



UNIVERSITI PUTRA MALAYSIA

***EFFECTS OF TAMOXIFEN-LOADED ERYTHROPOIETIN-COATED
NANOSTRUCTURED LIPID CARRIER ON BREAST CANCER CELLS
AND RAT MAMMARY GLAND TUMOUR***

BEH CHAW YEE

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By

BEH CHAW YEE

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

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Chairman : Professor Rasedee Abdullah, PhD
Faculty : Institute of Bioscience

Nanomedicine is an emerging and fast developing area in the medical field, especially in the treatment of cancers. Most chemotherapy drugs have the limitation of poor drug water solubility which hinders their drug efficacy. The incorporation of drugs into nanoparticulated carriers had improved the low water solubility and efficacy of anti-breast cancer drugs. In this study, the nanostructured lipid carrier (NLC) was loaded with tamoxifen (TAM) and coated with erythropoietin (EPO) to produce EPO-TAMNLC, and the anticancer effects of this drug delivery system was determined. For comparison the TAM-loaded NLC (TAMNLC) was also developed. These nanoparticulated carriers were produced by using the high pressure homogeniser method and physiochemically and morphologically characterised using the dynamic light scattering technique, zetasizer, and transmission electron microscopy. The thermodynamic interaction between EPO and TAMNLC was investigated through the fluorescent spectroscopy and isothermal titration calorimetry while their binding efficiency was obtained through sodium dodecyl sulfate polyacrylamide gel electrophoresis. The elucidation of oestrogen and erythropoietin receptors status on MCF-7 and LA7 cells was determined through immunocytochemistry staining. The cytotoxic effect, mode of cell death and cell cycle arrest caused by treatment with EPO-TAMNLC and TAMLC toward MCF-7 and LA7 cells was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay and flow cytometry. The *in vivo* effects of EPO-TAMNLC and TAMNLC were determined in the normal and mammary gland tumour rats by using doses 1.25 mg/kg BW, 2.5 mg/kg BW and 5 mg/kg BW and treated through intravenously. The rat mammary gland tumour was induced by injecting 6×10^6 LA7 cells into the mammary fat pad. Toxicity parameters included serum kidney and liver function parameters and the histology of the kidneys, liver, heart, lungs, spleen, and bone marrow. The EPO-TAMNLC formulation was stable with particle size of 55.39 ± 0.98 nm, zeta potential of -1.58 ± 0.47 mV, and

Polydispersity index of 0.19 ± 0.01 . Based on ultrastructural analysis, the nanoparticles were spherical. The binding interaction of EPO and TAMNLC shown was spontaneous with positive enthalpy. It was shown the binding efficiency of EPO to TAMNLC was highest at pH 7.2 at 55.43%. The immunocytochemistry staining revealed that these cells are positive for oestrogen (ER) and erythropoietin receptors (EpoRs). The *in vitro* toxic effect of EPO-TAMNLC and TAMNLC on MCF-7 and LA7 cells was time-dependent with the GI_{50} of 4.8 μM , 5.1 μM , 2.5 μM and 2.5 μM respectively which postulated to occur through the targeting of the ER and EpoRs in the cancer cells. However, both drug carrier systems did not significant ($P > 0.05$) affect the viability of the normal MCF-10A and HDFa cells. Flow cytometry study showed that EPO-TAMNLC induced apoptosis and G_0/G_1 cell cycle arrest in the cancerous MCF-7 and LA7 cell lines. For the *in vivo* part, normal rats treated with intravenous EPO-TAMNLC and TAMNLC did not show evidence of toxicity from the treatments, suggesting that EPO-TAMNLC and TAMNLC are safe for parenteral use and the LD_{50} exceed 5 mg/kg BW. Both EPO-TAMNLC and TAMNLC, while significantly ($p < 0.05$) reducing the mammary gland tumour size in rats, achieved sustain anti-tumour effect and are more effective anti-tumour agents than oral TAM. In conclusion, EPO-TAMNLC is a promising targeted anticancer drug formulation for treatment of ER-positive breast cancers.

