UNIVERSITI PUTRA MALAYSIA

TOXICITY AND ANTI-BREAST CANCER PROPERTIES OF THYMOQUINONE-LOADED NANOSTRUCTURED LIPID CARRIER IN MICE

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By

ONG YONG SZE

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirements for the Degree of Doctor of Philosophy

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

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April 2019

Chairman : Associate Professor Latifah Saiful Yazan, PhD
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Thymoquinone (TQ), the bioactive compound extracted from seeds of *Nigella sativa*, has exhibited anti-cancer properties against several cancer cell lines including breast cancer cells. Despite the promising anti-cancer activities, the clinical translation of TQ was hindered by its hydrophobic property. In order to overcome its low water solubility and poor bioavailability, TQ has been encapsulated into a lipid based nanocarrier known as nanostructured lipid carrier (NLC) in the previous study. TQ that was loaded into NLC (TQNLC) has shown *in vitro* anti-proliferative activity towards breast cancer cell lines (MDA-MB-231 and MCF-7) and cervical cancer cell lines (HeLa and SiHa). Towards realising the clinical translation of TQNLC, the toxicity and anti-breast cancer properties of TQNLC in 4T1-tumour bearing BALB/c mice were evaluated in the present study. After the production of TQNLC by hot high-pressure homogenisation, its physicochemical characteristics and cytotoxicity were determined. The oral acute and sub-acute toxicity studies were conducted in healthy BALB/c mice in accordance with Organisation for Economic Cooperation and Development (OECD) 420 and 407 Guidelines, respectively. The toxicological parameters including mortality, body weight change, haematological profile, biochemical profile and histological change were assessed. The anti-breast cancer properties of oral administration of TQNLC and TQ for 28 days in 4T1-tumour bearing female BALB/c mice were determined based on the tumour volume, tumour weight, survival time and survival rate. India ink staining was performed to assess the inhibition of lung metastases. Apoptosis in the tumour was evaluated by TUNEL assay. Effects of TQNLC on the expression of apoptotic-, metastatic- and angiogenic-related proteins were analysed by Western blot. TQNLC has excellent physicochemical characteristics such as particle size less than 50 nm, polydispersity index less than 0.2, high zeta potential, high encapsulation efficiency (98.96%), high drug loading (7.45%) and good stability. In the acute toxicity study, the encapsulation in NLC minimised the toxic effect of TQ based on the LD_{50} value. TQNLC is regarded safe at the dose

of 10 mg/kg in mice with human equivalent dose of 0.813 mg/kg/d for long term oral consumption. Both treatments at 50 mg/kg and 100 mg/kg of TQNLC and TQ reduced the tumour volume and weight via induction of intrinsic apoptotic pathway with down-regulation in the expression of Bcl-2 protein and up-regulation of Bax and caspase-8. Treatment with 25 mg/kg of TQ and 50 mg/kg of TQNLC significantly inhibited lung metastasis ($p<0.05$) by suppressing the expression of MMP-2. NLC enhanced the therapeutic effect of TQ by improving the survival rate of the tumour-bearing mice. In conclusion, TQNLC is a potential anti-breast cancer agent as compared to free TQ and doxorubicin (the standard chemotherapeutic drug) with reduced side effect and improved survival rate.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

