UNIVERSITI PUTRA MALAYSIA

ELUCIDATION OF BASIC MECHANISMS OF FLAVOKAWIN B INHIBITORY EFFECTS ON THE GROWTH OF SELECTED CANCER AND TRANSFORMED NORMAL CELL LINES

NASIR TSAFE UMAR

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BY

NASIR TSAFE UMAR

Thesis submitted in fulfilment of the requirements for the Degree of Master of Science in the Faculty of Medicine and Health Sciences Universiti Putra Malaysia

May 2008

DEDICATION

To all the de-humanised, oppressed,

starving, sub-dued and suppressed

peasants of the world.

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

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Chair: Associate Professor Dr Rozita Rosli, PhD

Faculty: Medicine and Health Sciences

Flavokawin B (FNB) is a hydroxychalcone isolated from a local plant species, Alpinia zerumbet of the Zingiberaceae family. It has been shown to have anti-oxidant and pro-immune properties. The aim of the present study was to assess the basic growth inhibitory mechanisms of FNB, on 21 cell lines, comprising tumour and transformed cell lines. The cells were treated with FNB at 0.1, 1, 3, 10, 30 and 100 µM. Over 3 to 4 days exposure, FNB selectively inhibited the growth of all the cell lines. Tumour cells were more sensitive to FNB than normal cells. MTT cytotoxicity assay was then conducted in order to assess the cytotoxic-indices of FNB. Caco-2, CEM-SS, MCF-7, T-47D and U87MG were found to be the five most sensitive cell lines, with IC50 values of 6.53 ± 0.81, 2.00 ± 0.41, 7.67 ± 0.71, 8.67 ± 1.40 and 14.00 ± 3.00 µM respectively. Tamoxifen (TMX) was used as a positive control for the 5 cell lines, with all the IC50 values found to be comparable to that of FNB. Cell survival analysis confirmed very significant patterns of FNB efficacy

(P<0.001 - P<0.05), in comparison to the untreated/negative controls in all the 5 cell lines. Apoptosis induction was then assessed by life-culture morphology in all the 21 cell lines, out of which 9 indicated apoptosis induction. Nucleoprotein fluorescence analysis was carried out in order to quantify and establish apoptotic frequencies. Combined image captures were used to analyse the apoptotic effects. FNB was found to induce apoptosis at the IC50 concentrations in 9 of the 21 cell lines. The most significant FNB-induced apoptotic frequencies compared to the untreated controls, were found at 70.67 ± 7.51% (P<0.01), 68.17± 6.81% (P<0.01), 49.33 ± 7.32% (P<0.01), 57.5 ± 4.82% (P<0.01) and 52.83 ± 3.62% (P<0.001), for the Caov-3, CEM-SS, CHO, HL-60 and MDA-MB-468 cell lines, respectively. The maximal apoptotic frequency effect induced by FNB was on the Caov-3 cell line, which was more significant than etoposide (ETS) positive control (P<0.05). Apoptosis induction was confirmed only in the 5 most significant (P≤0.01) cell lines, using agarose gel electrophoresis for DNA-laddering. The effect of FNB on oestrogen metabolism at IC50 concentrations, was tested using radioisotope enzymatic assays for oestrone sulphatase (E1STS) and oestradiol-17β hydroxysteroid dehydrogenase (oestrone → oestradiol, E2HD), on four selected human breast cancer cell lines (MCF-7, T-47D, MDA-MB-231, MDA-MB-468). FNB had significant inhibitory effects on the E1STS enzyme in the MCF-7 and T-47D cell lines, with 26.41 ± 0.69% (P < 0.01) and 18.53 ± 1.21% (P < 0.05), respectively at IC50 levels. FNB also significantly inhibited the E2HD enzyme in the T-47D cell line (36.40 ± 1.70%, P < 0.01). Confirmatory assays using the E1STS and E2HD ELISA kits were conducted on the MCF-7 and T-47D cell lines. Similar inhibitory effects of FNB on both enzymes were found in the MCF-7 and T-47D cell lines. On the other hand, FNB stimulated both enzymes in the non-oestrogen dependent cell lines, MDA-MB-