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

KESAN PEMBAWA LIPID NANOSTRUKTUR DIMUAT TAMOKSIFEN AND DISALUT ERITHROPOIETIN TERHADAP SEL KANSER PAYUDAYA DAN TUMOUR KELENJA MAMA TIKUS

Oleh

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Nanoperubatan merupakan suatu bidang baru wujud dan pesat membangun dalam perubatan, terutama sekali untuk rawatan kanser. Kebanyakan ubat kemoterapi lemah dengan kelarutan air yang rendah menyebabkan kesan ubat terjejas. Pemuatan ubat ke dalam pembawa nanozarah telah meningkatkan ubat kelarutan dalam air and kemujaraban drug anti-kanser payudara. Dalam kajian ini, pembawa lipid nanostruktur (NLC) telah dimuatkan dengan tamoksifen (TAM) and disalut dengan erithropoietin (EPO) untuk menghasilkan EPO-TAMNLC, dan kesan anti-kanser sistem penghantar drug ini ditentukan. Untuk bandingan, NLC termuat TAM (TAMNLC) juga dikembangkan. Pembawa nanozarah ini dihasilkan melalui kaedah penghomogenan tekanan tinggi dan diciri secara fisiokimia and morfologi menguna teknik sebaran cahaya dinamik, zetasizer, and mikroskopi elektron pancaran. Saling tindakan ikatan EPO dengan TAMNLC yang dinyatakan melalui spektroskopi pendarflour and kalorimetri pentitratian isoterma, manakala kekuatan ikatan EPO kepada TAMNLC ditentukan oleh elektraforesis gel natrium dodesil sulfat poliakrilamida. Status reseptor erythropoietin dan oestrogen juga ditentukan melalui imunositokimia. Kesan sitotoxic, cara kematian dan hentian kitaran sel yang disebabkan oleh perlakuan EPO-TAMNLC and TAMNLC terhadap MCF-7 dan LA7 sel telah ditentukan mengguna assai 3-(4,5-dimetiltiazol-2-yl)-2,5-difeniltetrazolium bromida and sitometri aliran. Kesan *in vivo* ditentukan pada tikus normal dan yang diaruh untuk memperolehi tumour kelenjar mama oleh EPO-TAMNLC and TAMNLC dengan menggunakan dos 1.25 mg/kg BW, 2.5 mg/kg BW and 5 mg/kg BW melalui intravena. Tumour kelenjar mama tikus ini diaruh dengan menyuntik 6×10^6 sel LA7 ke dalam pad mama. Parameter ketosikan termasuk fungsi ginjal dan hati serum and histologi ginjal, hati, jantung, peparu, limpa, and sumsum tulang. Formulasi EPO-TAMNLC adalah stabil bersaiz zarah 55.39 ± 0.98 nm, potensi zeta -1.58 ± 0.47 mV, and indeks kepoliserakan 0.19 ± 0.01 . Berasaskan analisis ultrastruktur, nanozarah ini berbentuk sfera. Saling tindakan ikatan EPO

