UNIVERSITI PUTRA MALAYSIA

CYTOTOXICITY AND MECHANISMS OF ACTION OF ZERUMBONE-LOADED NANOSTRUCTURED LIPID CARRIER IN TRIPLE NEGATIVE BREAST CANCER CELLS, MDA-MB-231

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By

MAHNAZ HOSSEINPOUR

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

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DEDICATION

THIS THESIS IS DEDICATED TO

MY BELOVED HUSBAND SAMAN MOHANDESAN

MY LOVELY PARENTS AND SIBLINGS

ALL MY GREAT AND KINDHEARTED LECTURERS AND

FRIENDS

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

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April 2015

Chairman: Ahmad Bustamam Abdul, PhD
Faculty: Institute of Bioscience

Breast cancer is a life-threatening disease mostly effecting women. According to the Malaysian National Cancer Registry (2006), breast cancer is one of the five most common cancers amongst Malaysian women. Currently, the most widely used breast cancer therapies include surgery, chemotherapy, radiotherapy, hormonal therapy and targeted therapy. Generally, most of these treatments can affect normal cells and tissues with several side effects. Therefore, scientists are applying new techniques and strategies, including the use of natural products in drug discovery for enhanced survival rates with fewer side effects. Zerumbone (ZER), a monocyclic, sesquiterpene crystalline compound, was isolated from a ginger herb, *Zingiber zerumbet* (L.) Smith, locally known as *lempoyang*. Several studies had demonstrated that ZER has the ability to inhibit abnormal proliferation of neoplastic colon, cervical, ovary, liver, breast and blood cells. In addition, ZER demonstrated selective antiproliferative effect towards cancer cells. Unfortunately, ZER is poorly soluble in water that leads to poor oral bioavailability and hence, delivery. For this reason, it is crucial to improve the delivery of this compound in blood by incorporation into nanostructured lipid carrier (ZER-NLC), which increases its water solubility. This study determined and compared the cytotoxicity of ZER-NLC with ZER towards triple negative breast cancer, MDA-MB-231, cells.

The IC_{50} of ZER and ZER-NLC on MDA-MB-231 cells were determined by 3-(4, 5-dimethylthiazol-2-yl) -2, 5-diphenyltetrazolium bromide assay. Mode of cell death was determined by microscopic observation of AO/PI double stained cells, annexin-V, TUNEL, caspases assays and cell cycle analysis. In addition, Western blot was used to determine the expression of Bcl-2, Bcl-xL, Bax, cytochrome c (Cyt-c) and proliferation cell nuclear antigen (PCNA) proteins.

ZER and ZER-NLC significantly ($P < 0.05$) decreased the proliferation of MDA-MB-231 cells in a time-dependent manner. The IC_{50} of ZER and ZER-NLC on the MDA-MB-231 cells at 72 hours, were 5.96 ± 0.13 and 6.01 ± 0.11 µg/mL,

respectively. Both ZER and ZER-NLC caused development of apoptotic features in MDA-MDB-231 cells, particularly nuclear condensation, cell shrinkage, chromatin cleavage and membrane blebbing. Treatment with ZER and ZER-NLC caused MDA-MB-231 cell cycle arrest at G2/M phase. Furthermore, annexin-V and tunnel assay also demonstrated that both ZER and ZER-NLC induced apoptosis in MDA-MB-231 cells. ZER and ZER-NLC induced apoptosis via intrinsic (mitochondrial) pathway through the activation of caspase-3 and caspase-9. The western blot analysis provided evidence of up-regulation of Bax, Cyt-c with down-regulation of Bcl-2, Bcl-xL and PCNA proteins in both ZER- and ZER-NLC-treated MDA-MB-231 cells. Finally, ZER and ZER-NLC treatment did not affect the proliferation of non-cancerous breast epithelial cells (MCF-10A), suggesting that they are cancer-cell specific.

In conclusion, the current study showed that the cytotoxic effect of ZER-NLC in the induction of apoptosis similar to that of ZER. Thus, NLC can be used as a carrier for ZER in the treatment of breast cancers.