231 and MDA-MB-468. Finally, genotoxicity study was conducted in order to establish a safety profile of FNB in the CHO normal cell line, using ethylmethanesulphonate (EMS) as a positive control. Antigenotoxicity was assayed using a combination of FNB and EMS. The results showed an insignificant FNB clastogenicity ($P>0.05$, at 45 μM) and a significant FNB anti-clastogenicity ($P<0.05$, at 45 μM), in the CHO cells. In conclusion, the overall cytotoxicity, selectivity, cell survival, apoptotic, anti-oestrogenic, non-genotoxic and anti-genotoxic properties of flavokawin B (which are comparably better than TMX, ETS and EMS), forms the basic inhibitory mechanisms, which make this compound potentially an interesting anti-neoplastic agent.

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

PENJELASAN MEKANISMA-MEKANISMA ASAS KESAN-KESAN PERENCETAN FLAVOKAWIN B KE ATAS PERTUMBUHAN TITISAN-TITISAN SE KANSER DAN TERTRANSFORMASI YANG TERPILIH

Oleh

NASIR TSAFE UMAR

Mei 2008

Pengerusi: Associate Professor Dr Rozita Rosli, PhD

Fakuti: Perubatan dan Sains Kesihatan

Flavokawin B (FNB) adalah kumpulan hydroxychalcone yang dipencilkan daripada spesis pokok tempatan, Aplinia zerumbet dalam keluarga Zingiberaceace. FNB telah dikenalpasti mempunyai ciri-ciri anti-oksida dan pro-imun. Kajian ini bertujuan untuk menilai mekanisma perencatan pertumbuhan asas oleh FNB, ke atas 21 titisan sel yang terdiri daripada tumor manusia, dan manusia tertransformasi. Sel-sel diperlakukan dengan FNB (0.1, 1, 3, 10, 30 and 100 μM). Berikutan pendedahan pada ketoksikan piawai selama 3-4 hari, FNB merencat pertumbuhan titisan-titisan sel tersebut secara selektif. Sel-sel tumor lebih sensitif terhadap FNB berbanding sel-sel normal. Asai sitotoksik MTT kemudian dijalankan untuk menentukan indeks sitotoksik bagi FNB. Caco-2, CEM-SS, MCF-7, T-47D dan U87MG merupakan 5 titisan sel yang paling sensitif dengan nilai IC50 6.53 ± 0.81, 2.00 ± 0.41, 7.67 ± 0.71, 8.67 ± 1.40 dan 14.00 ± 3.00 uM masing-masing. Tamoksifen (TMX) telah digunakan sebagai kawalan positif.

