UNIVERSITI PUTRA MALAYSIA

EFFECTS OF CO-LOADED DOXORUBICIN AND THYMOQUINONE CALCIUM CARBONATE NANOPARTICLES ON MDA MB231 BREAST CANCER STEM CELLS

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By

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

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DEDICATION

This thesis is dedicated to Almighty Allah, the All Merciful, the Most Merciful.

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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Chairman : Professor Md. Zuki bin Abu Bakar @ Zakaria, PhD
Institute : Biosciences

A subtype of cells, cancer stem cells (CSCs) within the breast cancer has been implicated for the metastasis, chemo/radiotherapy resistance and relapse resulting in poor prognosis. The main objective of the study was to determine the effects of drugs-loaded (thymoquinone, doxorubicin, and a combination of thymoquinone and doxorubicin-loaded) aragonite $CaCO_3$ nanoparticles (ACNP) on breast cancer stem cells. The formulated blank and drugs-loaded nanoparticles were characterized for physicochemical properties. The cytotoxic effect of blank and drug-loaded nanoparticles on MDA MB231 breast cancer cell line, 3D mammosphere, normal breast cells (MDF10A) and normal fibroblast (3T3) were also analysed. Morphological changes, sphere forming assay, cancer stem cell self-renewal capacity, ALDH activity analysis, CD44 and CD24 expression were carried out. The prepared nanoparticles were pleomorphic with sizes ranging from 53.65±10.29nm to 60.49±11.36nm and overall negative charge. The encapsulating efficiency of Dox and TQ in the dual loaded nanoparticles was found to be 95.8 and 41.6% respectively. The XRD patterns revealed strong crystallizations in blank and drug loaded formulation, while FTIR showed little alteration upon loading Dox and TQ. About 100% of drug release was noticed at pH 4.8, 70% at pH 6 while only 50% at pH 7.4. The blank nanoparticle was biocompatible, cell viability of 80% at a high concentration of 1000ug/ml. MDA MB231 IC_{50} dosages of drug-loaded nanoparticle were not toxic to the normal cells. For monolayer culture, the combination therapy showed enhanced apoptosis, reduction in cellular migration and invasion when compared to the single drug loaded nanoparticle and the free drugs. Scanning electron microscopy and transmission electron microscopy showed presence of cell shrinkage, cell membrane blebbing and disruption of cell membrane. For cancer stem cell enriched mammosphere, the combination therapy showed enhanced apoptosis, reduction in ALDH activity and expression of CD44 and CD24 surface maker, reduction in cancer stem cells metastatic capacity, inhibition of 3D sphere formation and cancer stem cell

self-renewal capacity when compared to the free drugs and the single drug loaded nanoparticle. Scanning electron microscope showed poor spheroid formation, cell membrane blebbing, presence of cell shrinkage, distortion in the spheroid architecture. Thus, the cockle shell-derived aragonite calcium carbonate nanoparticle system provides a simple and efficient platform for multiple drug delivery and pH sensitive release. The combined drugs-loaded cockle shell-derived aragonite calcium carbonate nanoparticles (Dox/TQ-ACNP) showed higher efficacy in MDA MB231 breast cancer cells at lower dose of doxorubicin or thymoquinone, efficiently destroyed the breast CSCs and may be a potential curative strategy for the management of breast cancer recurrence and metastasis.

Keywords: Cancer stem cells, Doxorubicin, Thymoquinone, Aragonite cockle shell derived calcium carbonate nanoparticles, Multi-Drug Delivery

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

KESAN NANOPARTIKEL KALSIUM KARBONAT YANG DIMUATKAN DENGAN DOXORUBICIN DAN THYMOQUINONE KE ATAS SEL-SEL STEM KANSER PAYUDARA MDA MB231