KETOKSIKAN DAN CIRI-CIRI ANTI-KANSER PAYUDARA TIMOKUINON YANG DIMUAT DALAM PEMBAWA LIPID BERSTRUKTUR NANO DALAM MENCIT

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Timokuinon (TQ), sebatian bioaktif diekstrak daripada biji benih *Nigella sativa*, telah menunjulkan ciri-ciri anti-kanser terhadap beberapa jujukan sel kanser termasuklah sel kanser payudara. Meskipun menunjukkan aktiviti anti-kanser, penterjemahan klinikal TQ dihalang oleh ciri-ciri tidak larut airmya. Bagi menyelesaikan masalah keterlarutan air and keterbiosediaannya yang rendah, TQ telah dimuat dalam pembawa berskala nano berasalkan lipid dikenali sebagai pembawa lipid berstruktur nano (NLC) dalam kajian sebelumnya. TQ yang dimuat dalam NLC (TQNLC) telah menunjukkan aktiviti anti-pertumbuhan terhadap jujukan sel kanser payudara (MDA-MB-231 dan MCF-7) dan sel kanser serviks (HeLa dan SiHa) secara *in vitro*. Pada kajian ini, ketoksikan dan ciri-ciri anti-kanser payudara TQNLC pada mencit betina BALB/c bertumor payudara (sel 4T1) dinilai. Setelah TQNLC dihasilkan melalui kaedah penghomogenan tekanan tinggi, ciri-ciri fizikokimia dan sitotoksiknya telah ditentukan. Kajian ketoksikan akut dan sub-akut TQNLC secara minum telah dilakukan, masing-masing, berdasarkan pedoman OECD 420 dan 407 pada mencit BALB/c yang sihat. Parameter ketoksikan termasuklah kematian, perubahan berat badan, profil hematologi, profil biokimia dan perubahan histologi telah dinilai. Ciri-ciri anti-kanser payudara TQNLC dan TQ secara minum selama 28 hari dalam mencit betina BALB/c bertumor payudara telah ditentukan berdasarkan isipadu tumor, berat tumor, tempoh hayat dan kadar hayat mencit. Pewarnaan India ink telah dilakukan untuk menilai perencatan metastasis ke paru-paru. Apoptosis dalam tumor telah dinilai melalui asai TUNEL. Kesan TQNLC terhadap pengekspresan protein berkaitan apoptosis, metastasis dan angiogenesis telah dianalisis dengan pemblotan Western. TQNLC telah menunjukkan ciri-ciri fizikokimia yang cemerlang seperti saiz zarah kurang daripada 50 nm, indeks penyebaran kurang daripada 2, potensi zeta yang tinggi, enkapsulasi (98.96%) dan pemuatan (7.45%) TQ yang tinggi dan kestabilan yang baik. Dalam kajian ketoksikan akut, pemerangkapan TQ dalam NLC telah mengurangkan kesan toksik TQ berdasarkan nilai LD_{50}. TQNLC dianggap selamat untuk diminum

pada jangka masa panjang pada dos 10 mg/kg dalam mencit bersamaan dengan dos 0.813 mg/kg/hari untuk manusia. Kedua-dua rawatan 50 mg/kg dan 100 mg/kg TQ dan TQNLC telah mengurangkan berat dan isipadu tumor melalui aruhan tapak jalan apoptosis dalaman dengan penurunan pengekspresan Bcl-2, peningkatan pengekspresan Bax dan caspase-8. Rawatan dengan 25 mg/kg TQ dan 50 mg/kg TQNLC dengan ketara telah merencat metastasis ke paru-paru ($p<0.05$) dengan penurunan pengekspresan MMP-2. NLC telah meningkatkan kesan penyembuhan TQ dengan menambah baik kadar hidup mencit yang mempunyai tumor. Kesimpulannya, TQNLC menunjukkan ciri-ciri anti-kanser yang lebih baik berbanding TQ dan doxorubicin (ubat kemoterapi yang standard) dengan kesan sampingan yang kurang dan kadar hidup yang lebih baik.

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

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ATCC	American Type and Culture Collection
CCAC	Canadian Council on Animal Care
DL	Drug loading
EE	Encapsulation efficiency
EPR	Enhanced permeability and retention
FDA	Food and Drug Administration
GHS	Globally Harmonized System
HED	Human equivalent dose
HPO	Hydrogenated palm oil
IACUC	Institutional Animal Care and Use Committee
IC_{50}	Half maximal inhibitory concentration
LD_{50}	Medium lethal dose
MMP	Matrix metalloproteinase
NLC	Nanostructured lipid carrier
NOAEL	No-observed-adverse-effect level
OECD	Organisation for Economic Co-operation and Development
PDI	Polydispersity index
SLN	Solid lipid nanoparticle
TNF	Tumour necrosis factor
TQ	Thymoquinone
TQNLC	Thymoquinone-loaded nanostructure lipid carrier
TUNEL	Transferase-mediated dUTP nick end-labeling
VEGF	Vascular endothelial growth factor

CHAPTER 1

INTRODUCTION

1.1 Background

"Tumours destroy man in a unique and appalling way, as flesh of his own flesh which has somehow been rendered proliferative, rampant, predatory and ungovernable. They are the most concrete and formidable of human maladies, yet despite more than 70 years of experimental study they remain the least understood."