dengan TAMNLC adalah berlaku secara spontan dengan entalpi positif. Kekuatan ikatan EPO kepada TAMNLC ditunjukkan paling tinggi pada kadar 55.43% pada pH 7.2.. Kajian imunositokimia menunjukkan kedua-dua sel ini positif untuk reseptor oestrogen (ER) dan eritropoietin (EpoR). Kesan toksik EPO-TAMNLC dan TAMNLC terhadap sel MCF-7 dan LA7 adalah bersandarkan masa dengan GI₅₀ 4.8 μ M, 5.1 μ M, 2.5 μ M and 2.5 μ M masing-masing yang mana dipostulatkan berlaku melalui menyasaran ER dan EpoR pada sel kanser. Bagaimanapun, kedua-dua sistem pembawa drug tidak secara ketara ($p>0.05$) memberi kesan terhadap kedayahidupan sel MCF-10A dan HDFa yang normal. Sitometri aliran menunjukkan EPO-TAMNLC mengaruh apoptosis dan hentian G₀/G₁ kitaran sel untuk sel kanser MCF-7 and LA7. Dalam kajian *in vivo*, Tikus normal yang diperlaku secara intravena dengan EPO-TAMNLC dan TAMNLC tidak menunjukkan sebarang ketoksikan akibat perlakuan, menunjukkan EPO-TAMNLC dan TAMNLC ini selamat untuk diguna secara parenteral dan LD₅₀ tidak melebihi 5 mg/kg BW. Kedua-duanya, EPO-TAMNLC dan TAMNLC, sambil secara tererti ($p<0.05$) mengurangkan saiz tumour kelenjar mama dapat tikus, mencapai kesan anti-tumour yang mengekalkan dan adalah lebih berkesan sebagai agen anti-tumour daripada TAM yang diberi secara oral. Kesimpulannya, EPO-TAMNLC adalah formulasi anti-kanser penyasar yang berpotensi tinggi untuk rawatan kanser payudara ER-positif.

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LIST OF ABBREVIATIONS

ΔG	Gibbs free energy of binding
ΔH	Enthalphy of binding
ΔS	Entropy of binding
A	Corrected absorbance
AE	Association efficiencies
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
ANOVA	One-way analysis of variance
AQ	Aqueous phase
ASR	Age-standardised rate
AST	Aspartate aminotransaminase
ATCC	American type culture collection
BSA	Bovine serum albumin
BW	Body weight
CDKs	Cyclin-dependent kinases
CH_3OH	Methanol
CK14	Cytokeratins 14
CKIs	Cyclin-dependent kinases inhibitors
DAPI	4, 6-diamidino-2-phenylindole
DCIS	Ductal carcinoma in situ
DLS	Dynamic light scattering
DMEM	Dulbecco's Modified Eagle Media
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EE	Entrapment efficiency
EGFR	Epidermal growth factor receptor
EpoRs	Erythropoietin receptors
EPO-TAMNLC	tamoxifen-loaded erythropoietin-coated nanostructured lipid carrier
EPR	Enhanced permeation and retention effect
EREs	Oestrogen-response elements
ER α	Oestrogen receptor α
FA	Folic acid
FasL	Fas Ligand
FITC	Fluorescein isothiocyanate
G ₁	Gap-1 checkpoint
GGT	Gamma-glutamyltransferase
GI ₅₀	Half-growth inhibitory concentration
H&E	Hematoxylin-Eosin
HA	Hyaluronan
HED	Human equivalent dose
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HER2	Human epidermal growth factor receptor 2
HPF	High-power field

HPH	High pressure homogenisation
HPLC	High performance liquid chromatography
IACUC	Institutional Animal Care and Use Committee
ITC	Nano-Isothermal titration calorimetry
K2-EDTA	Ethylenediaminetetraacetic acid
K ₂ HPO ₄	Dipotassium hydrogenphosphate
K _a	Association constant
K _{sv}	Stern-Volmer constant
LC ₅₀	Lethal concentration 50
LCIS	Lobular carcinoma in situ
	lipid carrier
M:E	Myeloid and erythroid ratio
M3-PALS	Laser doppler velocimetry and phase analysis scattering
MaSCs	Mammary gland stem cells
MCHC	Mean corpuscular haemoglobin concentration
MES	2-(N-morpholino)ethanesulfonic acid
MIA	Mitotic index analysis
MPS	Mononuclear phagocyte system
MRT	Mean residence time
MTT	Thiazolyl blue tetrazolium bromide
MUC-1	Tansmembrane glycoprotein Mucin 1
MWCO	Molecular weight cut-off
n	Stoichiometry
NaCl	Sodium chloride
NaI	Sodium iodide
NLC	Nanostructured lipid carrier
OS	Overall survival
PBS	Phosphate buffered saline
PCV	Packed cell volume
PEG	Polyethylene glycol
P-gp	P-glycoprotein
PI	Polydispersity index
PI	Propidium iodide
PR	Progesterone receptor
PSMA	Prostate specific membrane antigens
QE	Quenching efficiency
rhEPO	Recombinant human erythropoietin
Rnase A	Ribonuclease A
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SERDs	Selective oestrogen receptor down modulators
SERM	Selective oestrogen receptor modulator
SFEE	Supercritical fluid extraction of emulsification
SLNs	Solid lipid nanoparticles
TAM	Tamoxifen
TEM	Transmission electron microscope
TfR1	Transferrin receptor
TGI	Total growth inhibition concentration

