IN VITRO AND IN VIVO TOXICITY OF DOCETAXEL-LOADED POLYMERIC NANOPARTICLES FORMULATION FOR POTENTIAL TREATMENT OF LUNG CANCER

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by

FAISALINA BINTI AHMAD FISOL

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DEDICATION

This thesis is dedicated to

My beloved father, mother, husband and families

Dato' Hj. Ahmad Fisol b Md Nor

Datin Hjh. Suhailah bt Hj. Husin

Hafizul Hanif b Abu Bakar

Ahmad Firas Haziq b Hafizul Hanif

Fatimah Az Zahra bt Hafizul Hanif

Fahimah Hasna bt Hafizul Hanif

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LIST OF SYMBOLS

°c	Degree Celcius
%	Percentage
µg/ml	Microgram per mililiter
µm	Micrometer
M	Molar
µl	Microliter
ml	Miliiter
AU	Absorbance unit
mg	Miligram
mg/ml	Miligram per mililiter
min	Minute
hr	Hour
nm	Nanometer
pH	Scale of basicity and acidity
ppm	Parts per million
mg/kg	Miligram per kilogram

LIST OF ABBREVIATION

DTX	Docetaxel
FDA	Food and Drug Administration
NSCLC	Non-small cell lung cancer
IV	Intra venous
PTX	Paclitaxel
NP	Nanoparticle
PNP	Polymeric nanoparticles
EPR	Enhanced permeability and retention
PCL	Poly-ε-caprylactone
Vitamin E TPGS	d-α-tocopheryl polyethylene gycol 1000 succinate
PVA	Polyvinyl alcohol
PDI	Polydispersity index
HPLC	High performance liquid chromatography
PCLS	Precision cut lung slices
LDH	Lactate dehydrogenase
BAL	Bronchiol alveolar fluid
P-gp	Glycoprotein
SCLC	Small cell lung cancer
SCC	Squamous cell carcinoma
NCR	National Cancer Registry
WHO	World Health Organization
DDS	Drug delivery systems
PLGA	Poly(lactide-co-glycolide)

PEG	Polyethylene glycol
DMSO	Dimethylsulfoxide
MTD	Maximum tolerated dose
ACN	Acetonitrile
DLS	Dynamic Light Scattering
Mw	Molecular weight
NC	Nanocapsules
NS	Nanospheres
RI	Refractive Index
SEM	Scanning electron microscopy
DMEM	Dulbecco's Modified Eagle Medium
RPMI	Roswell Park Memorial Institute Medium
EMEM	Eagle’s Minimum Essential Medium
FBS	Fetal bovine serum
PMA	Phorbol 12-myristate 13-acetate
LDH	Lactate dehydrogenase
HBSS	Hanks’ balanced salt solution
BAL	Bronchoalveolar fluid
MPO	Myeloperoxidase
DPBS	Dulbecco's Phosphate Buffered Saline
NADH	Nicotinamide adenine dinucleotide (NAD) +(H) hydrogen
BSA	Bovine serum albumin
PBS	Phosphate buffer saline
B.W	Body weight
SLN	Solid lipid nanoparticle

CMC	Critical micelle concentration
SSM	Sterically stabilized micellar
SSMM	Sterically stabilized mixed micelles
DSPE–PEG	Poly(ethylene glycol)- grafted distearoylphosphatidy lethanolamine

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APPENDIX A Experimental Data

APPENDIX B Standard Calibration Curve of Docetaxel

TOKSISITI IN VITRO DAN IN VIVO FORMULASI NANOPARTIKEL POLIMERIK TERMUAT DOCETAXEL UNTUK RAWATAN KANSER PARU-PARU BERPOTENSI

ABSTRAK

Docetaxel merupakan salah satu drug kemoterapi yang telah digunakan untuk rawatan kanser paru-paru sejak dua dekad yang lalu. Walau bagaimanapun, keterlarutan yang lemah bagi drug ini memerlukan penambahan surfaktan dalam formulasi komersial sistem pelarut standard mereka seperti Cremophor EL, polysorbate 80 dan etanol. Ini telah menghasilkan beberapa kesan farmakologi dan biologi yang berkaitan dengan formulasi ubat ini seperti tindak balas hipersensitiviti akut yang berkaitan secara klinikal dan neuropati periferal. Justeru, tujuan kajian penyelidikan ini adalah untuk membangunkan, mencirikan, dan menilai tahap ketoksikan formulasi nanopartikel poli-ε-kaprolakton yang mengandungi drug docetaxel (DTX) untuk meningkatkan keberkesanan agen kemoterapi ini. Nanopartikel poli-ε-kaprolakton yang mengandungi DTX (DTX-PCL-NP) dibangunkan menggunakan kaedah terubah nanopresipitasi. Formulasi optimum DTX-PCL-NP dihasilkan dengan menyaring beberapa jenis tensioaktif (polivinil alkohol, vitamin E TPGS, natrium kaproil hyaluronat dan sodium oleil hyaluronat) menggunakan kaedah nanopresipitasi yang diubah suai. DTX-PCL-NP kemudian dicirikan fizikokimia seperti pengukuran saiz dan taburan saiz partikel (PDI), cas zeta potential dan morfologi partikel. DTX-PCL-NP yang dibangunkan dalam kajian ini memberikan pencirian optimum dan terbaik pada ukuran saiz partikel 154.30 nm dan