Analisis survival sel menentukan corak keberkesanan FNB yang sangat signifikan ($P < 0.001$ – $P<0.05$), dalam kelima-lima titisan sel. Aruhan apoptosis kemudian dinilai menggunakan morfologi kultur-hidup. Analisis floresen nukleoprotin telah dijalankan untuk mengira kuantiti dan menentukan frekuensi apoptotik. Gabungan imej-imej telah digunakan untuk menganalisa kesan apoptosis. FNB didapati telah mengaruh apoptosis pada kepekatan IC50 dalam 9 daripada 21 titisan sel. Frekuensi apoptosis aruhan FNB yang paling signifikan berbanding kawalan tanpa perlakuan adalah 70.67 ± 7.51% ($P<0.01$), 68.17± 6.81% ($P<0.01$), 49.33 ± 7.32% ($P<0.01$), 57.5 ± 4.82% ($P<0.01$), 52.83 ± 3.62% ($P<0.001$), masing-masing bagi Caov-3, CEM-SS, CHO, HL-60 dan MDA-MB-468. Kesan frekuensi apoptosis yang maksima adalah pada titisan sel Caov-3, yang mana lebih signifikan berbanding etoposid (ETS) sebagai kawalan positif ($P<0.05$). Aruhan apoptosis hanya ditentukan pada 5 titisan sel paling signifikan ($P\leq0.01$), menggunakan elektroforesis agaros untuk tetangga-DNA. Kesan FNB ke atas metabolisma estrogen pada kepekatan IC50 telah diuji menggunakan asai radioisotop enzimatik bagi estrone sulphatase (E1STS) dan oestradiol-17ß hydroxysteroid dehydrogenase (oestrone → oestradiol, E2HD), dalam 4 titisan sel terpilih (MCF-7, T-47D, MDA-MB-231 and MDA-MB-468). FNB mempunyai kesan perencatan signifikan ke atas enzim E1STS dalam titisan sel MCF-7 dan T47-D, 26.41 ± 0.69% ($P<0.01$) dan 18.53 ± 1.21% ($P<0.05$) masing-masing. FNB juga merencat secara signifikan enzim E2HD dalam titisan sel T-47D (36.40 ± 1.70%, $P<0.01$). Asai penentuan menggunakan kit ELISA E1STS dan E2HD telah dijalankan ke atas titisan sel MCF-7 dan T-47D. Kesan perencatan yang serupa ditemui ke atas kedua-dua enzim dalam titisan sel MCF-7 dan T-47D. Sebaliknya, FNB telah merangsang kedua-dua enzim dalam titisan sel

bersandar bukan-estrogen, MDA-MB-321 dan MDA-MB-468. Akhirnya, kajian genotoksik telah dijalankan bagi profil keselamatan dalam titisan sel normal CHO, dengan menggunakan ethylmethanesulphonate (EMS) sebagai kawalan positif. Asai antigenotoksik adalah secara gabungan FNB dan EMS. Ujian menunjukkan kesan klastogenik yang tidak signifikan ($P>0.05$, dengan 45 μM), dan anti-genotoksik yang signifikan ($P<0.05$, dengan 45 μM). Kesimpulannya, secara keseluruhan ciri-ciri sitotoksik, selektif, survival, apoptotik, anti-estrogenik, non-genotoksik dan anti-genotoksik oleh flavokawin B (secara bandingannya lebih baik daripada TMX, ETS dan EMS) menjadikan sebatian ini berpotensi sebagai agen anti-neoplastik yang menarik.

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I certify that an Examination Committee has met on 15 May 2008 to conduct the final examination of Nasir Tsafe Umar on his Master of Science thesis entitled "Elucidation of Basic Mechanisms of Flavokawin B Inhibitory Effects on the Growth of Selected Cancer and Transformed Normal Cell Lines" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the student be awarded the Master of Science.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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DECLARATION

I hereby declare that the thesis is based on my original work except for 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.

Signature

NASIR TSAFE UMAR

Date : 15 May 2008

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

Abbreviation	:	Full Term
%CV	:	Percentage coefficient of variation
%Ec	:	Percentage counting efficiency
%Rv	:	Percentage recovery value
α	:	Alpha
β		Beta
δ		Delta
µ	:	Micro
µg/L	:	Microgram per litre
µg/ml	:	Microgram per millilitre
µl	:	Microlitre
µM	:	Micromolar
^{14}C	:	Carbon-14
$^{14}CE_2$	:	Carbon-14-oestradiol
^{3}H	:	Tritium
^{3}H-^{14}C	:	Tritiated-carbon-14
$^{3}H\_^{14}C\_Xv$	:	Specific activity value of tritiated-carbon-14
$^{3}HE_1$	:	Tritiated-oestrone
$^{3}H\_Xv$	:	Tritiated specific activity value
ACS	:	American Cancer Society
AGE	:	Agarose gel electrophoresis
AO	:	Acridine orange
ATCC	:	American type culture collection

