UNIVERSITI PUTRA MALAYSIA

IN VITRO TREATMENT OF MCF-7 AND MDA-MB-231 BREAST CANCER CELL LINES WITH DOXORUBICIN AND VINCRISTINE SULPHATE CALCIUM CARBONATE NANOPARTICLES

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By

XUE MENGYI

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

August 2019

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DEDICATIONS

This thesis is dedicated to

My Dear Parents,

Mr. GENG ZHONGXIANG

Ms. XUE CHANGJUN

My Dear Friend

Mr.CAI CHAOJUN

My Dear Supervisor

Professor Dr Md Zuki bin Abu Bakar@Zakaria

Dr. Mokrish Md Ajat

For the precious encourage and great help they provided

Abstract of thesis presented to the senate of Universiti Putra Malaysia in fulfilment of the requirements for the degree of Master of Science

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August 2019

Chairman : Professor Md Zuki bin Abu Bakar, PhD
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Vincristine Sulphate (VCR) and Doxorubicin (DOX) are standard chemotherapy drugs widely used as a systemic treatment for cancer. As for most kinds of solid tumor, they can help achieve an acceptable anticancer activity respectively. The dose limiting side effects, narrow pharmaceutics index with unique pharmacological profile and multidrug resistance (MDR) largely limits the range of application. Breast cancer can be regarded as a public health issue which bears the highest rate of female malignant disease diagnose around the world. Thus, discovering a high-performance of anticancer drug delivery is important. In this study, cockle shell-derived calcium carbonate nanoparticles (ANPs) along with Vincristine Sulphate and Doxorubicin was synthesized as the passive targeting nanoparticles drug delivery system. The DOX/VCR-ANPs co-delivery system and VCR-ANPs delivery system were characterized and evaluated through TEM, FESEM, Zeta sizer and Zeta potential. Drug loading and control release study, MTT assay, cell uptake assay, cell morphology observation, and AO/PI double stain assay *in vitro* were also conducted. Through this study, the results revealed that the cockle shells-derived $CaCO_3$ aragonite nanoparticles (ANPs) are homogeneous spherical and porous particles with zeta potential negatively charge of -34.5 mV, and with a zeta size of 157.8 nm in diameter. After loading with DOX/VCR or VCR alone, the nanoparticle porosity become unclear and size increased. The result also shows that the Vincristine Sulphate and Doxorubicin can effectively be loaded into calcium carbonate nanoparticles and keep a fast release at pH 4.8 and a sustained release at pH 7.2 up to 3 days. After being loaded into ANPs, VCR exhibits a better anticancer efficiency in breast cancer treatment while the IC_{50} value of VCR-ANPs is half of that in free VCR. The synergistic effect of VCR and DOX significantly improves the antineoplastic efficient *in vitro*, which demonstrates the lowest combination index (0.0433 at 48 hours of treatment and 0.08325 at 72 hours of treatment) under the DOX/VCR ratio of 3/1. For the cell morphology examination under microscope and

florescence microscope, the cells showed an evident apoptosis *in vitro*. Overall, aragonite nanoparticles delivery system loaded with VCR and DOX can efficiently act against breast cancer with higher anticancer efficiency and significant synergistic effect which can be attribute to the ANPs control release ability and the unique physicochemical property of aragonite nanoparticles.

Key Words: anticancer, Vincristine sulfate, Doxorubicin, nanoparticle drug delivery system

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

RAWATAN IN-VITRO SEL KANSER PAYUDARA MCF-7 DAN MDA-MB-231 DENGAN PARTIKEL NANO CALCIUM CARBOANATE-DOXORUBICIN DAN VINCRISTIN SULFATE