Francis Peyton Rous, tumour virologist, Nobel lecture, 1966

Breast cancer, the malignant tumour that forms in breast tissue, was the most common cancer among women with estimated 1.7 million of cases reported worldwide, representing 25% of all the cancer diagnosed in women in 2012 (Ferlay et al., 2015). It was the leading cause of death among women with estimated 521,907 deaths, representing 14.7% of all the cancer mortality in women in 2012 (Ferlay et al., 2015). Breast cancer is initiated from the changes or mutations in DNA of normal breast cells that eventually lead to uncontrollable cell proliferation, formation of primary tumour and metastasis (the secondary tumour). Five to ten percent of the breast cancer cases are associated with genetic inheritance of mutated genes such as *BRCA1* and *BRCA2*; while the majority of breast cancer cases are related to acquired genetic changes caused by environmental factors, physical factors and hormones. The cause of death from breast cancer is not only due to the primary tumour but also the metastasis of cancer cells at distant sites (Weigelt et al., 2005).

The current treatment regimens for breast cancer are comprised of local regional treatment (radiotherapy and surgery) and systemic treatment (chemotherapy). Chemotherapy is the most common treatment for advanced and metastatic breast cancer. The treatment kills rapidly-dividing cells by interfering with DNA or key proteins that are responsible for cell division and cell cycle (Makin, 2014). As the risk of metastasis is impossible to be predicted, more than 80% of the breast cancer patients continue receiving chemotherapy even after the removal of primary tumour.

Natural products have been considered as a promising source for chemotherapeutic drug candidates (Khazir et al., 2014). Since ancient times, plants have been well documented for their medical uses in treating illnesses in mankind. Medicinal plants are able to produce novel pharmacologically-active compounds with unique and diverse structures as they have evolved to adapt and withstand the environmental challenges in nature (de Oliveira Júnior et al., 2018; Khazir et al., 2014). These plant-derived compounds are now being used as cancer therapeutics due to their ease of availability and cost effectiveness (Kuppusamy et al., 2013). The examples of plant-derived chemotherapeutic agents available for clinical use are vinca alkaloids

(vinblastine and vincristine) from *Catharanthus roseus* (BlaskÓ and Cordell, 1990), paclitaxel from the bark of *Taxus brevifolia* (Rowinsky and Donehower, 1995) and camptothecin from *Camptotheca acuminata* (Wall et al., 1966). A number of plants are still being actively researched for their potential for cancer treatment.

Nigella sativa (Family Ranunculaceae), commonly known as black cumin, is an annual herbaceous plant native to Middle Eastern Mediterranean, South Europe, Pakistan and India (Khare, 2004). Traditionally, its black seeds and seed oil have been used to treat various conditions such as cough, asthma, eczema, headache and fever (Darakhshan et al., 2015). Recent pharmacological investigations further revealed that the extracts from *N. sativa* seed possess a broad spectrum of therapeutic effects for the treatment of various type of cancers (Khan et al., 2011), diabetes (Mathur et al., 2011), gastrointestinal disorders (Bahmani et al., 2014), cardiovascular diseases (Shabana et al., 2013) and hypertension (Dehkordi and Kamkhah, 2008).

Most of the biological activities are mainly contributed by thymoquinone (TQ) (2-methyl-5-isopropyl-1,4-benzoquinone), the predominant bioactive component (30-48%) in the volatile seed oil (Gali-Muhtasib et al., 2008). TQ has been reported to exhibit promising *in vitro* anti-cancer properties against a variety of cancerous cell lines (Shoieb et al., 2003) such as A549 adenocarcinomic human alveolar basal epithelial cells (Farah et al., 2005), SH-SY5Y human neuroblastoma cells (Martin et al., 2006), SW-626 human colon cancer cells (Norwood et al., 2006), ES-2 human ovarian cancer cells (Wilson-Simpson et al., 2007), HeLa human cervical carcinoma cells (Latifah et al., 2009) and SiHa human cervical squamous carcinoma cells (Ng et al., 2011). The anti-cancer property of TQ has also been demonstrated in several *in vivo* animal models such as WEHI-3 leukemic mice (Ali Salim et al., 2014), C57BL/6 ovarian cancer mice (Wilson et al., 2015) and HCT116 colorectal cancer mice (Gali-Muhtasib et al., 2008).

TQ is claimed as a pleiotropic agent that targets multiple signalling pathways (Amin et al., 2009). The mechanisms of TQ in anti-cancer action include the induction of apoptosis (Amin et al., 2009), induction of cell cycle arrest (Acharya et al., 2014), inhibition of angiogenesis (Paramasivam, Raghunandhakumar, et al., 2012) and reduction of metastasis (Arumugam et al., 2016) through modulating multiple targets. Besides, the attractive feature of TQ is that it selectively targeted cancerous cells and exhibited low toxicity to normal cells (Amin et al., 2009).