Avrg.	:	Average
Bax	:	An oncogene involved in apoptosis/cell growth regulation
Bcl-2	:	An oncogene involved in apoptosis/cell growth regulation
Bcl-W	:	A factor involved in apoptosis/cell growth regulation
Bcl-XL	:	A factor involved in apoptosis/cell growth regulation
Bcl-XS	:	A factor involved in apoptosis/cell growth regulation
BHC	:	Biohazard cabinet
bp	:	Base pair
BQN	:	Benzoquinone
CA	:	Chromosome aberration
CCO	:	Corrected carry over
CGM	:	Complete growth medium
CI	:	Confidence interval
CLC	:	Chalcone
CnDF	:	Cell number dilution factor
CO_2	:	Carbon dioxide
contd		Continued
cpm	:	counts per minute
CRC	:	Corrected reference count
DAPI	:	4',6-diamidino-2-phenylindole
DF	:	Dilution factor
DMEM	:	Dulbecocco's modified Eagle's medium
DPH	:	Diphenylheptone
dpm	:	Disintegrations per minute
DTP	:	Diterpene

E_1DF	:	Oestrone-counts dilution factor
E_1S	:	Oestrone sulphate
E_1STS	:	Oestrone sulphatase
E_2	:	Oestradiol
E_2DF	:	Oestradiol-counts dilution factor
E_2HD	:	Oestradiol-17β hydroxysteroid dehydrogenase
E_2HSD		Oestradiol-17β hydroxysteroid dehydrogenase
E_3	:	Oestriol
ECACC	:	European collection of (Applied) cell cultures
EDTA	:	Ethylene diamine tetra acetic acid
ELISA	:	Enzyme-linked immunosorbent assay
EMS	:	Ethylmethanesulphonate
ER	:	Oestrogen receptor
ETS	:	Etoposide
FAS	:	A surface protein for apoptosis (programmed cell death)
Fas-L	:	A ligand surface protein for apoptosis (programmed cell death)
FBS	:	Foetal bovine serum
FCS	:	Foetal calf serum
FITC	:	Fluorescein isothiocyanate
FLV	:	Flavone/flavonol
fmol	:	Femtomole(s)
FNB	:	Flavokawin B
G	:	Gram
H	:	Hour(s)
HEPES	:	4-2-hydroxyethyl-1-piperazineethanesulphonic acid

HF-12	:	Ham's F-12
HPH	:	Hydroxyphenylheptone
i.e.	:	That is
IARC	:	International agency for research on cancer
IC	:	Interphase cells
IC_{50}	:	50 % inhibitory concentration of cell growth
IMDM	:	Iscove's modified Dulbecocco's medium
Kb	:	Kilobase
KPN	:	Kavapyrone
LL-15	:	Leivobitz's L-15
M	:	Molar
MC	:	Mitotic cells
Mc5A	:	McCoy's 5A
Mcl-1	:	A factor involved in apoptosis/cell growth regulation
MEGM	:	Mammary epithelial growth medium
MEME	:	Minimum essential medium Eagle's
mg/ml	:	Milligram per millilitre
MgCl2	:	Magnesium chloride
MI	:	Mitotic index
MIC_{100}	:	100 % of control mitotic index
MIC_{25}	:	25 % of control mitotic index
MIC_{50}	:	50 % of control mitotic index
Ml	:	Millilitre
mRNA	:	Messenger ribonucleic acid
MTD	:	Maximum tolerative dose