PDI 0.108. Cas zeta potential nanopartikel ialah -24.10 mV. DTX-PCL-NP didapati stabil selama tiga bulan dalam keadaan penyimpanan yang berbeza dengan kecekapan enkapsulasi maksimum 34.00% pada pemuatan drug DTX sebanyak 20.00%. Ujian sitotoksisiti seterusnya dijalankan pada sistem kultur sel *in vitro* dan sistem model *ex vivo* kepingan tisu paru-paru jitu (*precision cut lung slices*). Toksisiti DTX-PCL-NP selanjutnya dinilai secara *in vivo* pada tikus Wistar Han. Parameter biokimia untuk penilaian ketoksikan DTX-PCL-NP pada haiwan adalah kiraan sel pembezaan, analisis-analisis laktat dehidrogenase (LDH), myeloperoxidase (MPO), albumin dan analisis hemoglobin. Analisis-analisis ini dilakukan pada sampel cecair lavaj bronkooalveolar (BAL) dan darah / plasma paru-paru tikus Wistar Han yang diberikan dos DTX-PCL-NP dan DTX yang berbeza melalui kaedah instilasi intratrakea. Dalam kajian ini, hasil ujian sitotoksisiti dan toksisiti *in vivo* dan *ex vivo* menunjukkan bahawa DTX-PCL-NP pada dos 0.10 mg/kg B.W telah menunjukkan tahap ketoksikan yang sama / hampir sama dengan DTX bebas pada dos 1.00 mg / kg B.W. Penemuan ini menunjukkan bahawa pembawa nano yang dibangunkan dalam kajian ini tidak menyumbang kepada pertambahan tahap ketoksikan pada drug DTX. Oleh itu, formulasi nanopartikel polimerik ini mempunyai prospek besar sebagai pembawa nano kepada drug jenis hidrofobik seperti docetaxel untuk rawatan kanser paru-paru.

IN VITRO AND IN VIVO TOXICITY OF DOCETAXEL-LOADED POLYMERIC NANOPARTICLES FORMULATION FOR POTENTIAL TREATMENT OF LUNG CANCER

ABSTRACT

Docetaxel is one of chemotherapeutic drug that had been used for treatment of lung cancer for the past two decades. However, the poor solubility of these compounds necessitates the inclusion of surfactant vehicles in their standard solvent system commercial formulations such as Cremophor EL, polysorbate 80 and ethanol. These had led to a number of pharmacologic and biologic effects related to these drug formulations such as clinically relevant acute hypersensitivity reactions and peripheral neuropathy. Hence, the aim of the present study was to prepare, characterize, and evaluate toxicity of poly-ε-caprolactone nanoparticles containing anticancer drug, docetaxel (DTX) to in order to improve the efficacy of this chemotherapeutic agent. Poly-ε-caprolactone nanoparticles containing DTX (DTX-PCL-NP) were prepared by nanoprecipitation method. The optimized DTX-PCL-NP formulations were developed by screening the use of different tensioactives (polyvinyl alcohol, vitamin E TPGS, sodium caproyl hyaluronate and sodium oleyl hyaluronate) in modified nanoprecipitation method. DTX-PCL-NP were then characterized for their physicochemical characteristics such as size and size distribution (PDI), zeta potential and morphology. Optimised DTX-PCL-NP developed in this study gave the best results with particles size 154.30 nm and low PDI of 0.108. The zeta potential of the nanoparticles is -24.10 mV. DTX-PCL-NP were found stable up to three months in different storage conditions with maximum encapsulation efficiency of 34.00% at

20.00% loading of DTX drug inside DTX-loaded PCL NP system. Cytotoxicity was tested on *in vitro* cell culture system and *ex vivo* model system of precision cut lung slices. The toxicity of DTX-PCL-NP were further evaluated *in vivo* in Wistar Han rats. The biochemical parameters for toxicity evaluation of DTX-PCL-NP carried out were differential cells count, lactate dehydrogenase (LDH), myeloperoxidase (MPO), albumin and hemoglobin analyses. These analyses were done on bronchoalveolar lavage (BAL) fluid and blood/plasma samples of Wistar Han rat lung which were treated with different doses of DTX-PCL-NP and free DTX via intratracheal instillation. In this study, the *in vivo* and *ex vivo* cytotoxicity and toxicity results suggested that DTX-PCL-NP at 0.10 mg/kg B.W had produced same/similar toxicity with free DTX at 1.00 mg/kg B.W which showed that the nanocarrier developed in this study did not contribute to additional toxicity for DTX. In fact, less amount of DTX in the nanoparticles (i.e. only 0.037 mg/kg B.W) were needed to produce the same effect as 1.00 mg/kg B.W free DTX drug. Thus, this polymeric nanoparticles formulation had great prospect as nanocarrier for lung cancer hydrophilic drug such as docetaxel for lung cancer treatment.

CHAPTER 1

INTRODUCTION

1.1 Background

Cancer is a disease in which cells in the body grow out of control which can spread to other parts of the body. Cancer that begins in the lungs is called lung cancer (National Cancer Institute, 2021b). Thus, lung cancer occurs when cells divide in the lungs uncontrollably which leads to growth of tumors (Cleveland Clinic, 2019). Lung cancer can be caused by many factors but smoking poses a major risk factor for the cancer (Cleveland Clinic, 2019). Despite advances in diagnosis and therapeutics of lung cancer, many patients still develop advanced, incurable and progressively fatal disease of the cancer (Lim, 2016). Thus, it is not surprising that today, lung cancer is the most common cancer and is by far the leading cause of cancer deaths in the world (Sung et al., 2021).

Generally, lung cancer classified into two types which are small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). NSCLC is more commonly diagnosed than SCLC. There are three subtypes of NSCLC which are adenocarcinoma, squamous cell carcinoma and large cell carcinoma. Adenocarcinomas start in the cells that would normally secrete substances such as mucus. This type of lung cancer occurs

mainly in smokers and non-smokers. It is more common in women than in men, and it is more likely to occur in younger people compared to other types of lung cancer. On the other hand, squamous cell carcinomas commence in squamous cells, which are flat cells that line the inside of the airways in the lungs. They are often linked to a history of smoking and tend to be found in the central part of the lungs, near a main airway (bronchus). Whilst, large cell carcinoma can appear in any part of the lung. This lung cancer subtype tends to grow and spread quickly, which make it harder to treat (WebMD, 2020).

Currently, treatments for NSCLC include surgery, radiofrequency ablation (FRA), radiation therapy, chemotherapy and targeted drug therapy. For stage 0 NSCLC it is usually curable by surgery alone. No chemotherapy or radiation therapy is needed since the cancer is limited to the lining layer of the airways and has not invaded deeper into the lung tissue or other areas. However, for stage 1 NSCLC surgery is the prefered treatment option. This may be done either by taking out the lobe of the lung that has the tumor (lobectomy) or by taking out a smaller piece of the lung (sleeve resection, segmentectomy, or wedge resection). As for stage II NSCLC, surgery treatment is carried out by lobectomy or sleeve resection removal. Meanwhile, treatment for stage IIIA and IIIB NSCLC include some combination of radiation therapy, chemotherapy (chemo), and/or surgery. However NSCLC diagnosed at stage IVA or IVB where the tumour has spread, treatment options have to include surgery, chemotherapy (chemo), targeted therapy, immunotherapy, and radiation therapy (National Cancer Institute, 2021a).