Oleh

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Pengerusi : Profesor Md. Zuki bin Abu Bakar @ Zakaria, PhD
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Satu sub-sel sel, sel stem kanser (CSC) dalam kanser payudara telah dikaitkan dengan metastasis, rintangan kemoterapi/radioterapi dan tumbuh semula mengakibatkan prognosis yang buruk. Tujuan kajian ini adalah untuk menentukan kesan nanopartikel kalsium karbonat aragonit yang berasal dari kulit kerang yang dimuatkan dengan drug (doxorubicin (Dox) dan thymoquinone (TQ), bersendirian atau bergabung) ke atas sel stem kanser payudara. Nanopartikel yang kosong tanpa drug dan yang dimuatkan dengan drug telah dicirikan untuk ciri-ciri fizikokimia. Kesan sitotoksik nanopartikel yang kosong dan yang dimuatkan dengan drug ke atas sel kanser payudara (MDA MB231), 3D mammospehe, sel-sel payudara normal (MDF10A) dan fibroblast (3T3) juga telah dianalisa. Perubahan morfologi menggunakan mikroskop cahaya kontras, mikroskop elektron pengimbasan dan mikroskop elektron penghantaran, esei membentuk sfera, kapasiti pembaharuan CSC, analisis aktiviti ALDH, penanda permukaan CD44 dan CD24 telah dijalankan. Nanopartikel yang dihasilkan adalah berbentuk pleomorfik dengan saiz antara 53.65 ± 10.29nm hingga 60.49 ± 11.36nm dan bercaj negatif. Kecekapan muatan bagi kedua-dua drug, Dox dan TQ kedalam nanopartikel adalah masing-masing 95.8 dan 41.6. Corak XRD mendedahkan penghabluran yang kuat dalam nanopartikel yang kosong dan formulasi yang dimuatkan dengan drug, manakala FTIR menunjukkan sedikit perubahan apabila nanopartikel dimuatkan dengan Dox dan TQ. Hampir 100% pelepasan dadah diperhatikan pada pH 4.8, 70% pada pH 6 manakala hanya 50% pada pH 7.4. Nanoparticle kosong adalah biokompatibel, 80% daya tahan sel pada kepekatan tinggi 1000ug / ml. Dos MDA MB231 IC_{50} nanopartikel dimuatkan dengan drug adalah tidak toksik kepada sel normal. Untuk kultur monolayer, terapi dengan nanopartikel dimuatkan dengan kombinasi drug menunjukkan peningkatan apoptosis, pengurangan penghijrahan selular dan pencerobohan apabila dibandingkan dengan nanopartikel dimuatkan drug tunggal dan drug secara bersendirian. Pengimbasan mikroskop elektron menunjukkan kehadiran pengecutan sel, penunjulan sel membran, manakala

mikroskop elektron penghantaran menunjukkan pemecahan nukleus, kerosakan sel membran, badan-badan apoptosis, dan kerosakan pada mitokondria. Untuk sel stem kanser yang diperkayakan mamografi, terapi gabungan menunjukkan peningkatan apoptosis, pengurangan aktiviti ALDH dan ekspresi penanda permukaan CD44 dan CD24, pengurangan kapasiti metastatik sel stem kanser, menghalang pembentukan sfera 3D dan kapasiti pembaharuan sel stem kanser apabila dibandingkan dengan drug bebas dan nanoparticle yang dimuatkan dengan drug tunggal. Pengimbasan mikroskop elektron menunjukkan pembentukan sphera yang lemah, penunjulan sel membran, kehadiran pengecutan sel, dan kerosakan struktur sphera. Oleh itu, sistem nanopartikel kalsium karbonat aragonite berasal dari kulit kerang menyediakan pendekatan yang mudah dan efisien untuk penghantaran drug secara berganda dan juga berfungsi sebagai platform untuk pelepasan drug boleh kawal yang sensitif pH untuk beberapa agen terapeutik. Kombinasi nanopartikel kalsium karbonat aragonite dengan drug (Dox/TQ-ACNP) menunjukkan efikasi yang lebih tinggi pada sel-sel kanser payudara pada dos drug (doxorubicin atau thymoquinone) yang lebih rendah, dan dengan berkesan memusnahkan CSC payudara dan ini mungkin berpotensi tinggi untuk rawatan kanser payudara yang metastasis dan berulang.

Kata kunci: Sel stem kanser, Doxorubicin, Thymoquinone, nanopartikel kalsium karbonat aragonite berasal dari kulit kerang, penghantaran drug.