MTP	:	Moneterpene
MTT	:	Methylthiazoletetrazolium
MW	:	Molecular weight
Na	:	Sodium
NCI	:	National cancer institute
ng/ml	:	Nanogram per millilitre
Nm	:	nanometre
OECD	:	Organisation for economic corporation and development
P	:	Probability (statistical)
p53	:	A tumour suppressor gene involved in apoptosis/cell growth signal
PBS	:	Phosphate buffered saline
PCN	:	Proanthocynidine
pg/ml	:	Picogram per millilitre
PI	:	Propidium iodide
pMB	:	Promyeloblast
pMC	:	Promyelocytic
PNL	:	Phenolic
ppm		Parts per million
PR	:	Progesterone receptor
pS2		An Oestrogen inducible gene
rpm	:	Revolutions per minute
RPMI	:	Rosewell park memorial institute
SD	:	Standard deviation
SEM	:	Standard error of mean
SQP	:	Sesquiterpene

STDEV	:	Standard deviation
TC	:	Tissue culture
TtC	:	Total cells
TGF	:	Transforming growth factor
TLC	:	Thin layer chromatography
TMX	:	Tamoxifen
TNF	:	Tumour necrosis factor
TNFR	:	Tumour necrosis factor receptor
TNFα	:	Tumour necrosis factor-Alpha
TNFβ	:	Tumour necrosis factor-Beta
TRITC	:	Tetramethyl rhodamine isothiocyanate
UK	:	United kingdom
US	:	United States
USA	:	United States of America
UV	:	Ultraviolet
V	:	Voltage (volt)
v/v	:	Volume/volume
w/v	:	Weight/volume

1 INTRODUCTION

Plants provide an unlimited source of novel and complex chemical structures isolated as bioactive compounds, which could be developed continuously as therapeutic agents, with the goal of direct use as drugs (Fabricant and Farnsworth, 2001). Flavokawin B (FNB, section 2.2.4) is one such compound, a hydroxychalcone purified from a local herb, *Alpinia zerumbet (formerly known as A. nutans)*. The compound also exists in other species of the Zingiberaceae family, such as *A. japonica*, *A. mutica* and *A. rafflesiana* (Habsah, 2002) among others.

A. zerumbet is a perennial, erect herb, with numerous leafy stems usually up to 2 metres tall (Habsah, 2002), occurs widely and mainly in secondary vegetation of bamboo and teak forest under shady conditions of lowlands and hill slopes, throughout Southeast Asia. Locally named *lengkuas hutan*, this plant like other Zingiberaceae species is used as a component of spices, medicines, flavouring agents and as dye-sources (Burkill, 1966). In Malaysia, the *Alpinia* genus is one of the major ingredients of a traditionally prepared 'tonic' called *Jamu*. In northern Brazil however, the aqueous extract of *A. zerumbet* is traditionally used as a sedative (Dias and Takahashi, 1994). Its sister species (*A. speciosa*) is used as a diuretic to control hypertension (Mendonca *et al* 1991). As substantial 'germplasm' of *A. zerumbet* are not yet reported to exist (Oyen and Dung, 1999), this species is now being cultivated by the Laboratory of Phytomedicines and the Plant Genetic Centre, Institute of Bioscience, Universiti Putra Malaysia. Previous studies on *A. zerumbet* have so far only been reviewed and reported for its crude extract effects of anti-microbial, anti-oxidant (Habsah *et al* 2000) and anti-tumour promoting activity (Mackeen *et al* 2000; Habsah, 2002). Further investigations on *A. zerumbet* using bioactivity-guided

fractionation have yielded four compounds that were isolated and found to have antioxidant activities, with FNB as one of the most active (Habsah, 2002). It has been recently reported that FNB possess pro-immune properties of inhibiting nitrogen oxide production in macrophages *in vitro* (Syahida *et al* 2006), being a non-toxic kava-herbal component in rats (Disilvestro *et al* 2007) and as a strong antioxidant (Elzaaweley *et al* 2007).