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

KESITOTOKSIKAN DAN MEKANISME TINDAKAN PEMBAWA LIPID NANOSTRUKTUR TERMUAT ZERUMBON TERHADAP SEL KANSER PAYUDARA NEGATIF GANDA TIGA, MDA-MB-231

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Kanser payudara adalah penyakit yang paling banyak berlaku kepada wanita. Mengikut *Malaysian National Cancer Registry* (2006), kanser payudara merupakan antara lima kes kanser tertinggi dalam kalangan wanita Malaysia. Di kala ini, terapi kanser payudara yang kerap diguna termasuk pembedahan, kemoterapi, radioterapi, terapi homon dan terapi sasaran. Secara umum, rawatan ini boleh mendatangkan kesan terhadap sel dan tisu normal dengan beberapa kesan sampingan. Zerumbon (ZER), suatu sebatian monosiklik, seskuiterpen hablur, telah dipencilkan daripada herba halia, *Zingiber zerumbet (L.) Smith*, yang juga dikenali sebagai lempoyang. Beberapa kajian menunjukkan ZER mempunyai keupayaan untuk menghalang pemproliferatan abnormal neoplasia kolon, serviks, ovari, hati, payudara dan sel darah. Tambahan, ZER mempamerkan kesan antipemproliferatan pilihan terhadap sel kanser. Malangnya, ZER kurang larut dalam air dan ini menyebabkan penurunan bioavailabiliti oral dan seterusnya penghantaran. Dengan sebab ini, adalah genting untuk meningkatkan penghantaran sebatian ini dalam darah dengan memuatkannya dalam pembawa lipid nanostruktur (ZER-NLC), yang meningkatkan kelarutannya. Kajian ini menentu dan membandingkan antara kesitoksikan ZER-NLC dengan ZER terhadap sel kanser payudara negatif ganda tiga, MDA-MB-231.

IC_{50} ZER dan ZER-NLC pada sel MDA-MB-231 ditentukan melalui assai 3-(4, 5-dimetiltiazol-2-yl) -2, 5-difeniltetrazolium bromide. Cara kematian sel ditentukan melalui pencerapan mikroskopi terhadap sel terwarna berganda AO/PI, assai Annexin-V, TUNEL, dan kaspase dan analisis kitaran sel. Selain itu, sap Western digunakan untuk menentukan penyataan protein Bcl-2, Bcl-xl, Bax, cytochrome c (Cyt-c) dan antigen nucleus sel pemproliferatan (PCNA).

ZER dan ZER-NLC telah mengurangkan pemproliferatan sel MDA-MB-231 *in-vitro* secara tererti ($P<0.05$) dan bersandarkan masa. IC_{50} untuk ZER dan ZER-NLC terhadap sel MDA-MB-231 pada 72 jam, masing-masing adalah 5.96 ± 0.13 dan 6.01 ± 0.11 µg/mL. Kedua-dua ZER dan ZER-NLC menyebabkan

perkembangan ciri apoptosis pada sel MDA-MB-231, terutamanya pengenapan nucleus, pengecutan sel, pembelahan kromatin dan pembleban membran. Perlakuan dengan ZER dan ZER-NLC menyebabkan hentian kitaran sel MDA-MB-231 pada fasa G2/M. Tambahan pula, assai Annexin-V dan TUNEL menunjukkan yang kedua-duanya, ZER dan ZER-NLC mengaruh apoptosis terhadap sel MDA-MB-231. ZER dan ZER-NLC mengaruh apoptosis melalui arah laluan intrinsik (mitokondrion) secara pengaktifan kaspase-3 dan kapase-9. Analisis sap Western menunjukkan bukti berlaku pengawalan-naik protein Bax, Cyt-c dan pengawalan-turun protein Bcl-2, Bcl-xL dan PCNA dalam sel MDA-MB-231 yang diperlakukan dengan ZER dan ZER-NLC. Akhir sekali, perlakuan ZER dan ZER-NLC terhadap sel epitelium normal bukan kanser (MCF-10A) tidak memberi kesan pada pemproliferatannya, menyaran bahawa kesan kedua-duanya adalah khusus sel kanser.

Kesimpulannya, kajian ini menunjukkan bahawa kesan sitotoksik ZER-NLC dalam pengaruhan apoptosis adalah serupa dengan ZER. Dengan demikian, NLC boleh digunakan sebagai pembawa ZER dalam rawatan kanser payudara.

