COMBINATION TREATMENT OF DOXORUBICIN AND SAR405-LOADED CHITOSAN NANOPARTICLES FOR DUAL TARGETED ANTICANCER THERAPY ASSESSMENT IN A549 LUNG CANCER CELL LINE

By

ALAMASSI MOHAMMEDARFAT N K

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

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

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Chair : Associate Professor Mas Jaffri Masarudin, PhD
Faculty : Biotechnology and Biomolecular Sciences

Conventional anti-cancer drugs including doxorubicin are associated with high toxicity and non-specific distribution in the body resulting in acute side effects. targeting cancer cell survival pathways like autophagy can be enhanced the effective of therapeutic drugs at low concentration to decrease its side effects. Cancer cells can use autophagy to promote tolerance to the stress caused by anti-cancer agents, leading to resistance induction in advanced tumors. SAR405 is an inhibitor of autophagy activity due to its molecular interactions within the ATP binding site but is prematurely degraded extracellularly, therefore is poorly up taken by cells limiting its use. Chitosan nanoparticles (CNPs) are considered to be biologically degradable, non-toxic, and biocompatible as a drug delivery system to minimize and determine their side effects. In this study, we developed a therapeutic strategy to enhance doxorubicin efficiency while simultaneously inhibiting autophagy, by increasing cellular uptake of SAR405 loaded into CNPs. The synthesized nanoparticles were characterized for hydrodynamic diameters and polydispersity measurements. Encapsulation efficiency and drug loading was then elucidated and subsequent morphological of the nanoparticles were determined by electron microscopy. Cytotoxicity assessment and apoptosis were performed through MTT and Annexin-v apoptosis assays. Following encapsulation, the SAR405-loaded chitosan nanoparticles expanded from 54 nm to 161 nm at 10 μM SAR405, while the polydispersity index increased from 0.11 to 0.31. When cells were treated with IC50 values of doxorubicin and 10 μM of SAR405 encapsulated CNP, approximately 47% reduction in viability of cells were observed in Annexin V-FITC/PI assay, compared to doxorubicin alone. Inhibition of autophagy was shown to decrease the resistance of cancer cells to therapeutic drugs and increase their efficacy at low concentration. This study promises targeting of cancer cell survival pathways can be an effective way to Increasing the efficiency of chemotherapeutic drugs.

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

GABUNGAN RAWATAN DOKSORUBISIN DAN NANOPARTIKEL KITOSAN BERISI SAR405 UNTUK PENILAIAN TERAPI ANTIKANSER DWITARGET DALAM TITISAN SEL KANSER PARU-PARU A549

Oleh

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Ubat anti-kanser konvensional termasuk doxorubicin dikaitkan dengan ketoksikan tinggi dan pengedaran tidak spesifik dalam badan, mengakibatkan kesan sampingan akut. Dengan menyasar tapak jalan kemandirian sel kanser seperti autofagi, kesannya boleh dipertingkatkan dengan menggunakan ubat terapeutik pada kepekatan rendah untuk mengurangkan kesan sampingannya. Sel kanser boleh menggunakan autofagi untuk menggalakkan toleransi terhadap tekanan yang disebabkan oleh agen anti-kanser, yang menyebabkan induksi rintangan dalam tumor yang kritikal. SAR405 ialah perencat aktiviti autofagi kerana interaksi molekulnya dalam tapak pengikatan ATP, namun ia ternyahasli secara pramatang secara ekstraselular; oleh itu, ia kurang diambil oleh sel, lalu mengehadkan penggunaannya. Nanopartikel kitosan dianggap boleh terurai secara biologi, tidak toksik dan biokompatibel sebagai kargo penghantaran ubat. Dalam kajian ini, kami membangunkan strategi terapeutik untuk meningkatkan kecekapan doksorubisin sambil menghalang autofagi secara serentak, dengan meningkatkan pengambilan selular SAR405 yang dimuatkan ke dalam nanopartikel kitosan. Nanozarah yang disintesis telah dicirikan untuk diameter hidrodinamik dan ukuran polidispersi. Kecekapan enkapsulasi dan pemuatan dadah kemudiannya diperincikan dan morfologi nanozarah seterusnya ditentukan oleh mikroskop elektron. Penilaian sitotoksisiti dan apoptosis dilakukan melalui ujian apoptosis MTT dan Annexin-v. Berikutan pengkapsulan, nanozarah kitosan yang dimuatkan SAR405 berkembang daripada 54 nm kepada 161 nm, manakala indeks polidispersiti atau penyebaran meningkat daripada 0.11 kepada 0.31 Apabila sel dirawat dengan nilai IC50 doxorubicin dan 10 μM SAR405, kira-kira 47% pengurangan daya tahan sel dalam ujian Annexin V-FITC/PI, berbanding dengan doksorubisin sahaja. Perencatan autofagi mengurangkan rintangan sel kanser terhadap ubat terapeutik dan meningkatkan keberkesanannya pada kepekatan rendah. Kajian ini menjanjikan bahawa menyasarkan laluan survival sel kanser sebagai cara yang mujarab untuk meningkatkan kecekapan ubat kemoterapi.