Cytotoxicity is the degree of harmfulness that causes damage, acute or eventual lethality at general or specific cellular levels. Cytotoxicity is established on the basis of the ability of cells to survive a toxic insult *in vitro* or *in vivo* (Dean and Danford, 1984; Butler and Dawson, 1992; Roper, 1994). It is a destruction capacity that produces structural or functional change in the target cell, which is potentially harmful to the cells or its descendants (Lewis and Besterman, 1998). The general principle of cytotoxicity assays is based on the assessment of a special characteristic of most cytotoxic agents (such as drugs, hormones, nutrients and irradiation). These agents exert cell killing in a manner which may be non-specific, but selective towards cellular proliferative activities, often with acute death, cell cycle blockages and other specificities (Darzynkiewicz *et al* 1995; Skehan, 1995). In other words, these assays are based on the principle that cytotoxic agents inhibit mammalian cell division in culture, so that at effective concentrations of the agent in the culture medium, cells plated in sparse culture, will not grow to confluency in the wells of a microtitre tray (Shier, 1991).

The phrase 'cytotoxicity assay' has traditionally been used to describe methods of measuring the intensity of death resulting from treatment with compounds that cause

cell death (Freshney, 1987; Wilson, 1992). Measurement of toxicity *in vitro* is a purely cellular event that can only mimic the complex pharmaco-kinetics of drug exposure through clearance and excretion (Freshney, 1994). Although it is possible to stimulate most of the *in vivo* parameters (including complex tissue and systemic reactions), most *in vitro* studies concentrate solely on a direct cellular response, thereby gaining the simplicity to be measured by changes in cell survival, metabolism and other effects. In essence therefore, depending on the parameter in question, numerous cytotoxicity assays are widely available for applications to both normal and cancer cells *in vitro* and *in vivo*.

Cancer is a dreaded disease that is due to what is generally understood to be constituent 'mutational' activations, that leads to the 'loss of control' of the network of activities, regulating normal 'life and death' pattern of an individual's own cells (characterised by net tissue expansion), in a particular part of the body (Lowe, 1996; Vogelstein and Kinzler, 1998). Walt Kelly, cited in Pratt *et al* (1994), described cancer as 'an enemy within'.

'Apoptosis', a Greek word originally describing the dropping of leaves from trees (Sluyser, 1996), which was first defined by Kerr and co-authors (Kerr *et al* 1972), is described as a normal, programmed and actively specialised process or mode of cell killing, that occurs in a similar way as normal growth, through proliferation and differentiation, featured by original tissue-localisation (*in vivo*), to becoming eventually contagious, *in vitro* (Wyllie *et al* 1980). In apoptosis, single cells are deleted in the midst of normal living tissue, within an adherent or suspension cell populations, *in vitro* (Kerr *et al* 1994).

Apoptosis, alias ‘suicide’, ‘rescue’, or ‘honour’ death, has been understood to result in all normal cellular developments, for the survival of the neighbourhood populace (Fesus, 1991). Apoptosis is implicated in the steady-state kinetics of healthy adult tissues and accounts for focal deletion of cells, where it serves to clear expired cells during normal embryonic development and metamorphosis (Wyllie *et al* 1980). It is responsible for the fashioning of many tissue structures such as digits-shaping (spaces between our fingers and toes at the very early stage of foetal development) and the hollow cavity development and completion of fusion processes.

The spaces between our fingers and toes, at the very early stage of foetal development, the metamorphosis of tadpole tail to frog, the surplus cell eliminations in the neuron-brain connections or synapses, and the sloughing off of the inner lining of the uterus (endometrium), at the beginning of every regular menstrual process, are all understood to occur by apoptosis. It is an agent of physiological death in normal tissue turnover, and also provides the accomplishing criteria for ‘natural selection’ of the immune system (Wyllie, 1985; 1992; Gregory, 1996).