Despite the promising therapeutic properties, the translation of TQ to clinical phase is still a challenge. TQ exhibited low oral bioavailability as it is either eliminated via a first-pass metabolism in liver or intestines due to its high hydrophobicity or accumulated in other tissues due to its high lipophilicity. There is no clinical study reported in human regarding the anti-cancer therapeutic effect of TQ due to insufficient drug concentration reaching the tumour site (Ballout et al., 2018).

Therefore, researchers have attempted several approaches to enhance the bioavailability of TQ such as synthesis of soluble TQ analogs and encapsulation of TQ in nanoformulations. The latter approach is of preference as nanotechnology provides a more efficient way in altering the physicochemical properties of poor aqueous soluble drugs. The application of nano-drug delivery system generally improved the drug pharmacokinetics such as prolonged circulation time, increased half-life, protection from external factors and reduced rapid metabolism and clearance (Schneider-Stock et al., 2014; Brigger et al., 2002). In addition, the nanocarrier is claimed to be more efficient in delivering and accumulating drug in tumour site by "passive-targeting" (Danhier et al., 2010; Puri et al., 2009). With the unique features of solid tumour which are hypervasculature and impaired lymphatic drainage, long-circulating nanocarriers will selectively extravasate and accumulate in tumour by enhanced permeability and retention (EPR) effect (Maeda et al., 2000).

So far, several TQ nanoformulations have been reported such as poly(lactide-co-glycolide) acid (PLGA) (Ravindran et al., 2010), liposomes (Odeh et al., 2012), solid lipid nanoparticles (SLNs) (Singh et al., 2013), chitosan nanoparticles (Alam et al., 2012) and nanostructured lipid carriers (NLCs) (Ng et al., 2015). Lipid-based nanoparticles are the most extensively-studied drug delivery carrier due to their biocompatibility, biodegradable, structural simplicity and safety (Tang et al., 2018). Nanostructured lipid carrier (NLC) is the latest generation of lipid-based nanoparticle, which is modified from solid lipid nanoparticle (SLN). NLC is a partially crystallized lipid carrier that incorporates liquid and solid lipids in the core matrix to cause imperfections. It overcomes the limitations of SLN and liposomes by improving the drug loading capacity, reducing the drug repulsion during storage, increasing the drug encapsulation efficiency and maintaining the carrier stability (Müller et al., 2002a).

Considering the limitations of TQ and outstanding features of NLC, TQ has been encapsulated into NLC (hereinafter referred to TQNLC) in the previous study with excellent physiochemical properties such as high encapsulation efficiency, particle diameter less than 50 nm and good stability up to 2 years. TQNLC exhibited anti-proliferative activity towards breast cancer cell lines (MDA-MB-231 and MCF 7) and ovarian cancer cell lines (HeLa and SiHa). Moreover, this nanoformulation holds a great potential for treatment of breast cancer with the induction of apoptosis and cell cycle arrest in MDA-MB-231 breast cancer cells (Ng et al., 2015). Although TQNLC has been documented to exhibit cytotoxic effects in breast cancer cell lines, the *in vivo* oral safety and anti-breast cancer activities of TQNLC have not been investigated.

1.2 Hypotheses

The encapsulation of TQ in NLC will improve the anti-breast cancer properties of TQ by inhibition of 4T1 tumour growth, reduction of tumour metastasis, reduction of angiogenesis and improvement of survival rate of 4T1-tumour bearing BALB/c mice at the dosage that will not cause adverse side effects to the mice.

1.3 Objectives

1.3.1 General Objective

The general objective of the study was to determine the toxicity and anti-breast cancer properties of thymoquinone-loaded nanostructured lipid carrier (TQNLC) in BALB/c mice.

1.3.2 Specific Objectives

The specific objectives were:

(1) to synthesize and determine the physiochemical characteristics of TQNLC,
(2) to determine the safe dosage of TQNLC in the mice,
(3) to evaluate the inhibitory effect of TQNLC on the 4T1 primary tumour,
(4) to determine the anti-metastatic and anti-angiogenic properties of TQNLC and
(5) to evaluate the survival rate of 4T1-tumour bearing mice after treatment with TQNLC.

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