Cancer chemotherapeutics must be able to penetrate cancer tissues in an effective concentration necessary to kill the cancer cells. Most of these anticancer drugs are delivered via intravenous (IV) administration. Although IV administration may produce immediate therapeutic effect of cancer, the dose required may be high (Senapati et al., 2018). This is due to the fact that IV administration of the anticancer drug might cause a considerable proportion of the drugs to be widely distributed in various organs. Such high doses can cause severe adverse effects, especially at the sites of rapidly dividing cells such as hair, skin, spleen and liver, among others. These toxicity might compromise the treatment efficacy and patient compliance (Mangal et al., 2017). Thus, many efforts have been directed towards new anticancer drug discovery or new drug delivery for existing anticancer to overcome these toxicity problems. One of it is the utilization of nanomedicine systems of drug delivery. Nanomedicine systems have gained much attention in recent years, due to the fact that these nano-systems are easily manipulated to increase the efficiency of drug delivery as well as the bioavailability of anticancer (Wicki et al., 2015).

One of the anticancer chemotherapeutic drugs which has received substantial attention in nanomedicines application is docetaxel. Docetaxel is an important therapeutic component in the treatment of advanced-stage NSCLC, either as part of a combination therapy or as monotherapy.

1.2 Problem Statement

Docetaxel (DTX) is a United State Food and Drug Administration (FDA)-approved anticancer chemotherapy drug derived from plant alkaloid. DTX acts as an

antimicrotubule agent in cancer therapy for non-small cell lung cancer (NSCLC) (Georgoulias, 2002). It has also been used in the treatment for other types of cancers including gastric, breast, ovarian and prostate cancers. Despite the clinically meaningful benefits of DTX, the anti-mitotic effect of DTX is associated with high cytotoxicity and severe adverse effects. These adverse effects have limited its therapeutic applications in terms of dosage and treatment duration, especially among older patients (Ho and Mackey, 2014).

It has been well documented that the usage of organic solvent in DTX formulation and DTX low selective distribution has caused many severe side effects (Zhang et al., 2019). Moreover, due to its hydrophobicity (3.00 μg/ml, biopharmaceutical classification system class IV) and low biocompatibility, most clinically administered DTX is formulated with polysorbate 80 as solubilising agent and ethanol (50:50, v/v). Table 1.1 shows the current DTX formulations developed and commercialized in the market for NSCLC treatment. Polysorbate 80 has been shown to cause hypersensitivity reactions in cancer patients such as cumulative fluid retention, hypersensitivity reactions, mouth sores and nausea leading to a reduce uptake of DTX by tumour tissues (Steele et al., 2005). The solubilizing agent also exposes DTX to other body compartments, thus leading to harmful effects on normal tissues (Lee et al., 2009). Furthermore, the drug itself has too many side effects such as neurotoxicity, musculo-skeletal toxicity and neutropenia (Engels et al., 2007, Zheng et al., 2010). Ethanol has also been used as solubilizing agent in the formulations of DTX. Commercial DTX formulations available in the market that utilize these solubilizing agents include Taxotere, Docefrez and Docetaxel Injection as listed in Table 1.1 (Fries et al., 2019). Although the utilisation of ethanol has overcome the

hypersensitivity reactions caused by polysorbate 80, its uses in DTX formulation is also not without any limitations, where one of them is the risk of intoxication (Cheng et al., 2019). Ethanol intoxication may cause mild sedation, decreased coordination and severe effect can lead to complication such as seizures and low blood sugar. Therefore, with regards to the exposed formulation side effects, substantial effort had been focused on developing alternative, less intoxicating (ethanol free) and better tolerated polysorbate 80-free formulations for DTX. Such efforts include the incorporation of DTX in nanomedicine systems.

Table 1.1: DTX formulations commercialized in the market

Brand	**Company**	**Content**
Taxotere®; 1996	Sanofi-Aventis	a polysorbate 80 (Tween 80)-based formulation (50:50 of Tween 80 and ethanol with 4.0 g of ethanol per dose
Docetaxel Injection; 2011	Sandoz Inc.	using high concentration of ethanol; 5.5 g of ethanol per dose
Docetaxel Injection; 2011	Accord Healthcare Inc.	using high concentration of ethanol; 4.0 g of ethanol per dose
Docetaxel Injection; 2011	Hospira Inc.	using high concentration of ethanol; 3.7 g of ethanol per dose
Docefrez; 2011	Sun Pharma Global	lowest ethanol concentration-based formulation; 2.9 g per dose
Docetaxel Injection; 2013	Pfizer Inc.	using high concentration of ethanol; 6.4 g of ethanol per dose
Docetaxel Injection Concentrate; 2013	Actavis Inc.	using high concentration of ethanol; 4.0 g of ethanol per dose
Non-alcohol formulation of DTX; 2015	Eagle Pharmaceuticals	contained higher amount of Tween 80

The delivery of DTX via nanomedicine drug delivery system for lung cancer has been considered as an attractive alternative to conventional formulation

(Chenthamara et al., 2019, Wang et al., 2019). Nanomedicine typically fabricated with biocompatible and biodegradable polymers which can facilitate targeted and/or controlled delivery. For DTX loaded nanoparticle systems, among the most commonly used polymers include polylactic acid (PLA) (Keum et al., 2011, Mishra and Dey, 2018, Sanna et al., 2011a), polylactic-co-glycolic acid (PLGA) (Poltavets et al., 2019b, Pradhan et al., 2013, Poltavets et al., 2019a, Jose et al., 2019, Rafiei and Haddadi, 2017a, Shi et al., 2015, Bowerman et al., 2017, Noori Koopaei et al., 2014), poly-ε-caprolactone) (PCL) (Kong et al., 2018, Liu et al., 2012, Ma et al., 2011, Mei et al., 2009), alginate (Chiu et al., 2020), chitosan (Mu et al., 2020, Nair and Velmurugan, 2018, Jain et al., 2014) and gelatin base (Kumar and Jain, 2018, Niknejad and Mahmoudzadeh, 2015). Besides the nature of the polymers that give different physicochemical characteristics, the fabrication methods used will give distinct features of nanoparticles produced. In addition, they can be modified in their chemical and surface properties in order to make them an efficient drug delivery carrier (Bennet, 2014).