Oleh

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Pengerusi : Profesor Md Zuki bin Abu Bakar, PhD
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Vincristine sulfat (VCR) dan doxorubicin (DOX) merupakan ubat kemoterapi yang digunakan secara meluas untuk merawat kanser secara sistemik. Rawatan berasingan yang dilaksanakan atas kebanyakan sel pepejal tumor dapat mencapai tahap aktiviti anti kanser yang dikehendaki. Terapi gabungan vincristine sulfat bersama doxorubicin sering digunakan di klinik untuk menyembuh neoplasma hematologi (hematologic neoplasms) kerana mempunyai fungsi mekanisme berbeza yang menghasilkan kesan sinergi serta tiada kesan sampingan yang bertindih. Kesan-kesan sampingan mengikut dos kemoterapi, profil farmakologi unik dengan indeks farmaseutikal yang terhad dan perintang pelbagai dadah (MDR) mengehadkan aplikasi prosedur ini.. Kanser payudara boleh dianggap sebagai satu isu kesihatan awam adalah antara penyakit merbahaya yang paling tinggi didiagnos dalam kalangan wanita di seluruh dunia. Oleh itu, kajian mengenai cara penghantaran ubat anti kanser yang efektif amat penting. Dalam kajian ini, kaedah penghantaran nano secara pasif menggunakan gabungan partikel nano kalsium karbonat dari kulit kerang (ANPs) bersama vincristine sulfat dan doxorubicin. Penghantaran secara gabungan DOX/SV-ANPs dan sistem penghantaran VCR-ANPs telah dikategorikan dan dinilai melalui TEM, FESEM, Zeta sizer dan Zeta potential. Kajian-kajian lain juga dilaksanakan seperti pemuatan dan pembebasan terkawal dadah, ujian MTT, ujian penyerapan sel, pemerhatian morfologi sel, dan ujian dwipewarna AO/PI secara *in vitro*. Melalui kajian ini, keputusan menunjukkan bahawa partikel nano aragonite CaCo3 dari kulit kerang berbentuk sfera secara seragam dan berporos dengan potensi zeta bercaj negatif pada -34.5 mV, serta saiz zeta berdiameter 157.8 nm. Setelah dimuatkan dengan DOX/SV ataupun SVkeporosan partikel nano tidak dapat dikesan dan saiz bertambah. Hasil kajian juga menunjukkan bahawa vincristine sulfat dan doxorubicin mampu dimuatkan secara efektik ke dalam partikel-partikel nano kalsium karbonat dan dapat mengekalkan sifat pembebasan pantas pH 4.8 dan pembebasan kekal pada pH 7.2

selama tiga hari. Selepas dimuatkan ke dalam ANPs, IC_{50} VCR_ANP adalah separuh daripada VCR bebas menunjukkan ia adalah antikanser yang lebih berkesan untuk merawat kanser payudara. Kesan gabungan VCR dan DOX mendatangkan hasil yang signifikan bagi meningkatkan keberkesanan antineoplastik *in vitro* yang demonstrate indeks gabungan terendah (0.0433 pada 48 jam rawatan dan 0.08325 setelah 72 jam rawatan) menggunakan nisbah DOX/SV pada 3/1. Bagi kajian morfologi sel melalui permerhatian mikrosop serta mikroskop fluoresen, didapati sel-sel menunjukkan apoptosis *in vitro*. Kesimpulannya, system penghantaran nano partikel aragonite yang dimuatkan dengan VCR dan DOX boleh bertindak untuk melawan kanser payudara dengan keberkesanan anti kanser yang tinggi dan kesan sinergi dengan signifikan yang disebabkan oleh keupayaan pembebasan terkawal ANPs dan ciri-ciri fizikal dan kimia nano partikel aragonite yang distingtif.

Kata Kunci: anti kanser, Vincristine Sulfate, Doxorubicin, sistem penghantaran ubat nano partikel

ACKNOWLEDGEMENTS

First, I really want to thank my father who encourages me to apply to University Putra Malaysia and study overseas. During my 3 years' study in Malaysia, my parents continuously support me financially and spiritually.

I also want to express my deepest gratitude and respect to my supervisor Prof. Dr Md Zuki Abu Bakar and co-supervisor Dr. Mokrish. I was born to be a shy kid with more or less sociality dread. I'm so lucky have chance to be their student and learn a lot good research habit from them. During the most tough time of my life, I don't feel so alone because of their courage, guidance and help without reservation, and they drive me to complete my master project with their knowledgeable ideas, constructive criticism and trust on me.

I should express the appreciation to our lab staffs (Mr. Jamile, Miss Nor Hayati) and my dear friends (Mustaffa Saddam Ghaji, Saffanah Kuder, Sharifah Zakiah, Fadzly Salleh and Fu Wenliang). Thanks for all of the warm help and care I receive in this alien land.

Lastly, I want to thank UPM for giving me the chance to meet all these dear persons and complete my master journey.