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

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Declaration by Members of the Supervisory Committee

This is to confirm that:

- the research and the writing of this thesis were done under our supervision;
- supervisory responsibilities as stated in the Universiti Putra Malaysia (Graduate Studies) Rules 2003 (Revision 2015-2016) are adhered to.

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LIST OF ABBREVIATIONS

A549	Adenocarcinomic human alveolar basal epithelial cell line
ULK	Unc-51-like Autophagy-Activating Kinase
A430nm	Absorbance at 430nm
A570nm	Absorbance at 570nm
A335	Absorbance at 570nm
BIF-1	Bax Interacting Factor-1
CNP	Chitosan nanoparticle
CNP-SAR405	Chitosan nanoparticle encapsulated SAR405
CNT	Carbon Nanotube
Conc.	Concentration
CS	Chitosan
Mwt	Molecular weight
dH2O	Deionized distilled water
DLS	Dynamic Light Scattering
NNMT	Nicotinamide N-methyl transferase
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
DIW	Deionized Water
EE	Encapsulation Efficiency
EDTA	Ethylenediaminetetraacetic acid
ERK	Extracellular Signal-regulated Kinase
FBS	Feta bovine serum
FDA	Food and Drug Administration
TEM	Transmission Electron Microscopy

FITC	Fluorescein isothiocyanate
GFP	Green Fluorescence Protein
HCl	Hydrochloric acid
HUVEC	Human Umbilical Vein Endothelial Cells
IC_{50}	Half maximal inhibitory concentration
ERK	Extracellular single-regulated kinase
MTT	3-(4 5-dimethylthiazol-2-yl)-2 5-diphenyltetrazolium bromide
MW	Molecular weight
NaOH	Sodium hydroxide
NH_2	Amine group
$NACO_3$	Sodium Carbonate
TNBS	Trinitobenzenesulfonic acid
NSCLC	Non-small cell lung carcinoma
PBS	Phosphate buffer saline
PCA	Protocatechuic acid
pCNP	Palmitoyl chitosan nanoparticle
TPP	Tripolyphosphate
pCS	Palmitoyl chitosan
PDI	Polydispersity Index
PEG	Polyethylene Glycol
PI	Propidium Iodide
PSD	Particle Size Distribution
RNA	Ribonucleic acid
ROS	Reactive Oxygen Species
rpm	Revolutions Per Minute
RPMI	Roswell Park Memorial Institute