While cancer is said to result out of abnormal cell regulation, apoptosis being a natural cell homeostatic tool or end-point is a genetically controlled system, which operates through a `network’ or ‘cascade’ of factors. In this process, unrepaired or mis-repaired cell damages are the major, committing and critical determinants that trigger the normal but induced cell death process, sparing the neighbourhood cell populace. The apoptotic homeostasis feature in particular, is what makes apoptosis a very important process that plays a vital role in the kinetics of tumour survival and death (Wyllie,

1992; Lavin and Watters, 1993; Sluyser, 1996; Gregory, 1996), to such an extent that its effective presence basically means cancer suppression and its absence translates in to cancer cell survival. A major problem that appears to be the most serious of all is the prevalence of cancer in terms of 'resistance to treatment' and or 'metastasis'. Considering both genetic and non-genetic factors on the aetiology of cancer, the biggest problem, however, dramatically remains 'nobody is above cancer' and that the exact cause of many cancers remain largely unknown.

It has now been fully reviewed that the general understanding of the regulation of the molecular machinery of apoptosis *in vitro,* has witnessed major advances, in which molecules linking proliferation and apoptosis in healthy cells are being identified, as the apoptotic cell death provides the 'fail-safe' mechanism to counteract excess proliferation. Furthermore, since tumour specificity as ever, is the main issue to be resolved, inhibition of apoptosis in particular, remains a major mechanism of chemotherapeutic resistance (Makin and Dive, 2003; Duffy, 2007; Leen *et al* 2007).

Agents such as FNB that are found to induce cell death through apoptosis in multiple cell lines, are thus expected to provide contributions of paramount importance, towards the clarification of the mechanisms of drug-induced apoptosis, of tumour response. Such contributions are increasingly believed to be capable of providing renewed confidence, where therapeutic approaches based on drug targets within the apoptotic pathways will improve the treatment of cancer patients (Duffy, 2007; Leen *et al* 2007).

Oestrogen are generally understood to play an essential role in regulating the growth and differentiation of mammary gland (Lai, 2002; Reed *et al* 2005). The higher

concentrations of the biologically active oestrogen, oestradiol (E_2), have been shown to be closely related to breast cancer development in breast tumour tissue than in blood. In pre-menopausal women, nearly all the oestrogens are of ovarian origin. Yue *et al* (1998) have observed that after menopause, direct ovarian oestrogen production ceases as a result of which most oestrogens are derived exclusively from the peripheral conversion of androstenedione to oestrone (E_1) by the aromatase enzyme complex. This kind of aromatase activity was markedly observed in stromal cells around the carcinomatous glands in up to 78% of invasive breast carcinoma, where much of the E_1 formed is converted to oestrone sulphate (E_1S) by oestrone sulphotransferase (Sasano *et al* 1996). Several recent studies, have enumerated how the regulation of metabolism, in addition to the mitogenic properties of E_1STS and E_2HSD enzymes plays a chemotherapeutically vital role in the growth and death of oestrogen dependent cancer cells, like the MCF-7 and T-47D cell lines (Abraham *et al* 2006; Olofsson *et al* 2007; Stute *et al* 2007).

The description of the cell lines used in this study are as, indicated in the respective material data sheets provided by the suppliers, the American Type Culture Collections (ATCC) and the European Collections A Cell Culture (ECACC). These are: A-172 (human, brain cancer), Caco-2 (human, colon cancer), Caov-3 (human, ovarian carcinoma), CEM-SS (human, T-cell lymphoblastic leukaemia, Chang (human, transformed liver epithelium), CHO (Chinese hamster, transformed normal ovary), DU-145 (human, prostate carcinoma), HCN-2 (human, transformed glial epithelium), HeLa (human, cervical carcinoma), HepG2 (human, liver carcinoma), HL-60 (human, myelogenous leukaemia), MCF-7 (human, mammary carcinoma, positive for estrogen receptor), MCF-10A (human, transformed mammary

epithelium), MCF-12A (human, transformed mammary epithelium), MDA-MB-231 (human, mammary carcinoma, negative for estrogen receptor), MDA-MB-468 (human, mammary carcinoma, negative for estrogen receptor), PNT2 (human, transformed prostate epithelium), SK-BR-3 (human, mammary carcinoma, negative for estrogen receptor), T-47D (human, mammary carcinoma, positive for estrogen receptor), U87MG (human, Glioblastoma) and VERO (African Green Monkey, transformed normal kidney).