In this study, the aim is to formulate DTX in a suitable nanoparticle system fabricated with poly-ε-caprolactone (PCL). PCL is United States Food Drug Administration (FDA) approved biodegradable and biocompatible polyester. In the family of polyesters, PCL occupies a unique position: it is at the same time biodegradable and miscible with a variety of polymers. (Seppa¨la¨, 1996). PCL is suitable for controlled drug delivery due to its high permeability and lack of toxicity. This characteristics has made PCL as one of biodegradable polymer widely use in medical applications (Seppa¨la¨, 1996). This polymer has been broadly used to produce nanoparticles and micelles for the encapsulation of hydrophobic anticancer

drugs (Gaucher et al., 2010). In fact, as mentioned earlier, PCL has also been used to encapsulate DTX in a number of studies (Kong et al., 2018, Liu et al., 2012, Ma et al., 2011, Mei et al., 2009). This brings attraction and novelty to the field of NP drug delivery and leaves more space to explore (Rafiei and Haddadi, 2017b). Thus, it is hoped that poly-ε-caprolactone DTX formulation developed in this study might contribute to the current efforts and current understanding in the search of suitable nanoformulations as alternative to the current commercialized DTX formulations.

1.3 Objectives of the Study

In this study we aimed to develop a potential drug delivery of polysorbate 80 and ethanol free docetaxel formulation by formulating a biodegradable polymer, poly-ε-caprylactone (PCL) with docetaxel drug for treatment of non-small cell lung cancer. Since high amount of polysorbate 80 and ethanol content in the commercialized DTX formulation had contributed too many side effects for patients, it is hope with this project better tolerated DTX formulation can be developed. The objectives of this study are as listed below:

General objective

To develop polysorbate 80 and ethanol free docetaxel formulation by incorporating PCL polymer with docetaxel drug for treatment of non-small cell lung cancer.

Specific objectives

Objective 1: To develop and characterize docetaxel-loaded nanoparticles formulation.

Objective 2: To evaluate *in vitro* and *ex vivo* cytotoxicity of docetaxel-loaded nanoparticles formulation with cell lines and animal tissue.

Objective 3: To investigate *in vivo* toxicity of docetaxel-loaded nanoparticles formulation in animals (rats).

1.4 Content of the Thesis

This project was developed to formulate a polysorbate 80 and ethanol-free in docetaxel drug formulation for the treatment of non-small cell lung cancer. This cancer ranked among top 5 cancers most suffered by Malaysians. DTX has been used as the 2nd line drug and maintenance drug in the treatment regime for lung cancer in Malaysia by Ministry of Health. Most docetaxel formulations in the market contained either polysorbate 80 or ethanol, which have been known to cause adverse effects and severe side effects to patients.

Thus, this project was developed with the aim to produce docetaxel formulation without polysorbate 80 and ethanol to reduce the side effects of the current commercialized docetaxel formulation in the market with development of nanocarrier polymeric nanoparticles as nanotechnology tools. Over the past century, nanotechnology has increasingly acquired a crucial role in drug delivery area of research. Employing nanoparticles as delivery system in medical applications has been

widely studied and developed due to their biocompatibility, control and targeted release abilities (Singh and Lillard, 2009, Gelperina et al., 2005). Polymeric nanoparticles can modulate the pharmacokinetic properties of various active substances due to the subcellular size of nanoparticles (Deng et al., 2020). Depending on their internal structure, polymeric nanoparticles may further be classified as nanospheres or nanocapsules. Hence, the early stage of this project was focused on pre-screening of FDA approved tensioactives (surfactants) with PCL to produce optimized polymeric nanocapsules formulation. The selection of tensioactives in the pre-screening phase was to elude the usage of polysorbate 80. Only small amount of ethanol was used to dissolve the hydrophobic docetaxel drug at the initial step to produce polymeric nanocapsules and the solvent was removed in the finished formulation.

Then the loading and encapsulation efficiency of DTX in the nanoparticles system were studied to produce a formulation with acceptable drug dosage but better safety profile than the present reported formulations. HPLC method was used so that precise quantification of docetaxel loaded into the nanoparticles system can be produced and quantitated. The optimised formulation with highest loading efficiency of docetaxel was then undergone cytotoxicity study in human normal lung cells and NSCLC cells. The cytotoxicity study also was carried out via *ex vivo* system using mice lung tissue slices. This approach was chosen since *ex vivo* system derived data are reported as having more similarity with *in vivo* rather than single cell line cytotoxicity study. In order to further investigate the toxicity of the developed formulation, animal toxicity studies were carried out to generate preliminary toxicity

data of this optimized nanoparticles formulation by measuring a few toxicity parameters such as differential cell count, level of LDH, myeloperoxidase, albumin and haemoglobin of bronchiol alveolar fluid (BAL) of rat lung organ.

CHAPTER 2

LITERATURE REVIEW

2.1 Lung Cancer

Cancer is a major public health problem worldwide and is one of leading cause of death in the world. Cancer is caused by cells that divide uncontrollably which can result in development of tumours, impairment to the immune system and other deficiency that can be fatal to the body system (Baba, 2007). Lung cancer is one of the most life threatening disease in the world for several decades. In 2020 alone, it is estimated that there were 19.3 million new lung cancer cases and 9.9 million cancer deaths worldwide. Hence, it is not surprising that lung cancer has been listed as the most commonly diagnosed cancer (11.4% of the total cases) and the leading cause of cancer death (18.0% of the total cancer deaths) worldwide (Sung et al., 2021).