^{o}C	Degree Celsius
SDS	Sodium Dodecyl Sulphate

CHAPTER 1

INTRODUCTION

1.1 Background

Despite recent advancements in medicine, cancer is still considered one of the most researched illnesses owing to its complexity and steadily rising incidence rate over the years. Globally, 19.3 million new cancer cases were diagnosed, with an estimated 295.3 cases of cancer per 100,000 people in areas with very high human development, compared with 115.7 in areas with low human development in 2020. The most often diagnosed malignancies are lung cancer (11.4%), colorectal cancer (10%), prostate cancer (7.3%), and stomach cancer (5.6%). According to estimates, the global cancer burden will reach 28.4 million cases in 2040, up 47% from 2020, with a greater increase in developing (64%) than developed (32%) countries. Despite being the most widespread cancer in the world, lung cancer is also the greatest cause of cancer-related fatality, with just a 15% five-year survival rate (Sung et al., 2021a). Due to this worrying incidence, an efficient therapy must be developed.

Chemotherapy is the primary therapy for cancer due to its high efficacy in killing cells. Doxorubicin (DOX), a non-specific cell cycle inhibitor, was one of the two first anthracycline antibiotics identified from *Streptomyces paucities* and is one of the most commonly used chemotherapeutic agents to date. DOX stops cell proliferation by intercalating into DNA; inhibiting DNA synthesis and function via inhibition of topoisomerase II and formation of free radicals (Shoshan & Linder, 2008). As the pathway exists in all cell types, DOX-induced cytotoxicity also leads to induction of cell death pathways in not only cancer cells but also in normal cells soliciting unwanted side effects such as severe toxicity in normal organs and rapidly dividing cells. Chemotherapeutic persistence has also been reported in cancers treated with regular dosages of doxorubicin, where neoplastic cells develop drug resistance due to their pro-apoptotic ability to activate a number of mechanisms as a resistance to the chemotherapy treatment (Dandoti, 2021). These has included including drug inactivation, inhibition of cell death (apoptosis suppression), alterations in drug metabolism, enhanced DNA repair, gene amplification, and autophagy stimulation. Therefore, these recent findings have led to the notion that therapeutic regimes against cancer should be streamlined towards addressing a more holistic view towards this dynamic disease. Increasing the effectiveness of anticancer therapies is no longer a subject of producing more potent chemotherapy drugs, but instead designing combinatory therapies containing multiple moieties that target more than one aspect of cancer cell biology.

Autophagy is a well conserved response to nutrient restriction, in which starving cells use it as a recycling mechanism to temporarily compensate for the absence of external nutrients (Limpert et al., 2018). Autophagy also plays a role in the

elimination of long-lived or superfluous proteins and organelles. Autophagy, for example, is responsible for the removal of paternal mitochondria following conception. Another key role of this process is the destruction of defective mitochondria, which limits the release of reactive oxygen species and other harmful proteins into the cytosol. Protein aggregation clearance is also important for protecting cells from hazardous cellular waste. Autophagy is required for maintaining cellular homeostasis in addition to its function as a response to cellular stress. As a result, autophagy is a critical catabolic process in cell formation, differentiation, and survival.

However, autophagy has been shown to be triggered in certain cancer cells as a reaction towards the cellular stress caused by exposure to an anticancer medication. In this example, inhibiting autophagy can potentially render cancer cells more sensitive to treatment of cisplatin and increases the lethal effects of chemotherapeutic drugs to 40% (Schlütermann et al., 2018). Autophagy has been characterized as a pro-survival process seen in most advanced malignancies, allowing tumors to adapt to various stressors such as hypoxia and food restriction, and therefore influencing tumor development (Limpert et al., 2018). As a result, many advanced malignancies have more basal autophagic activity than normal tissues, and some have been labelled "autophagy-dependent," such as pancreatic cancer or activated Ras tumors (Pérez-Hernández et al., 2019). This basal autophagy also aids cancer cell adaptation to treatment-induced stressors, resulting in therapy resistance in malignant malignancies, which is a significant clinical problem. Autophagy has also been linked to the survival of latent cancer cells and the recurrence of metastatic tumors. As a result, inhibiting autophagy may not allow cancer cells to adapt to stress when they treat with chemotherapy leading to increasing the cell death pathway. This can open a window for improving chemotherapeutic efficacy and clinical outcomes in the treatment of malignant cancers.