Since cancer is a multifactorial, dreadful disease which is common worldwide, with wide ranging implications of multistage progressive processes, treatment and metastasis, its prevention and eradication efforts therefore requires coherently systematic approaches that are as wholly as the continuous developmental strides for new or novel anti cancer agents. This is a similar view to those shared by Raina and Aggarwal (2007) and Xu, *et al* (2007), on the chemotherapeutic role of apoptosis. With such a problem statement, this study was designed with a hypothesis-driven approach in order to elucidate some of the efficacious mechanisms involved in the proliferative inhibitory potential of FNB. Up to four hypothetical pathways were identified in this study. These are the FNB induction of cytotoxicity and its selectivity patterns, the apoptosis cell death induction pathway, the anti-oestrogenicity pathway and the direct genotoxicity and anti-genotoxicity pathway. The last pathway mainly serves as the pathway for safety potential. Each of these four hypothetical pathways were elucidated using three separate experimental approaches. These are [1] the basic assays: MTT cytotoxicity (Mosmann, 1983; Monks *et al* 1991), live cell apoptosis morphology (D'Herde *et al* 2003), the radioisotopic E_1S to E_1 activity (Ng *et al* 2000; Elsadig *et al* 2001) and mitotic index

(Dean and Danford, 1984), [2] the reproducibility assays: the cytotoxic selectivity (Freshney, 1994; Zachary, 2003) , the AO/PI apoptosis morphology (Singh *et al* 1994; Singh *et al* 1996a; 1996b; Al-Rubeai and Singh, 1998; Ali *et al* 2001), the reductive $E_1 \rightarrow E_2$, coupled with the oxidative $E_2 \rightarrow E_1$ activities (Wong *et al* 2001) and the clastogenic indices modulation (Dean and Danford, 1984), [3] the confirmatory assays: cell survival (Wilson, 1992; Zachary, 2003), apoptosis DNA laddering (Wyllie *et al* 1980; Chomcyznski *et al* 1997; Ali *et al* 2001), the E_1 and E_2 ELISA assays (El-sadig *et al* 2001; Wong, 2002) and genotoxicity and anti-genotoxicty assays (Dean and Danford, 1984; Umar-Tsafe *et al* 2004).

In this research project, it has been hypothesised that due to the medicinal, the antioxidative and the pro-immune response properties of FNB, as highlighted above and reviewed in the literature review section, the compound possesses a strong anticancer cytotoxicity potential. Investigation of the basic mechanisms of its cell growth inhibitory properties, thus require hypothesis-driven objectives as follows:

(i) To evaluate the basic cytotoxic potential of FNB, through the establishment of dose-responses and IC_{50} values for each of the 21 cell lines, as well as to assess the reproducible *in vitro* cytotoxic-selectivity of FNB among the panel of 21 cancer and normal cell lines, using the NCI selective-efficacy guidelines for chemotherapeutic potential.

(ii) To analyse the confirmatory cell survival effects of FNB in the most sensitive and most significant cell lines, using standard guidelines.

(iii) To enumerate the mechanism of apoptosis (as the mode of cell death) in the cell lines that are sensitive to FNB, using basic classical morphology, quantitatively reproducible and confirmatory analysis.

(iv) To elucidate the mechanistic effect of FNB on oestrogen synthesis in four breast cancer cell lines [2 hormone-dependent (MCF-7 and T-47D) and 2 hormone-independent (MDA-MB-231 and MDA-MB-468)], along with two normal-transformed breast epithelial cell line (MCF-10A and MCF-12A).

(v) To establish the safety and mechanistic-efficacy profile of FNB in the CHO normal cell line, using the basic and reproducible MI analysis, and the confirmatory genotoxicity and anti-genotoxicity CA analysis.

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