Lung cancer is one of the chief causes of cancer morbidity and mortality in men, whereas, it ranks third in morbidity, after breast and colorectal cancers, and second for mortality, after breast cancer, for women. In Malaysia lung cancer accounts for 16.6% of all cancers in males and females. According to Globocon 2020 (Sung et al., 2021), 48,639 people have been diagnosed with lung cancer in 2020 from Malaysians population of 32,365 988 people. From the statistic 29,530 cancer death was recorded due to lung cancer, in the same year. When ranked by gender and cases, lung cancer has been diagnosed as the leading cancer disease for male in the top 5 most frequent

cancers followed by colorectum cancer, prostate cancer, nasopharynx cancer in Malaysia in 2020. However,in female, it is outside of the five most frequent cancers diagnosed.

2.2 Types of Lung Cancer

There are two main types of lung cancer which are non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). According to the American Cancer Society (ACS), NSCLC accounts for 80–85% of lung cancer cases (American Cancer Society, 2019). The three main subtypes of NSCLC are:

- **Adenocarcinoma.** Around 40% of people with lung cancer have adenocarcinoma. It usually develops in the outer parts of the lung and tends to grow slower than the other two subtypes. This means that there is a better chance of finding and treating a tumour before it has spread.
- **Squamous cell carcinoma.** This accounts for about 25–30% of lung cancers. It grows from the cells that line the inside of the airways. Squamous cell carcinoma usually develops at the center of the lung.
- **Large cell carcinoma.** This makes up around 10–15% of lung cancers. It can grow in any part of the lung and tends to grow faster than the other subtypes.

Historically, squamous cell carcinoma was the most common lung cancer cell type. However, over the years, adenocarcinoma has now replaced squamous cell carcinoma as the most common lung cancer cell types. The reason for this shift is not well understood and could be contributed to factors such as diagnostic advances,

switch of smoking from high-tar to low-tar filtered cigarettes and changes in smoking patterns (Liam et al., 2006). An eight-year retrospective study carried out at University of Malaya Medical Center revealed that adenocarcinoma is the most common cell type in all age groups in Malaysia with a comparatively higher incidence in younger patients (less than 40 years old) (Liam et al., 2000). In another study done by the same group, it was revealed that the percentage of patients diagnosed with adenocarcinoma increased from 25% during the period of 1967–1976 to 43% during the period of 1991–1999 with a corresponding drop in the incidence of large cell carcinoma from 12% to 3%. There was no significant shift in the incidence of squamous cell carcinoma (Liam et al., 2006). Small cell lung cancer (SCLC) accounted for about 12% of all lung cancer cases. The incidence of SCLC seems to be on the decline. Two-thirds of cases of SCLC were diagnosed with advanced stage disease (Liam et al., 2006).

There are 5 stages for NSCLC: stage 0 (zero) and stages I through IV (1 through 4) described in Table 2.1 below. Diagnosis of the stages of NSCLC takes into account a combination of several factors, including the size and location of the tumour and whether it has spread to the lymph nodes and/or the spread (metastasis) to other parts of the body (Figure 2.1). One way to determine the stage of NSCLC is to detect whether the cancer can be completely removed by a surgeon. To completely remove the lung cancer, the surgeon must remove the cancer, along with the surrounding healthy lung tissue.

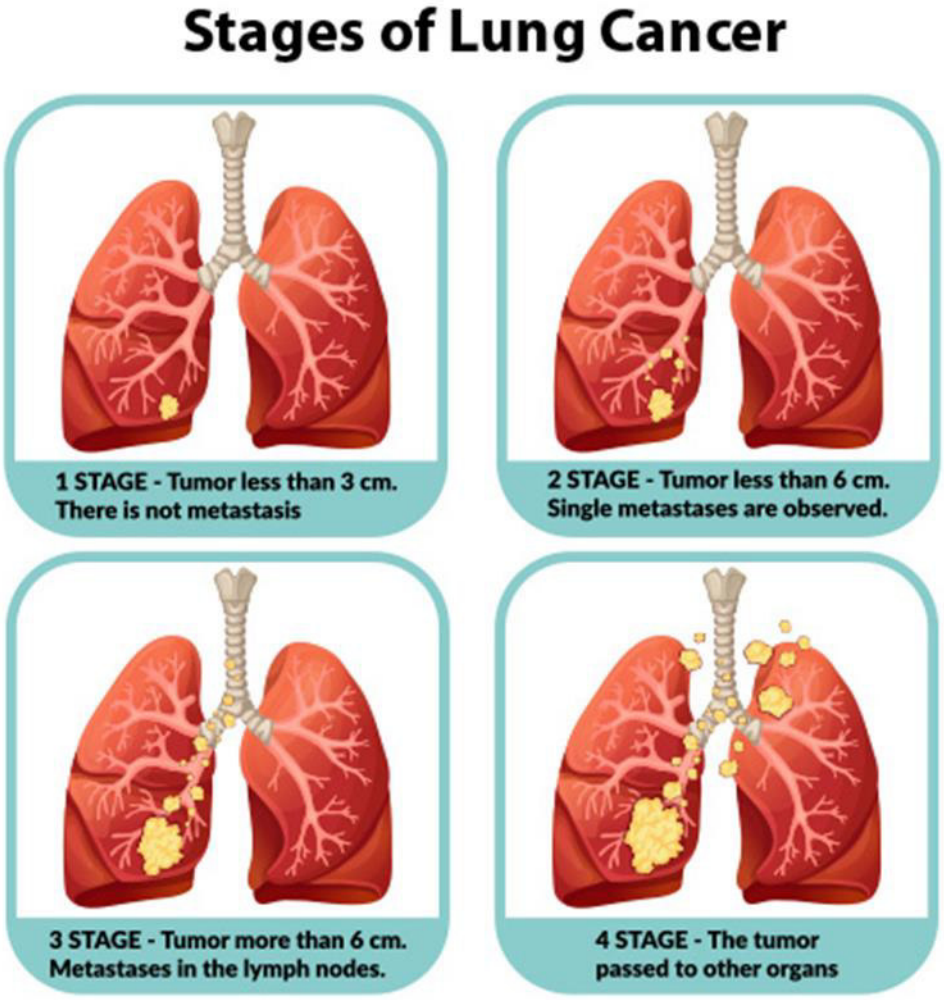


Figure 2.1 : Stages of lung cancer.