SAR405 is an autophagy inhibitor compound that was synthesized as a low-molecular-mass with a high specificity for Vps34, a critical protein in the autophagy mechanism. A study was shown the selectivity of SAR405 binding with ATP binding site of Vps34 affects vesicle trafficking between early and late endosomes and prevents autophagy (Pasquier, 2015). However, this compound is administered naked, it has been shown to have poor cellular absorption and stability in vivo. Increasing its cellular uptake and accumulation in cancer cells is one potential technique for increasing its efficiency to increase the autophagy inhibition level at which cancer cells use it as a pro-survival pathway. The emphasis of research has been on helping or improving existing medication delivery methods. Over the years, several researchers have reported on the use of nanoparticles to encapsulate cargos such as different medicinal medicines or chemicals, as well as genetic elements.

In this study a nanoparticle-mediated delivery system was adopted to increase the intracellular uptake of SAR405 into A549 lung cancer cell models. Encapsulation within a delivery vector protects the inhibitor from extracellular enzymatic degradation, and repacking it within nano-sized dimensions may

facilitate greater uptake into cells. Chitosan nanoparticles (CNPs) were selected as a delivery system for SAR405, being an inert polymeric nanoparticle system widely applied in drug administration applications (Fu et al., 2016). In contrast to other nanomaterials requiring surface modifications prior to *in vivo* and *in vitro* use, CNPs are a polymer-based organic nanomaterial conferring easier preparation approaches , biologically-compatible , and possess improved *in vivo* distribution (Fu et al., 2016). The SAR405-encapsulated nanoparticle system was formed through complexation of the chitosan polymer (CS) with SAR405, and subsequent cross-linking with sodium tripolyphosphate (TPP) initiating particle formation through ionic gelation processes (Figure 1.1).

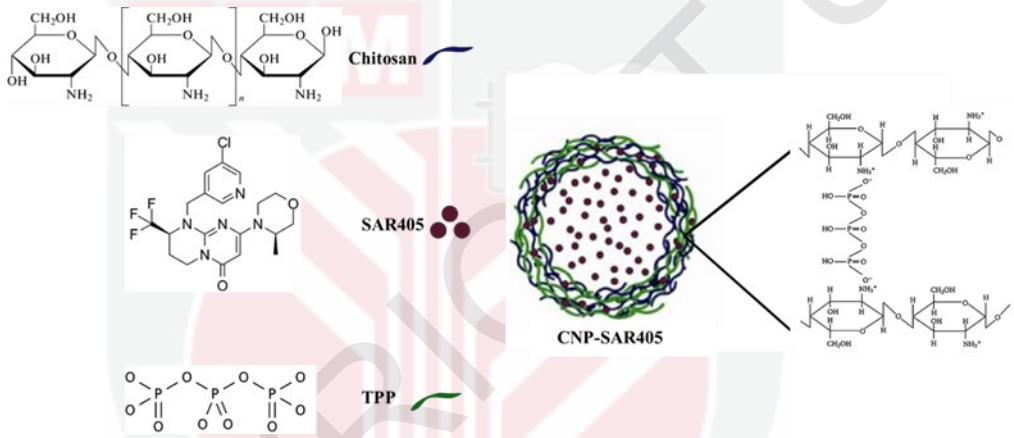

Figure 1.1 : A Schematic representation of the preparation of chitosan-TPP nanoparticles and SAR405-loaded chitosan

The system was then used in a binary treatment regime containing doxorubicin and the SAR405-encapsulated CNPs against A549 lung cancer cells, where the combination incurs apoptotic responses by targeting both cellular-death by apoptotic pathways and sequential inhibition of autophagy. By preventing cancer cells undergoing apoptosis to activate autophagy as a pro-apoptotic response, this system was able to increase the effectiveness of doxorubicin as an anticancer agent (Figure 1.2). Consequent of this, similar cell death rates are achievable at lower doxorubicin concentrations – a proof of concept elucidating the importance of employing a multi-facet approach towards anticancer therapies in the future.