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

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DECLARATION

Declaration by the student

I hereby confirm that:

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LIST OF ABBREVIATIONS

AI	Aromatase inhibitors
AIF	Apoptosis-inducing factor
AO	Acridine orange
Apaf-1	Apoptosis protease activating factor
ATCC	American Type Culture Collection
Bad	Bcl-2 associated death promoter
Bak	Bcl-2 homologous antagonist killer
Bax	Bcl_2 associated x protein
B-cell	B lymphocyte
Bcl-2	B cell lymphoma 2
Bcl-xL	B cell lymphoma extra large
BCPT	Breast Cancer Prevention Trial
BRCA1/2	Breast cancer susceptibility genes 1 and 2
BSA	Bovine serum albumin
CAD	Caspase-activated DNase
CAM	Complementary and alternative medicine
Caspase	Cysteine-aspartic protease
c-FLIP	Cellular (FADD-like IL-1β-converting enzyme)-inhibitory protein
COX-2	Cyclooxygenase -2
DCIS	Ductal carcinoma *in situ*
DISC	Death-inducing signalling complex
EBV	Epstein-Bar virus
EGF	Epidermal growth factor
EPR	Enhanced permeability and retention
ER	Estrogen receptor
FADD	Fas-associated protein with death domain
GSH	Glutathione
HER-2	Human Epidermal Growth Factor Receptor 2
IAP	Inhibitor of apoptosis protein
IDC	Invasive ductal carcinoma
ILC	Invasive lobular carcinoma
LCIS	Lobular carcinoma *in situ*
NADPH	Nicotinamide adenine dinucleotide phosphate
NCI	National Cancer Institute
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural killer
NLC	Nanostructured lipid carrier
p53	Tumour protein p53
PARP	Poly ADP ribose polymerase
PCNA	Proliferating cell nuclear antigen
PI	Propidium iodide
PR	Progesterone receptor
PUMA	p53 upregulated modulator of apoptosis

SERMs	Selective estrogen receptor modulators
SGM	Specific growth media for MCF-10A
SLN	Solid lipid nanoparticle
Smac	Second mitochondria-derived activator of caspases
TdT	Terminal deoxynucleotidyl transferase
TNBC	Triple negative breast cancer
TNF	Tumour necrosis factor
TNFR1	Tumour necrosis factor receptor 1
TPBC	Triple positive breast cancer
TRADD	TNFR1 associated death domain
TRAF	Tumour necrosis factor receptor-associated factor
TRAF1	TNF receptor-associated factor 1
TRAF2	TNF receptor-associated factor 2
TRAIL	TNF related apoptosis-including ligand
TUNEL	Tdt-mediated dUTP Nick End labelling
XO	Xanthine oxidase
ZER	Zerumbone
ZER-NLC	Zerumbone loaded nanostructured lipid carrier

CHAPTER 1

INTRODUCTION

A life-threatening disease and the most prevalent cancer amongst women is breast cancer. In 2002, this disease caused more than 400,000 deaths worldwide and accounted for 30.4% of all malignancies among women of all ethnic groups in Malaysia (Lim, 2003). The presence of genetic expressions of estrogen receptor (ER), progesterone receptor (PR) and amplification of human epidermal growth factor receptor 2 (HER-2 or HER 2/neu) provide three categorisations for breast cancer tumours, including estrogen receptor positive tumour (triple positive breast cancer, TPBC), estrogen receptor-negative tumour involving HER-2 amplified tumours and triple negative breast cancer (TNBC), in which there is no expression of ER, PR and HER-2 amplification (Lam *et al*., 2013). In general, chemotherapy, hormonal therapy, radiotherapy and surgery are examples of cancer treatment methods, unfortunately, they have drawbacks of affecting the normal cells and infuriating side effects. Several studies demonstrated the important selective toxocity of drugs originated from natural sources such as ginger, red grapes, and tea in human disease treatment, especially for cancer and infectious diseases (Yang *et al*., 2001; Mulakayala *et al*., 2013).

Compounds extracted from microorganisms, plants and animal sources have played important roles for treatment of human ailments. Approximately 60% and 75% of new drugs were obtained from natural sources in the field of cancer and infectious disease, between 1981 and 2002 (Lam, 2007). Natural product compounds, which have been applied in most clinical trials, mostly originate from plant sources (Harvey, 2008). The use of herbal medicine has been famous as one of the oldest methods for human healthcare (Spiteri *et al*., 2013). Phytomedicines are defined as herbal medicine that derived from all or part of plants due to their pharmacologically active compounds for disease treatment (Pribitkin, 2005). As reported by the World Health Organization (WHO), about 75% of the worldwide population have used plant products for medicinal purposes (Barrett *et al*., 1999).