(Figure adapted from Beacon Hospital (Beacon Hospital, 2017)

Table 2.1: Stages of non-small cell lung cancer adapted from the American College of Surgeons, Chicago, Illinois. The original and primary source for this Information is the AJCC Cancer Staging Manual, Eighth Edition (2017) published by Springer International Publishing.)

Stages	**Description**
Stage 0	This is called in situ disease, meaning the cancer is "in place" and has not grown into nearby tissues and spread outside the lung.
Stage I	A stage I lung cancer is a small tumour that has not spread to any lymph nodes, making it possible for a surgeon to completely remove it. Stage I is divided into 2 substages based on the size of the tumour: • Stage IA tumours are 3 centimeters (cm) or less in size. Stage IA tumours may be further divided into IA1, IA2, or IA3 based on the size of the tumour. • Stage IB tumours are more than 3 cm but 4 cm or less in size.
Stage II	Stage II lung cancer is divided into 2 substages: • A stage IIA cancer describes a tumour larger than 4 cm but 5 cm or less in size that has not spread to the nearby lymph nodes. • Stage IIB lung cancer describes a tumour that is 5 cm or less in size that has spread to the lymph nodes. A stage IIB cancer can also be a tumour more than 5 cm wide that has not spread to the lymph nodes. Sometimes, stage II tumours can be removed with surgery, and other times, more treatments are needed.
Stage III	Stage III lung cancers are classified as either stage IIIA, IIIB, or IIIC. The stage is based on the size of the tumour and which lymph nodes the cancer has spread to. Stage III cancers have not spread to other distant parts of the body. For many stage IIIA cancers and nearly all stage IIIB cancers, the tumour is difficult, and sometimes impossible, to remove with surgery. For example, the lung cancer may have spread to the lymph nodes located in the center of the chest, which is outside the lung. Or the tumour may have grown into nearby structures in the lung. In either situation, it is less likely that the surgeon can completely remove the cancer because removal of the cancer must be performed bit by bit.
Stage IV	Stage IV means the lung cancer has spread to more than 1 area in the other lung, the fluid surrounding the lung or the heart, or distant parts of the body through the bloodstream. Once cancer cells get into the blood, the cancer can spread anywhere in the body. But, NSCLC is more likely to spread to the brain, bones, liver, and adrenal glands. Stage IV NSCLC is divided into 2 substages: • Stage IVA cancer has spread within the chest and/or has spread to 1 area outside of the chest. • Stage IVB has spread outside of the chest to more than 1 place in 1 organ or to more than 1 organ. In general, surgery is not successful for most stage III or IV lung cancers. Lung cancer can also be impossible to remove if it has spread to the lymph nodes above the collarbone. It can also be impossible to remove if it has grown into vital structures within the chest. These vital structures include the heart, large blood vessels, or the main breathing tubes leading to the lungs.

Stages	Description
Recurrence Stage	Recurrent cancer is cancer that has come back after treatment. If the cancer does return, there will be another round of tests to learn about the extent of the recurrence. These tests and scans are often similar to those done at the time of the original diagnosis.

2.3 Cause of Lung Cancer

Smoking tobacco products such as cigarettes, is the most critical risk factor for the development of NSCLC. Tobacco use was reported to be the main causative factor of 90% of male and 70-80% female lung cancers (Walser et al., 2008) with 90% of lung cancer deaths are attributed to be due to smoking (Rao et al., 1999, Saremi et al., 2013). It is reported by Ibrahim and co-workers, that 20% of smokers had developed pulmonary carcinoma and 90% of lung cancer diagnosed patients are with smoking history. Only 2.0% of lung cancer patients were non-smokers (Ibrahim et al., 2019). The risk of pulmonary carcinoma in smokers increases depending on smokers' age commencement, quantity of cigarettes consumed daily and the depth of smoke inhalation (Ibrahim et al., 2019, Surapaneni et al., 2012, Sanna et al., 2011a). Moreover, studies reported by (Freitas and Müller, 1999, Chu et al., 2005, Hainsworth et al., 2000, Schuette et al., 2005, Baker et al., 2005, Dumontet and Jordan, 2010, Herbst and Khuri, 2003) showed that geographical variations and gender differences in the incidence of lung cancer are also related to the frequency of tobacco use. Besides tobacco, exposure to radon, asbestos or carcinogenic chemicals may also increase lung cancer risk.

Understanding the causative factors such as smoking, exposure to radon, asbestos or carcinogenic chemicals in the lung cancer occurrence in individual had also lead to investigation in molecular mechanism and genetic of how these factors play significant role in the occurrence of the disease in a person. Recent advances of cell signalling pathways that control cell survival have identified genetic and regulatory aberrations that suppress cell death, promote cell division, and induce tumorogenesis. One such discovery from the advances is the discovery of the role of epidermal growth factor receptor (EGFR) in lung cancer (Rodak et al., 2021).

EGFR is a transmembrane receptor tyrosine kinase protein that is expressed in some normal epithelial, mesenchymal, and neurogenic tissue. EGFR is also a transmembrane glycoprotein encoded by a gene located at the short arm of chromosome 7 and have functions to stimulate a wide range of cellular functions such as cell proliferation, differentiation, migration and survival (Inamura et al., 2010). Specific mutations in the tyrosine kinase (TK) domain of the epidermal growth factor receptor (EGFR) are associated with improved responses in NSCLC patients receiving EGFR targeting tyrosine kinase inhibitors (TKIs). Overexpression of EGFR has been reported and implicated in the formation of tumour in many human malignancies, including NSCLC (Inamura et al., 2010, Ohsaki et al., 2000). Some studies have shown that EGFR expression in NSCLC is associated with reduced survival (Scagliotti et al., 2004, Veale et al., 1993, Volm et al., 1998), primary mechanism for tumour movement usually at lymph node which cause metastasis and poor chemosensitivity (Ogawa et al., 1993, Fontanini et al., 1998).

