hours post treatment.

4.18: TUNEL micrographs of MCF-10A cell line treated with Flavokawin B and Tamoxifen at 48 hours post treatment.

4.19: Mean number of apoptotic cells in control and treated MCF-7 cell line.

4.20: TUNEL micrographs of MCF-7 cell line treated with Flavokawin B and Tamoxifen at 24 hours post treatment.

4.21: TUNEL micrographs of MCF-7 cell line treated with Flavokawin B and Tamoxifen at 48 hours post treatment.

4.22: Mean number of apoptotic cells in control and treated MDA-MB-231 cell line.


4.26: Cell cycle analysis of MCF-10A treated with Tamoxifen at 24-hour post treatment.

4.27: Cell cycle analysis of MCF-7 treated with Flavokawin B at 24-hour post treatment.

4.28: Cell cycle analysis of MCF-7 treated with Tamoxifen at 24-hour post treatment.


4.30: Cell cycle analysis of MCF-MB-231 cell line treated with Tamoxifen at 24-hour post treatment.

4.31: A representative of IL-6 levels determination in MCF-10A cell lines treated with Flavokawin B.

4.32: A representative of IL-6 levels determination in MCF-10A cell line treated with Tamoxifen.

4.33: A representative of IL-6 levels determination in MDA-MB-231 line treated with Flavokawin B.

4.34: A representative of IL-6 levels determination in MDA-MB-231 line treated with Tamoxifen.

4.35: Immunofluorescence micrographs of MCF-10A cell line treated with Flavokawin B at concentrations IC_{20} (B), IC_{50} (C) and IC_{80} (D) at 24-hour post treatment.

4.36: Immunofluorescence micrographs of MCF-10A cell line treated with Tamoxifen at concentrations IC_{20} (B), IC_{50} (C) and IC_{80} (D) at 24-hour post treatment.

4.37: Immunofluorescence micrographs of MCF-7 cell line treated with Flavokawin B at concentrations IC_{20} (B), IC_{50} (C) and IC_{80} (D) at 24-hour post treatment.
4.38: Immunofluorescence micrographs of MCF-7 cell line treated with Tamoxifen at concentrations IC_{20} (B), IC_{50} (C) and IC_{80} (D) at 24-hour post treatment.

4.39: Immunofluorescence micrographs of MDA-MB-231 cell line treated with Flavokawin B at concentrations IC_{20} (B), IC_{50} (C) and IC_{80} (D) at 24-hour post treatment.

4.40: Immunofluorescence micrographs of MDA-MB-231 cell line treated with Tamoxifen at concentrations IC_{20} (B), IC_{50} (C) and IC_{80} (D) at 24-hour post treatment.
## LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>FCS</td>
<td>Fetal Calf Serum</td>
</tr>
<tr>
<td>IC</td>
<td>Inhibition Concentration</td>
</tr>
<tr>
<td>MTT</td>
<td>Microculture Tetrazolium Assay</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin 6</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>rpm</td>
<td>rotation per minute</td>
</tr>
<tr>
<td>SPSS</td>
<td>Statistical Package for Social Sciences</td>
</tr>
<tr>
<td>ER</td>
<td>estrogen receptor</td>
</tr>
<tr>
<td>AO</td>
<td>Acridine orange</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulphoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoksiribonucleic acid</td>
</tr>
</tbody>
</table>
Cancer is a genetic disease that undergoes clonal evolution of transformed cells that arise through the accumulation of mutations; either inherited (germline) or acquired (somatic), in critical proto-oncogenes and tumour suppressor genes. Carcinogens may be chemical, physical or biological in nature and interacts directly or indirectly with DNA and they are ubiquitous.

In countries such as Europe, USA, Canada, South America, breast cancer represents 25–30% of the total incidence of cancers in women and accounts for 15–18% mortality. The risk of a woman developing breast cancer during her lifetime is 1 in 8 in the United States, 1 in 12 in the European Community and 1 in 80 in Japan. Two-thirds of breast cancers are detected in postmenopausal women. Most breast cancers (about 95%), whether in pre- or postmenopausal women, are initially hormone-dependent, where the hormone estradiol plays a crucial role in their development and progression. The hormone and estrogen receptor (ER) complex can mediate the activation of proto-oncogenes and oncogenes (Pasqualini, 2004).

There are several new approaches towards cancer therapy. Breast cancer is estrogen responsive and is treated by hormonal therapy using Tamoxifen, an
anti estrogenic drug. Anti-cancer drugs used in chemotherapy, destroy cancer
cells and these drugs work by interfering with the ability of cancer cells to
divide and reproduce itself. The affected cells thus become damaged and
eventually die. Unfortunately, most chemotherapeutic drugs also affect normal
cells. The traditional approach in cancer therapy aimed at improving the overall
survival of metastatic breast cancer include multiple lines of non-cross-resistant
hormonal therapies, increasing the duration, the dose, and the dose intensity of
chemotherapy, the use of non-cross-resistant polychemotherapy, and the
addition of maintenance hormonal therapy. Thus far, the results have not been
very rewarding despite prolongation of time to progression, improvements in
overall survival were difficult to obtain, suggesting that these strategies do little
to alter the natural history of breast cancer once it has metastasized (Awada, et
al., 2003).

The scientific evidence that plant based diets, in particular those rich in
vegetables and fruits, protect against cancers of various sites has been found to
be strong and consistent (Marchand, 2002). Flavonoids, which are structurally
similar to estrogens, are able to bind to the estrogen receptor and possess either
estrogenic or anti-estrogenic activities (Bail, et al., 1998).

Elimination of tumour cells by the induction of apoptosis has become an
important and new approach in cancer therapy. Apoptosis known as genetically
programmed physiological form of cell death is not only involved in the
development of tumours but also plays an essential role in their treatment (Noteborn, et al., 1998). Most of these bioactive substances exert their cancer chemotherapeutic activity by blocking cell cycle progression and triggering apoptotic cell death. Therefore, induction of apoptosis in tumour cells has become an indicator of the tumor treatment response in employing a plant derived-bioactive substance to reduce and control human mortality due to cancer (Smets, 1994; Paschka, et al., 1998).

Recently natural plant researches have been contributing to drug innovation by providing plant derived anti-cancer agents. Since, nature has been provided with many effective anticancer agents, clinical plant based research has made progress in anticancer therapies (De Smet, 1997).