Several studies have demonstrated that herbal medicine plays a unique role in the developing field of complementary and alternative medicine (CAM). CAM can be defined as a category of various medical and health care techniques, methods, exercises and products for the purpose of prevention, diagnosis and treatment of illnesses. However, they are not recognised as part of conventional medicine (Issa *et al*., 2006).

People in both developed and undeveloped countries have utilised plants for medicinal purposes since 6000 years ago. The Egyptian pharmacopoeia of Ebers Papyrus, in 1500 BC, described the application of plant extracts such as oil of Castor beans and poppy of Opium for treatment of diseases (Gossell-Williams *et al*., 2006). It means that, botanical medicine has been used since ancient times. Seven of eight plant specie's pollen that were found from the 60000-year-old

Neanderthal burial site were conspicuous phytomedicinals (Barrett *et al*., 1999). This provided clear evidence that plants demonstrated significant medicinal role since the birth.

In Malaysia, there seems to be an ever-growing number of herbal plants claimed to induce anti-proliferative effects on cancers (Mohan *et al.*, 2010a; Mohan *et al.*, 2010b; Mohan *et al*., 2011). For example, several bioactive compounds isolated from *Boesenbergia rotunda*, a perennial herb found in Asian countries like Malaysia, showed the therapeutic potential in various cancers including Boesenbergin A for lung cancer (Isa *et al*., 2012), and Panduratin A for colon cancer (Yun *et al*., 2005) and prostate cancer (Yun *et al*., 2006). It has been demonstrated that *Typhonium flagelliforme*, a local herbal plant known as rodent tuber, inhibited the proliferation of leukaemia cells (Mohan *et al.*, 2010a; Mohan *et al.*, 2010b). *Elephantopus scaber* and *Murraya koenigii* are other plants that possess anticancer properties towards breast (Ho *et al*., 2011) and liver cancer (Syam *et al.*, 2011), respectively.

The rhizomes of the Zingiberaceae family, which consists of the most prolific plants in the tropical rainforests, have been widely applied in many Asian countries as traditional recipes with medical functions (Singh *et al*., 2011). Approximately 161 species from 18 genera of this family could be found in Peninsular Malaysia. Members of Zingiberaceae are usually rich in terpenoids, because they are aromatic and used as spices (Chen *et al*., 2008). *Zingiber zerumbet* (L.) Smith, locally known as wild edible ginger or '*lempoyang*', is one of the important species of this family, has been traditionally applied for toothache, swelling, muscle sprain and sores (Sulaiman *et al*., 2009). The milky, mucilaginous content of the pine cones is usually utilised for natural shampoo and hair conditioner production, while the rhizomes and leaves are used as flavouring agents and medicine (Sabu, 2003). The rhizome of *Zingiber zerumbet* (RZZ) has high medicinal values due to the presence of many bioactive compounds such as humulene, monoterpenes, and zerumbone. For this reason, recent several studies have been performed to isolate and identify its phytochemical content for use as a medicine (Yob *et al*., 2011).

Zerumbone, which is the major sesquiterpenoid component of essential oil in *Zingiber zerumbet*, has been reported to inhibit the proliferation of the cervix, ovary, neoplastic colon, breast and liver cancer cells (Abdelwahab *et al*., 2011). However, poor solubility characteristic of zerumbone and consequently, its poor oral bioavailability and delivery have been the main reason to find alternatives to overcome this problem. Thus, application of nanoparticles and nanocarriers including solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC) were proposed to improve drug-delivery by increasing solubility of zerumbone (Jens and Rainer, 2008; Mistry *et al.*, 2011).

Hypothesis

Zerumbone and zerumbone-loaded nanostructured lipid carrier induce death of human breast cancer line (MDA-MB-231) through the intrinsic apoptosis pathway.

Objectives of the Study

General Objective

To evaluate the *in vitro* anti-breast cancer activity of zerumbone and zerumbone-loaded nanostructured lipid carrier on the triple negative breast cancer, MDA-MB-231 cells.

Specific Objectives

To determine the;

1. Effect of zerumbone and zerumbone-loaded nanostructured lipid carrier on MDA-MB-231 breast cancer cells.
2. Apoptosis mechanism in MDA-MB-231 cells treated with zerumbone and zerumbone-loaded nanostructured lipid carrier.
3. Expression of Bax, Bcl-2, Cytochrome c, Bcl-xL, and PCNA protein in MDA-MB-231 cells treated with zerumbone and zerumbone-loaded nanostructured lipid carrier.

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