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

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USA	United States of America
5-FU	fluorouracil
TNBC	triple-negative breast cancer
P-gp	P-glycoprotein
ABC	ATP-binding cassette
MDR	multidrug- resistant
DC	Loading content
EE	Encapsulate efficiency
FESEM	Field emission scanning electron microscopy
ANPs	Aragonite nanoparticles
VCR-ANPs	Vincristine sulfate-loaded Calcium Carbonate nanoparticles
DOX/VCR-ANPs	Doxorubicin /Vincristine sulfate-loaded Calcium Carbonate nanoparticles
PBS	Phosphate Buffer Saline
TEM	Transmission electron microscopy
Nm	Nano meter
mL	Milliliter
µ	Micro meter
mg	milligram
MRP	multidrug resistance-associated protein
EPR	enhanced permeability and retention
DOX	Doxorubicin
VCR	Vincristine sulfate
DDS	Drug delivery system
RES	Reticuloendothelial system
MCF-7	Michigan Cancer Foundation-7
PR	Progesterone
ER	Estrogen
HER-2	Human epidermal growth factor receptor 2
AIs	Tamoxifen /aromatase inhibitors
DCIS	Ductal carcinoma in situ
MBC	Metastatic breast cancer

mAbs	Monoclonal antibodies
DNA	Deoxyribonucleic acid
MAP	Microtubule-associated proteins
NACT	Neoadjuvant chemotherapy
pCR	Pathological complete response
DFS	Disease free survival
OS	Overall survival
NATC	North American Trials Council
ERP	Permeation and retention effect
API	Pharmaceutical ingredients
MPS	Mononuclear phagocyte system
CaCO3	Calcium carbonate
BS-12	Amphoteric surfactant BS-12
RNA	Ribonucleic Acid
VIPN	Vincristine-induced peripheral neuropathy
ALL	Acute lymphocytic leukemia
MBD	Microtubule-binding drugs
MRP1	Multidrug resistance-associated protein 1
ATP	Adenosine Triphosphate
PSD	Particle size distributions
CHOP	(C)yclophosphamide, (H)ydroxydaunorubicin, (O)ncovin (vincristine), (P)rednisone)
DMEM	Dulbecco's modified eagle medium
FBS	Fetal bovine serum
CGM	Complete growth media
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide
CO_2	Carbon dioxide
IC_{50}	Lethal concentration, 50%
CI	Combination index
DRI	Dose-reduction index
AO/PI	Acridine orange (AO) and propidium iodide (PI)
h	Hour (s)
µg/mL	Microgram per Milliliter

CHAPTER 1

INTRODUCTION

1.1 General Background

Breast cancer can be regarded as a public health issue which demonstrates the highest rate of female malignant disease diagnose around the whole Malaysia. There are 1.4 million new cases emerging worldwide and the corresponding age-standardized rate (ASR) is 47.3 per 100 000 per year in Malaysia. Breast cancer occurred in situ is milder and easier to be cured compared to ductal carcinoma, thus ductal carcinoma draws more concern in clinical cancer therapy. In Malaysia, 30%-60% of breast cancer patients tend to in the local advance and metastatic stage. The breast cancer's 5-year-survival rate decreases dramatically from 100% (in situ) to 83.6% (early invasive), 57% (locally advanced), and 23% (metastatic) respectively. Among all new emerging breast cancer cases, around one-tenth will be diagnosed as in incurable stage, and about a third to a half of the rest will progress into incurable metastasis cancer during years followed (Gradishar Anderson *et al.*, 2017; *Nunez et al*., 2016; Ewertz & Jensen 2011; Roche & Vahdat 2011; Yadav *et al*,. 2014; Maughan *et al.*, 2010).

Combinations of chemotherapy, surgery, radiotherapy and endocrine therapy are used as modern clinical breast cancer therapeutic method. Normally, chemotherapy is routinely administrated on metastatic cancers' patients. In order to control the progressed breast cancer after first-line chemotherapy (anthracyclines and taxanes), 5-FU, vinblastine, vincristine, methotrexate, mitomycin C, and platinum agents are commonly used (Roche & Vahdat 2011). Till now, there is no standard treatment guild line for triple-negative breast cancer (TNBC), and the only systemic treatment for TNBC is chemotherapy (Yadav *et al.*, 2014).

Chemotherapy is one of the standard methods to treat carcinoma, and besides, it demonstrates a lot of advantages in clinical usage: convenience in injection administration, age-independent efficacy, less invasive. Traditional small molecular chemotherapy agents have the adverse features however. The dose intensive of cytotoxic drug influences clinical survival rate and guarantee the curative effect. In clinical, actual administrate chemotherapy is always underdosed because of the lacking specificity and selectivity related cumulative toxicities, wide drug distribution in organs and tissues, and short half-life in blood serum. For the small molecular drug, it diffuses easily back to the blood circulation showing a lower retention time on tumor microenvironment. Some anticancer drugs also meet the problem of free drug stability and solubility.