In Malaysian patients with NSCLC, the EGFR mutation rate was found to be similar to that in other Asian populations but higher compared to Western populations (Shi Yeen et al., 2013). In a study conducted by Liam and co-workers, all of the EGFR mutations were found in adenocarcinoma tumours except one that was in squamous cell carcinoma. The mutation rate from adenocarcinoma was 46% (221/484) and was more frequent in women (61%, $p<0.001$) (Liam et al., 2013).

2.4 Lung Cancer Cost and Treatment in Malaysia

Treatment regime for patients with clinical stage I or II NSCLC usually is curative surgical resection treatment. Three quarters of patients with NSCLC are diagnosed at the last stage (stage III or IV) of the disease and therefore curative surgery is an infrequent option (Liam et al., 2002). Table 2.2 summarized different types of lung cancer, stages and treatment. Moreover, there is a factor of significant delay in the diagnosis of lung cancer whereby the median patient delay being 60 days (interquartile range, 30-150 days) and the median doctor delay being 33 days (interquartile range, 18-72 days) (Loh et al., 2006). Patient delay is the period from first onset of symptoms to first medical consultation while doctor delay is the period from first consultation with a health care professional to initiation of investigations for cancer related symptoms (Makwakwa et al., 2014). Some of patient factors contributing to the delay in diagnosis of lung cancer include the failure to recognise their symptoms as serious and warrant medical attention, “shopping” for doctors and hospitals, patients’ own personal beliefs in traditional complementary medicine and patients not wanting investigations to be carried out while among the causes of delay

encountered in the healthcare system are a “loose” private sector primary healthcare system where patients hop from one primary care practice to another, low doctor: patient ratio in the public sector and delays in investigations due to long queues.

Table 2.2: Different types of lung cancer, stages and treatment adapted from L. Wu et al. 2017 (Wu et al., 2017).

Type of lung cancer	**Stages**	**Treatment**
NSCLC	I	Surgery or radiotherapy
	II	Radiotherapy or surgery followed by chemotherapy, combined therapy, targeted therapy
	IIIA	Surgery combined with chemotherapy, chemoradiation, combined therapy, targeted therapy
	IIIB	Chemoradiation
	IV	Chemotherapy, combined therapy, targeted therapy
	Recurrent	Chemotherapy, palliative radiation therapy
SCLC	Limited stage LCSC	Chemotherapy, radiotherapy, combined therapy, targeted therapy
	Extensive stage LCSC	Combined therapy, targeted therapy, prophylactic cranial irradiation
	Recurrent	Chemotherapy, palliative therapy
Metastases		Systemic surgery, radiosensitization, radiotherapy

In Malaysia the standard care for NSCLC patients is based on recommendations from Malaysia Ministry of Health; where platinum doublet-based therapy as the first line, maintenance and second line for non-squamous and squamous NSCLC patients (Table 2.3). Most patients with either non-squamous or squamous disease relapse or

develop recurrent disease following the first line treatment. These patients are divided into two categories: refractory/resistant disease (primary progression or recurrence within 3 months of initial therapy) or relapsed/sensitive disease (recurrence > 3 months after initial therapy). In patients who relapse > 6 months from the completion of initial therapy, reinitiating the previously administered first-line therapy is recommended.

Table 2.3: Advanced NSCLC treatment regime in Malaysia adapted from Prof. Dr Liam Chong Kin, Lung Cancer Specialist, Department of Medicine, Faculty of Medicine, University of Malaya, Kuala Lumpur 2016 and (Rajadurai et al., 2020).

<table>
<tr><th></th><th>EFGR Mut (40%)</th><th>ALK +/ROS1+ (~5%)</th><th colspan="2">Pan –ve NSCLC (55-60%)</th></tr>
<tr><th></th><th></th><th></th><th>Non-squamous</th><th>Squamous</th></tr>
<tr><td>1st-line</td><td>EGFR TKI*
Gefitinib
Erlotinib
Afatinib
Osimertinib
Alectinib
Pembrolizumab **</td><td>Crizotinib **
Certinib**</td><td>Platinum doublet
±Bevacizumb**
Pemetrexed **
Gemcitabine
Vinorelbine
Paclitaxel</td><td>Platinum doublet
Gemcitabine
Vinorelbine
Paclitaxel</td></tr>
<tr><td>Maintenance</td><td colspan="2">Treatment until progression</td><td rowspan="2">Continuation:**
Pemetrexed
Gemcitabine</td><td rowspan="2">Continuation:**
Gemcitabine
Switch :
Docetaxel</td></tr>
<tr><td></td><td colspan="2">Oligo-progression:
Cont. TKI + local therapy</td></tr>
<tr><td>2nd -line</td><td>2ndgen EFGR TKI:Afatinib

3rd gen EFGR TKI:osimertinib (early access program)</td><td>Ceritinib **</td><td>Docetxel</td><td>Docetaxel</td></tr>
<tr><td></td><td></td><td></td><td colspan="2">Pembrolizumab **</td></tr>
<tr><td></td><td></td><td></td><td colspan="2">Atezolizumab
Nivolumab (early access program)</td></tr>
</table>

*Precribed only in 2nd-line in Ministry of Health Hospitals
**Not prescribed in Ministry of Health Hospitals

Single agent chemotherapy docetaxel is approved and recommended options for use with one of drug in platinum-doublet based therapy for maintenance stage treatment while as a single agent chemotherapy in 2nd line treatment of NSCLC. The cost for these three phase of treatment regime in Malaysia is described in Figure 2.2. According to Rajudurai et al. 2020, only gefitinib and erlotinib are available for free in public hospitals in Malaysia while other drugs cost can be reimbursed through private insurance (Rajadurai et al., 2020). In 2016, maintenance treatment of up to 6 cycles costs around RM 5,250.00 and is subsidized by the government to treat NSCLC patients. Whilst for 2nd line treatment, patients may have to undergo six to twenty four months RM 21, 000.00 per patient which need to be borne by the patient via reimbursement through private insurance.

