UNIVERSITI PUTRA MALAYSIA

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

MAHNAZ HOSSEINPOUR

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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

April 2015
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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

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

By

MAHNAZ HOSSEINPOUR

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 IC50 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 IC50 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

Oleh

MAHNAZ HOSSEINPOUR

Pengerusi: Ahmad Bustamam Abdul, PhD
Fakulti: Institut Biosains


IC50 ZER dan ZER-NLC pada sel MDA-MB-231 di tentukan 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 kasapse 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. IC50 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 menyeabakan

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

First of all, I would like to thank God for giving me the opportunity to complete my master study in this delightful and beautiful country, Malaysia. I would like to express my deepest respect, gratitude and admiration to my main supervisor, Dr. Ahmad Bustamam Abdul, for his excellent guidance, motivation, care, encouragement and patience. I thank him for his great help and support during each and all steps of this project. It was a great chance for me to have his endless guidance all the time and certainly without that I would not be able to complete my thesis. I would like to express my utmost gratitude for the commitment, kind treatment and enthusiasm of my co-supervisor, Professor Dr. Rasedee Abdullah. I truly appreciate his invaluable help and support.

I wish to express my appreciation to all staff in the UPM-MAKNA Cancer Research Laboratory, the Laboratory of Immunotherapeutic and Vaccines, Institute of Bioscience, Universiti Putra Malaysia for their invaluable assistance. In addition, I always feel blessed for having my helpful and super kind friends especially Dr. Heshu Sulaiman Rahman. It would not have been possible to finish my research project without their great support and help.

I also wish to express my deepest appreciation to my beloved parents and my precious husband, Saman Mohandesan. In every moment of my life, I feel my family is the greatest gift of God. They always provide the best situation for my success.
I certify that a Thesis Examination Committee has met on 27 April 2015 to conduct the final examination of Mahnaz Hosseinpour on her thesis entitled "Cytotoxicity and Mechanisms of Action of Zerumbone-Loaded Nanostructured Lipid Carrier in Triple Negative Breast Cancer Cells, MDA-MB-231" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.

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The molecular weight of B-action (A) and Bcl-2, Bcl-xL, PCNA, Bax and cytochrome-c (B) proteins expressed in MDA-MB-231 cells treated with ZER-NLC.
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<td>AI</td>
<td>Aromatase inhibitors</td>
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<td>AIF</td>
<td>Apoptosis-inducing factor</td>
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<td>AO</td>
<td>Acridine orange</td>
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<td>Apaf-1</td>
<td>Apoptosis protease activating factor</td>
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<td>ATCC</td>
<td>American Type Culture Collection</td>
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<td>Bad</td>
<td>Bcl-2 associated death promoter</td>
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<td>BSA</td>
<td>Bovine serum albumin</td>
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<td>CAD</td>
<td>Caspase-activated DNase</td>
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<td>CAM</td>
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<td>EGF</td>
<td>Epidermal growth factor</td>
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<td>EPR</td>
<td>Enhanced permeability and retention</td>
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<td>Fas-associated protein with death domain</td>
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<td>Invasive ductal carcinoma</td>
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<td>ILC</td>
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<td>LCIS</td>
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<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
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<td>NCI</td>
<td>National Cancer Institute</td>
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<td>NF-κB</td>
<td>Nuclear factor kappa-light-chain-enhancer of activated B cells</td>
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<td>NK</td>
<td>Natural killer</td>
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<td>NLC</td>
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<td>p53</td>
<td>Tumour protein p53</td>
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<td>PARP</td>
<td>Poly ADP ribose polymerase</td>
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<td>PCNA</td>
<td>Proliferating cell nuclear antigen</td>
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<td>PI</td>
<td>Propidium iodide</td>
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<tr>
<td>PR</td>
<td>Progesterone receptor</td>
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<td>PUMA</td>
<td>p53 upregulated modulator of apoptosis</td>
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<td>Smac</td>
<td>Second mitochondria-derived activator of caspases</td>
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<td>TdT</td>
<td>Terminal deoxynucleotidyl transferase</td>
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<td>TNBC</td>
<td>Triple negative breast cancer</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
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<tr>
<td>TNFR1</td>
<td>Tumour necrosis factor receptor 1</td>
</tr>
<tr>
<td>TPBC</td>
<td>Triple positive breast cancer</td>
</tr>
<tr>
<td>TRADD</td>
<td>TNFR1 associated death domain</td>
</tr>
<tr>
<td>TRAF</td>
<td>Tumour necrosis factor receptor-associated factor</td>
</tr>
<tr>
<td>TRAF1</td>
<td>TNF receptor-associated factor 1</td>
</tr>
<tr>
<td>TRAF2</td>
<td>TNF receptor-associated factor 2</td>
</tr>
<tr>
<td>TRAIL</td>
<td>TNF related apoptosis-including ligand</td>
</tr>
<tr>
<td>TUNEL</td>
<td>Tdt-mediated dUTP Nick End labelling</td>
</tr>
<tr>
<td>XO</td>
<td>Xanthine oxidase</td>
</tr>
<tr>
<td>ZER</td>
<td>Zerumbone</td>
</tr>
<tr>
<td>ZER-NLC</td>
<td>Zerumbone loaded nanostructured lipid carrier</td>
</tr>
</tbody>
</table>
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 toxicity 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 species’ 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 \textit{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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