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LIST OF ABBREVIATIONS

%	Percentage
µg	Microgram
3D	Three dimensional
ABC	ATP-binding cassette
ACNP	Aragonite calcium carbonate nanoparticle
ALDH	Aldehyde dehydrogenase
ATCC	American Type Culture Collection
BRCA 1	Breast cancer type 1 susceptibility protein
BS-12	Dodecyl dimethyl betaine
$CaCO_3$	Calcium carbonate
CD	Cluster of differentiation
CI	Combination index
CSCs	Cancer stem cells
CXCR	C-X-C chemokine receptor
DEAB	Diethylaminobenzaldehyde
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
Dox	Doxorubicin
Dox/TQ-ACNP	Doxorubicin and thymoquinone loaded aragonite calcium carbonate nanoparticle
Dox-ACNP	Doxorubicin loaded aragonite calcium carbonate nanoparticle
EGFR	Epidermal growth factor receptor
EMT	Epithelial–mesenchymal transition
ER	Oestrogen reeptor
FBS	Fetal bovine serum

FESEM	Field emission scanning electron microscopy
FTIR	Fourier transform infrared spectroscopy
FOXO3a	Forkhead box O3
GSK3β	Glycogen synthase kinase 3 beta
hEGF	Human epidermal growth factor
HER 2	Human epidermal growth factor receptor 2
HIF-α	Hypoxia-inducible factors α
HMLE	Human mammary epithelial cells
IC_{50}	Half maximal inhibitory concentration
IL	Interleukin
mg	Milligram
ml	Millilitre
MTT	3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NICD	Notch intracellular domain
^{o}C	Degree celcius
PBS	Phosphate buffer saline
pH	Potential of Hydrogen
PI	Propidium Iodide
RNA	Ribonucleic acid
RNase	Ribonuclease
ROS	Reactive oxygen species
rpm	Revolutions per minute
TCGA	The Cancer Genome Atlas
TEM	Transmission electron microscopy
TNF-α	Tumour necrosis factor α

TQ	Thymoquinone
TQ-ACNP	Thymoquinone loaded aragonite calcium carbonate nanoparticle
UV/vis	Ultraviolet visible
v/v	Volume/volume
WST	2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt
XRD	X-ray dispersity
β catenin	Beta catenin

CHAPTER 1

INTRODUCTION

1.1 Background of the Study

The leading cause of death in women is cancer of the breast, mainly due to metastasis to distant site, and this is usually associated with failure of therapy (Croker and Allan, 2012; Dai *et al.,* 2015). Breast cancer burden is on the rise in all countries with more burdens in the developing regions (population ratio of 1:4 developed and developing regions respectively) (Sudeshna *et al.,* 2013).

A subtype of cells (cancer stem cells, CSCs) within the breast cancer tumour has been implicated for the metastasis, chemo/radiotherapy resistance and relapse resulting in poor prognosis (Liu and Wicha, 2010; Croker and Allan., 2012). CSCs have characteristics that are identical to normal stem cells in their capability of self-renewal, the ability to develop into differentiated cells found in malignancy (Liu and Wicha, 2010). However, CSCs are able to resist chemotherapy and radiation by increasing the production of proteins involved in drug resistance, commencement of DNA repair, and activation of pathways like Notch, Hedgehog, Wnt/ß-catenin (Liu and Wicha., 2010). These stem like cells (CSCs) have been identified based on high ALDH1 activity and/ or $CD44^{+}CD24^{-/low}$ phenotype in breast tissue samples of cancer patients and breast cancer cell lines (Croker and Allan., 2012) The aldehyde dehydrogenase (ALDH) enzymes are important for certain processes in nature, importantly, detoxification throμgh the NAD $(P)^{+}$-dependent oxidation of aldehyde. Increase in the percent of $CD44^{+}/CD24^{-}$ cells as well as ALDH1 activity was noted after exposure to chemotherapy and radiotherapy (Croker *et al.,* 2009).

Recently, nanotechnology has shown a great advantage in drug delivery for cancer treatment by enhancing build ups of cytotoxics in tumour tissue, specificity in tumour targeting, reducing the cytotoxics side effect on normal cells, reducing systemic side effect, increasing drug solubility, and increasing maximum tolerated dose (Kamba *et al.*, 2013). Interestingly, calcium carbonate ($CaCO_3$) nanoparticles are biocompatible, biodegradable, highly porous, and pH-sensitive. Calcium carbonate ($CaCO_3$) is much abundant in nature. Among the polymorphs of $CaCO_3$ (calcite, aragonite, and vaterite), aragonite has got immense attention because it is the most biocompatible; this makes aragonite an excellent biological drug delivery systems of anticancer drugs (Islam *et al.*, 2012).