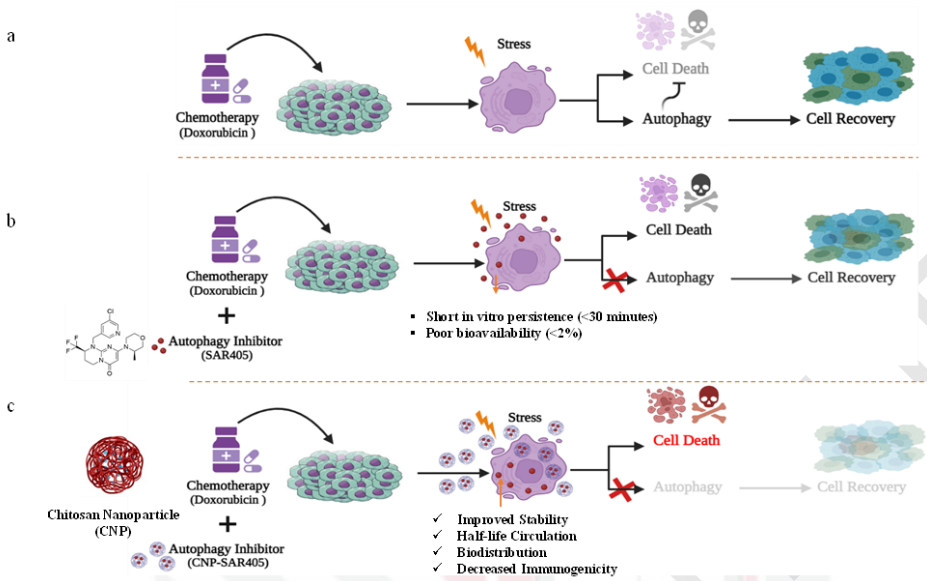

Figure 1.2 : A schematic representation of the binary treatment approach using CNPs as a delivery vector for SAR405. Doxorubicin, a potent cellular apoptotic agent may induce autophagic responses in cancer cells. As a result, this pro-apoptotic event leads to (a) cancer cell recovery and causes doxorubicin resistance in advanced cancers. Alternatively, autophagy inhibitors such as SAR405 may be able to prevent activation of autophagy but instead (b) suffers from limitations including short in vivo persistence as well as poor bioavailability. Therefore, (c) adoption of a nanoparticle delivery system such as CNPs increase the stability, intracellular uptake, and biodistribution of SAR405 in cells. As a consequence, autophagy is arrested in the cancer cells, enhancing the efficacy of doxorubicin by inhibiting autophagy activation leading to increased cell death.

The purpose of this project is to develop a dual treatment system for anticancer therapy which targets both cell death and autophagy pathways of A549 lung cancer cells for enhancing the efficacy of the chemotherapy drugs by inhibiting of autophagy pathway. This involves use of doxorubicin + SAR405, in this project SAR405 is delivered via CNP.

1.2 Objectives

1.2.1 General objective

To develop a dual-treatment system for anticancer therapy which targets both the apoptotic and autophagic pathways of A549 lung cancer cell using Doxorubicin + CNP-SAR405.

1.2.2 Specific objectives

1. To characterize the formation of SAR405-encapsulated chitosan nanoparticles, CNP-SAR405.
2. To determine the efficacy of Doxorubicin in co- treatments with CNP-SAR405 toward A549 lung cancer cells.
3. To assess autophagy progression in A549 cells treated with Doxorubicin in co-treatments with CNP-SAR405.

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