Concerning the working mechanism of some anticarcinogen, the pharmacological desired locations are cytoplasm or the nucleus. There are several barriers in a cellular level including cytomembrane, nuclear envelope of cancer cell, efflux related P-gp

over-expression, and the detention in endosome or lysosome. As the cell membrane is lipoid, small molecular lipid-soluble drugs show a quicker diffusion and penetration than relatively lipid-insoluble larger drugs. But the hydrophobicity of drug is always followed by low hydrophilic influencing the dissolve and distribution of free drug.

The overwhelming multidrug resistance is widely observed in various type of cancer cells to weaken chemotherapy anticancer efficiency. The inhibition of the apoptosis pathway and over-expression of ATP-binding cassette (ABC) transporters are two of the most common underlying origins of MDR (Wang *et al*., 2013). P-glycoprotein (P-gp) which encode by MDR-1 gene or survivin, has been studied the most among all the other ATP-binding cassette transporters. P-gp exists as a potential target in breast cancer treatment to overcome MDR. There are two reasons: the first reason is around half of the breast cancer cases over express P-gp. The second reason is that over expression of the multidrug resistance transporters P-glycoprotein (PGP) or multidrug resistance-associated protein (MRP) are the part of self-defensive system with many cytotoxic agents as substrate, including VCR and DOX, which effectively transports the chemotherapy drug extracellular and decrease the intracellular accumulation drug concentration (Nunez *et al*., 2016). More frequent injections or higher dosage in single dose is the solution of chemotherapy underdose, but it always followed by dose-dependent toxicity. The sever side effect and MDR (multidrug resistant) are the two main barriers restricting the applicability of chemotherapy dosage through intravenous injection.

Nanoparticle has a broader area of applications and has increasingly becoming a new alternative pharmaceutical transporting application to overcome MDR, it helps decrease the systemic toxicity, maximize the anticancer efficiency and optimize therapeutic index which draw a lot of researchers' attention in recent years. These biocompatible (or biodegradable) particles can be divided into several different group: prodrugs, polymeric nanomicelles, liposomes, solid lipid nanoparticles, nanoparticles of biodegradable polymers, nanohydrogels, inorganic nanoparticles and dendrimers.

Comparing to free drug, nanodrug is an alternative pharmaceutical form which bears a lot of advantages in clinical usage, including: increase drug solubility by encapsulating poorly soluble drugs, specific tumor targeting, enhanced drug intracellular accumulation concentration around tumor, protect therapeutic molecules by avoiding inactive of drugs before reaching the established site through release control, decrease systemic toxicity of chemotherapy agent and increased maximum tolerate dosage because of the enhanced permeability and retention (EPR) effect and passive targeting mechanism (Bertrand *et al*., 2014; Kamba *et al*., 2013).

Co-delivery of two toxicity agents was developed with the advances in nanotechnology, and it demonstrates that co-delivery with certain magnitude can significantly enhance the anticancer efficiency and generate synergistic effect comparing with two separate drug administration when treating same population of

cells. The requirement of co-delivery construction is that two drugs shall bear different pharmacological mechanisms or with non-overlapping toxicities and side effects, which are normally designed based on the clinical manifestation.

Combination administration of co-delivery has several potential benefits compared to the separate delivery of single agent. More specifically the benefits area illustrated as below: administration convenience, patient compliance, synchronized pharmacokinetics, avoiding the uncertainty caused by dose fractionation, and defining the comparative formulation of both drug in same site (Nunez *et al*. 2016).

Calcium carbonate bears the potential to be a good candidate material for drug delivery in the future, which helps deliver the small molecule drugs to the target sites and improve therapeutic efficacy.

As a drug delivery system, calcium carbonate nanoparticle has several common advantages when comparing with other similar drug delivery system and overcome the shortcomings of conventional chemotherapy, which can be describe below: promote the dug accumulation in tumor related area, increase the anticancer efficiency and the maximal tolerance dose, reduce non-selectivity toxicity of cytotoxic substances (Kamba et al. 2013). The theoretical basis of nanoparticle anticancer delivery system is that the enhanced permeability and retention (EPR) effect will increase the site-specific drug accumulation and evade the reticuloendothelial system (RES) (Kamba et al. 2014).

Compare with the assembled organic polymers, calcium carbonate nanoparticles based drug delivery system in clinical usage bears several unique advantages, including calcium carbonate biocompatibility, low cost in raw material and synthesis process, high affinity for bone uptake, a tissue penetration ability, prolonged plasma circulation, optimized therapeutic index via modifying the pharmacokinetics parameter, and pH stimuli response degradation (Danmaigoro et al., 2017).