Thymoquinone (TQ) is one of the active constituent of black seeds (*Nigella sativa*). The seeds have been used to treat a range of ailments in traditional medicines. TQ has been shown to have antineoplastic effects in both *in vitro* and *in vivo* studies (Padhye *et al.*, 2008; Randhawa and Alghamdi, 2011; Khan *et al.*, 2011; Mostofa *et al.*, 2017). TQ inhibits IL-8 expression and it receptors activities particularly CXCR1 (Ashour *et al.*, 2014), Notch1 expression (Ke *et al.*, 2015) in hepatocellular cancer derived cell

lines, suppressing Akt activation and inducing apoptotic cell death (Khan *et al.*, 2011; Singh *et al.*, 2012). Akt regulates breast stem cell self-renewal by phosphorylating GSK3β, which results in the stimulation of Wnt pathway (Ginestier *et al.*, 2010). The growth restrictive effect of TQ was restricted to cancer cells and it is less toxic to the normal cells (Gali-Muhtasib *et al.*, 2006).

Doxorubicin (Dox) is a common cytotoxic drug used in the treatment of breast, leukaemia, and other types of cancer. Dox acts by inhibiting enzyme topoisomerase II. Topoisomerase I and II alter DNA topography throμgh DNA strand cleavage, strand passage and relegation (Wei *et al.*, 2015). Moreover, Thymoquinone enhanced the cytotoxic properties of ionizing radiation (Velho‐Pereira *et al.*, 2011) and doxorubicin in multi-drug resistant variant of MCF-7 cells (Effenberger-Neidnicht and Schobert, 2011). TQ was shown to reduce the toxicity of other cytotoxic drugs by up-regulating antioxidant mechanisms (Alenzi *et al.*, 2010).

Althoμgh, there have been advancement in breast cancer research, death from breast cancer is still on the rise. About 13 million new cases of breast cancer were diagnosed worldwide in 2008 with 7.6 million deaths and the incidence is anticipated to increase to about 26.4 million by 2030 with 17 million deaths (Akarolo-Anthony *et al.*, 2010). However, to reduce mortality and the burden of breast cancer, it is important to develop treatment options which will include drugs that can target CSCs, and drug delivery systems which is cancer cell specific with less or no toxicity to normal cells.

There are so far very limited identified therapeutic agents that could effectively target CSCs. TQ has attracted significant attention in recent years but research to assess the use of TQ in targeting CSCs surviving or self-renewal and chemotherapy resistance is limited. There is little to no information on the effect of TQ on CSCs and also the use of aragonite nanoparticles as a nanocarrier for TQ and co-loading with Dox has not been reported. A CSCs targeting approach using combined doxorubicin and thymoquinone-loaded aragonite nanoparticles (Dox/TQ-ACNP) may provide a good approach to targeting breast CSCs.

1.2 Hypothesis

i. Cockle shell-derived aragonite calcium carbonate nanoparticles (ACNP) have a high loading capacity for doxorubicin and thymoquinone.
ii. The blank and drugs-loaded ACNP is safe to the normal cells.
iii. Doxorubicin/Thymoquinone-loaded ACNP (Dox/TQ-ACNP) show higher efficacy in breast cancer cells at lower dose.
iv. Dox/TQ-ACNP induced cytotoxicity on breast cancer stem cell (CSCs).
v. Dox/TQ-ACNP reduce CSCs self-renewal capacity, surface marker expression, ALDH activity and metastatic potential.

1.3 General Objectives

The main objective of the study was to determine the effects of drugs-loaded (thymoquinone, doxorubicin, and a combination of thymoquinone and doxorubicin-loaded) aragonite $CaCO_3$ nanoparticles (ACNP) on breast cancer stem cells.

1.3.1 Specific objectives

i. To prepare and determine the physicochemical characteristic of blank and drugs-loaded ACNP.
ii. To determine the cellular uptake and the biocompatibility of blank and drugs-loaded ACNP.
iii. To evaluate the ultrastructure changes and antitumor effect of drugs-loaded ACNP on breast cancer cell line.
iv. To evaluate morphological changes and antitumor effects of drugs-loaded ACNP on breast CSCs.
v. To evaluate the effects of drugs-loaded ACNP on CSCs self-renewal capacity, surface marker expression, ALDH activity and metastatic potential.

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LIST OF PUBLICATIONS

Ibiyeye, K. M., Nordin, N., Ajat, M., and Zuki, A. B. Z (2019). Ultrastructural Changes and Antitumor Effects of Doxorubicin/ Thymoquinone-Loaded CaCO3 Nanoparticles on Breast Cancer Cell Line. *Front. Oncol*. 9, 599.

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