Tris-HCl
Trp
ZP

Tris-hydrochloride
Tryptophan
Zeta potential



CHAPTER 1

INTRODUCTION

Breast cancer is one the most feared diseases among women. The disease causes psychological disturbances and imposes huge economic burdens on patients and their families. The most common treatments for breast cancers are surgical removal and chemotherapy. However, these conventional chemotherapies are highly toxic, non-specific, lack cancer-targeting, and often prolonged (Harrington & Smith, 2008).

Many chemotherapeutic drugs commonly used in the treatment of cancers have low bioavailability and distribution. These drugs are poorly water-soluble that have hindered efficient delivery to target tissues and organs. To fully benefit from the therapeutic effects of these drugs, there is need to modify the delivery system. One of the ways to improve current chemotherapeutic schemes is by adopting an effective drug delivery system using nanoparticle technology. Nanoparticles have great promise as an efficacious anticancer drug-delivery system that can overcome the limitations associated with inadequate pharmacokinetics and biodistribution of current anticancer drugs. Nanoparticle delivery systems involve the loading of drugs into carriers as ligand-nanoparticle conjugates and the complexes exhibit disease-targeting and sustained release characteristics without compromising efficacy of the load therapeutic compound (Orive *et al.*, 2005).

Oestrogen is steroid hormone responsible for the development and maintenance of female sexual and reproductive functions. Certain breast cells, such as the MCF-7, are oestrogen receptor α (ER α)-positive. These cells differentiate and proliferate through stimulation of the ERs by oestrogens. Thus, treatment of breast cancers often employs the selective oestrogen receptor modulators (SERM), such as tamoxifen (TAM). This drug acts by inhibiting oestrogen-receptor binding and curtailing breast cancer proliferation and spread (Heldring *et al.*, 2007).

One of the common manifestations of cancers is anaemia. Anaemia may also develop as the result of chemotherapy. Erythropoietin (EPO) is a hormone of renal origin that functions to stimulate erythrocyte production by the bone marrow. Although, the effects are debatable, EPO is used as adjunct therapeutic compound for the treatment of anaemia of cancers. Incidentally, human breast cancer cells have also been shown to express EPO receptors (EpoRs) (Arcasoy *et al.*, 2002). Like ER α , EpoRs is absent in normal breast cells (Lappin, 2003). The presence of EpoRs on breast cancer cells provides an opportunist target for EPO.

This study, for the first time ever, developed a nanoparticulated delivery system that is simultaneously loaded with TAM and EPO. In this study, the nanostructured lipid

carrier (NLC) was used as the carrier for TAM and EPO. The innovation in the study is not drug double-loading only, but also the positioning of the drugs in the NLC. Tamoxifen was incorporated in the matrix of NLC while EPO was loaded by coating the TAM-loaded NLC. The TAM-loaded and EPO-coated NLC, designated the EPO-TAMNLC, is now patent pending (PI 2015704030).

In this study, it is hypothesised that EPO-TAMNLC improves the anti-mammary gland cancer effects of TAM.

1.1 Objectives

The objectives of the study were to

1. Develop and characterise TAM-loaded EPO-coated nanostructured lipid carrier (EPO-TAMNLC).
2. Determine expression of the oestrogen and EPO receptors on breast and rat mammary gland cancer cells.
3. Determine the mode and mechanism of *in vitro* toxicity of EPO-TAMNLC on breast and rat mammary gland cancer cells.
4. Determine the effect of EPO-TAMNLC on LA7 cell-induced mammary gland tumour in rats.

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