The more research of inorganic material conducted, the more we realized that aragonite calcium carbonate contain several unique properties comparing other inorganic nanoparticles. More specifically, the corresponding advantages of the calcium carbonate nanoparticle are illustrated as below: the size and the shape of ANPs is variable and adjustable during the forming process with different experimental conditions, calcium carbonate is a natural material with minimum cellular toxicity, and the pH degradation ability can guarantee the precise targeting drug fast release to increase the anticancer efficiency.

The theoretical basis of nanoparticle anticancer delivery system lies in that the enhanced permeability and retention (EPR) effect will increase the site-specific drug accumulation and evading the reticuloendothelial system (RES) (Kamba et al., 2014).

Although many great breakthroughs of drug delivery system happened and there are several types of delivery systems came into the market in the past decades, the current drug delivery systems still have some problems and barriers which need further improvement in the future. The problems and barriers are listed below: the nanosurface toxicity of nanoparticles for healthy cells, the complicate and time-consuming nanoparticle production process combining with some poisonous surfactant or catalyst, the lacking of intracellular organelle-level targeting of current drug delivery system. In the cellular level, inflammation, oxidative stress, or mitochondrial dis-function could be introduced by nanoparticles.

1.2 Problem Statements

With the high incidence, low recovery rate of human breast cancer and the corresponding short life expectation, systemic therapy (endocrine treatment and chemotherapy) is widely used in breast cancer therapy from early-stage invasive to metastatic stage. Both doxorubicin and vincristine are the candidate option in breast cancer therapy and exist as the substrate of MDR.

In the conventional no-selective delivery systems, DOX is difficult to achieve hypothetical drug concentration and dosage, resulting in narrow therapeutic index and largely increasing the dose distribution to normal healthy tissues (Kamba *et al*., 2013). The therapy application of free VCR is quite limited, normally used to treat hematological diseases. This kind of limited clinical applicability mainly lies in the poor pharmaceutical properties of VCR (including quick initial plasma clearance, short initial plasma halve life, and extensive volume of distribution *in vivo*), severe neurological/hematological side effect of VCR which is dose-limiting toxicities and narrow therapeutic index, and wide interpatient variable pharmacologic characteristics. In general, the poor drug pharmaceutical form, chemotherapy side effect, multidrug resistant largely limits the usage in clinical. Thus an efficient, no-toxicity, stable DDS is in urgent.

1.3 Hypothesis

i. VCR and DOX can be efficiently loaded into the ANPs.
ii. VCR-ANPs and DOX/VCR-ANPs can be used as nano-carrier in controlling drug release.
iii. Both VCR-ANPs and DOX/VCR-ANPs bear an effective resistance to breast cancer *in vitro* reducing drug administration dosage when comparing to free drug.
iv. DOX/VCR-ANPs have the synergistic effect in breast cancer therapy.

1.4 Research Questions

i. What is the *in vitro* drug release profile of VCR-ANPs and DOX/VCR-ANPs?
ii. How effective VCR-ANPs and DOX/VCR-ANPs are in the treatment in MCF-7 and MDA-MB-231 cell line, and what are the differences between them?
iii. How VCR-ANPs and DOX/VCR-ANPs can reduce the dose of VCR and DOX?

1.5 Objective of study

1.5.1 Main Objective of the Study

This study was conducted with the aim of investigating the anticancer effectiveness of VCR-loaded calcium carbonate nanoparticles and VCR/DOX-loaded calcium carbonate nanoparticles in MCF-7 and MDA-MB-231 cell line.

1.5.2 Specific Objectives

i. To synthesis and characteristic of the free ANPs, VCR-ANPs and DOX/VCR-ANPs separately using TEM, FESEM, zeta sizer, zeta potential.
ii. To evaluate drug loading content and encapsulation efficiency of VCR-ANPs and DOX/VCR-ANPs separately.
iii. To evaluate the release profile of VCR-ANPs and DOX/VCR-ANPs using drug release study in physiological (pH 7.2) and acidic pH (4.8) environment.
iv. To evaluate the anticancer efficiency of VCR-ANPs and DOX/VCR-ANPs in the treatment MCF-7 and MDA-MB-231 cell line *in vitro* through MTT assay when comparing the anticancer efficiency of free DOX/VCR solution.
v. To evaluate the synergistic effect of VCR and DOX in DOX/VCR-ANPs by calculating the combination index and dose-reduction index

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