



UNIVERSITI PUTRA MALAYSIA

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

AJANTHA SINNI AH

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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,
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By

AJANTHA SINNIAH

MARCH 2005

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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} (\pm S.E.M) value of Flavokawin B on MCF-7 cell line was determined to be $11.5 \pm 0.015 \mu\text{M/ml}$ whilst the IC_{50} with Tamoxifen was at $10.2 \pm 0.012 \mu\text{M/ml}$. The IC_{50} value of Flavokawin B on MDA-MB-231 cell line was determined to be $17.5 \pm 0.019 \mu\text{M/ml}$ whilst the IC_{50} value



of Tamoxifen was at $32.5 \pm 4.2 \mu\text{M/ml}$. The MTT assay results on normal epithelial cell line, MCF-10A treated with Flavokawin B demonstrated that the IC_{50} value was $38.0 \pm 0.032 \mu\text{M/ml}$ whereas MCF-10A treated with Tamoxifen had an IC_{50} value of $28 \pm 0.021 \mu\text{M/ml}$. All values were statistically significant ($p < 0.05$), as analysed using one sample T-test. The breast cancer cell lines treated at IC_{50} 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.



Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia
sebagai memenuhi keperluan untuk ijazah Master Sains

**KAJIAN ANTI-TUMORIGENIK FLAVOKAWIN B KE ATAS SEL-
SEL SELANJAR PAYUDARA MCF-7 DAN MDA-MB-231**

Oleh

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Sebatian semulajadi Flavokawin B yang diasingkan dan ditulinkan daripada ekstrak *Alpinia zerumbet* telah dikaji bagi menentukan fungsi sebagai antikanser terhadap sel-sel selanjara 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 selanjara epithelial payudara digunakan sebagai kawalan negatif. Asai MTT digunakan untuk mengkaji kesan sitotoksik rawatan. Keputusan menunjukkan bahawa nilai IC_{50} (\pm S.E.M) untuk rawatan Flavokawin B ke atas sel selanjara MCF-7 ditentukan sebagai $11.5 \pm 0.015 \mu\text{M/ml}$ sementara nilai IC_{50} bagi rawatan Tamoxifen ialah $10.2 \pm 0.012 \mu\text{M/ml}$. Nilai IC_{50} bagi Flavokawin B ke atas sel selanjara MDA-MB-231 ditentukan sebagai $17.5 \pm 0.019 \mu\text{M/ml}$



sementara nilai IC_{50} bagi rawatan Tamoxifen ialah $32.5 \pm 4.2 \mu\text{M/ml}$. Keputusan asai MTT ke atas sel selanjar MCF-10A menunjukkan nilai IC_{50} bagi Flavokawin B ialah $38.0 \pm 0.032 \mu\text{M/ml}$ sementara nilai IC_{50} bagi rawatan Tamoxifen ialah $28 \pm 0.021 \mu\text{M/ml}$. Semua nilai IC_{50} adalah signifikan setelah dianalisis menggunakan satu sampel T-test ($P < 0.05$). Kajian mikroskop konfokal diteruskan bagi semua sel-sel selanjar dirawat dengan Flavokawin B dan Tamoxifen pada kepekatan IC_{50} masing-masing. Tiada perubahan yang signifikan dilihat pada kumpulan kawalan. Walaubagaimanapun, ciri-ciri apoptosis telah dilihat seperti pengembangan 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 selanjar MCF-10A berbanding dengan rawatan Tamoxifen. Tahap IL-6 bagi sel selanjar MDA-MB-231 berkurangan 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 selanjar MCF-7 dan MDA-MB-231



ditahan pada fasa G1 apabila dirawat dengan Flavokawin B dan Tamoxifen. Ini menunjukkan bahawa kedua-dua rawatan mempunyai mekanisma yang sama untuk menahan sel pada fasa tersebut. Kesimpulannya, sebatian semulajadi Flavokawin B merangsang apoptosis ke atas sel-sel selanjara MCF-7 dan MDA-MB-231 membawa kepada penemuan rawatan alternatif baru bagi rawatan kanser payudara.



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LIST OF ABBREVIATIONS

FCS	Fetal Calf Serum
IC	Inhibition Concentration
MTT	Microculture Tetrazolium Assay
IL-6	Interleukin 6
PBS	Phosphate Buffered Saline
rpm	rotation per minute
SPSS	Statistical Package for Social Sciences
ER	estrogen receptor
AO	Acridine orange
PI	Propidium iodide
ATCC	American Type Culture Collection
DMSO	Dimethyl sulphoxide
DNA	Deoksiribonucleic acid



CHAPTER 1

INTRODUCTION

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).