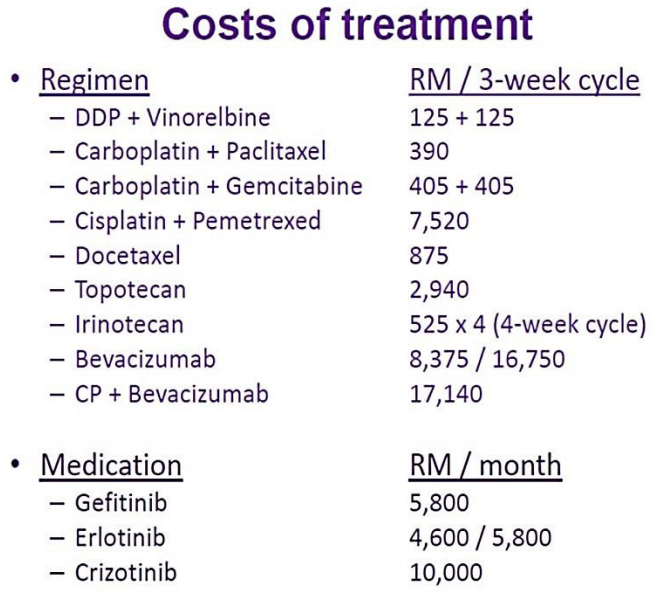
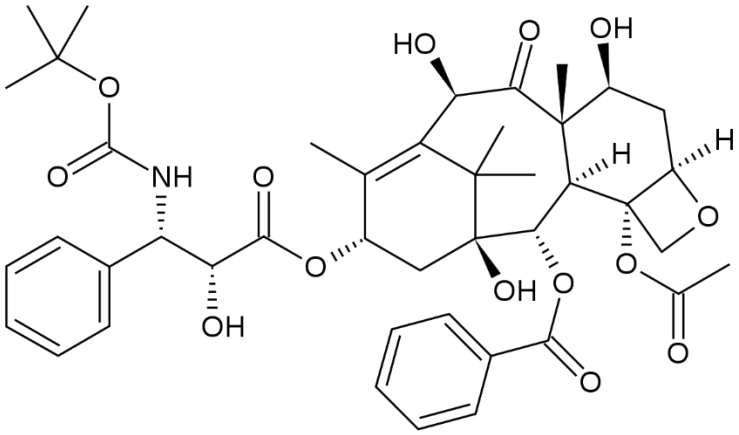


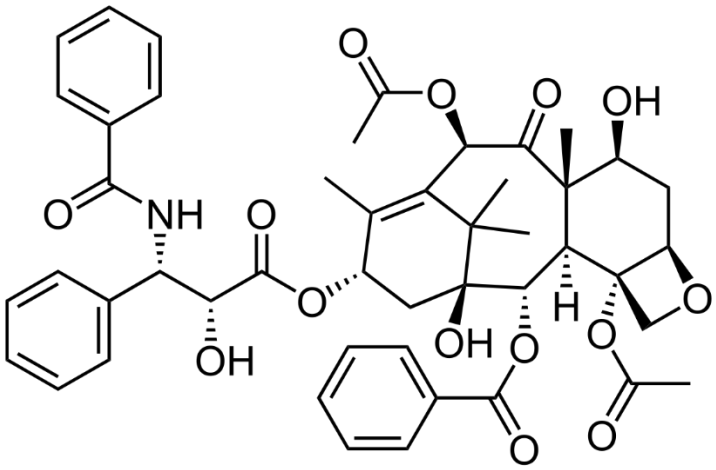
Figure 2.2 : Cost of advanced NSCLC treatment regime in Malaysia adapted from Prof. Dr Liam Chong Kin, Lung Cancer Specialist, Department of Medicine, Faculty of Medicine, University of Malaya, Kuala Lumpur 2016 (unpublished data).

2.5 Docetaxel (DTX)

Taxanes are cytotoxic diterpenes used clinically to treat patient especially with breast cancer and non small cell lung cancer. The taxane family includes paclitaxel, docetaxel and analogues with the taxane skeleton. Docetaxel (Figure 2.3A) very limited solubility is reflected by its bulky and fused ring structure with several lipophilic substituents which contributed to its high lipophilicity and high lattice enery. The structural differences of docetaxel makes it about 10-fold more soluble in water (3–25 Ig/ml) than paclitaxel (Du et al., 2007, Ali et al., 1997, Maryjane, 2004). Although slightly more hydrophilic than paclitaxel, DTX also exhibits limited water solubility. For this reason, Rhône-Poulenc Rorer Inc. (now Sanofi), formulated DTX in a 50:50 ethanol: polysorbate 80 mixture and commercialized it under the brand name Taxotere. A DTX dose of 100 mg/m^2 is often administered upon dilution, every 3 weeks within 1–2 hr of intravenous infusion (Airoldi et al., 2003). In phase III trials, Taxotere has shown similar or superior efficacy compared to Taxol in platinum combination therapy for the treatment of ovarian cancer and as single agent for treatment of anthracycline-resistant metastatic breast cancer respectively (Saloustros et al., 2008, Vu et al., 2008). Therefore, patients undergoing therapy with Taxotere have to be pre-treated with antihistamines and/or corticosteroids to temper severe, possibly fatal allergic reaction to polysorbate 80. Polysorbate 80 in particular can also induce fluid retention and often requires additional treatment with diuretics (ten Tije et al., 2003). This has somewhat limited the extensive used of Taxotere in clinic, but also stimulated scientific interest in developing alternative formulations using more biocompatible excipients in order to administer DTX in a safer fashion.



(A)



(B)

Figure 2.3 : Structures of (A) docetaxel (B) paclitaxel

DTX inhibits cell growth by binding to microtubules, stabilizing them, and preventing their depolymerisation (Ringel and Horwitz, 1991, Horwitz, 1992). Since the binding affinity of DTX to microtubule is 1.9-fold higher than that of paclitaxel, DTX is approximately twice as potent as paclitaxel (Ringel and Horwitz, 1991, Guéritte-Voegelein et al., 1991, Díaz and Andreu, 1993). The higher *in vitro* and *in vivo* anticancer potency of DTX may not only be attributed to its higher affinity for

microtubules, but also to its superior cellular accumulation. An *in vitro* study of the uptake and efflux of radiolabeled docetaxel and paclitaxel on P388 leukemia cells demonstrated that intracellular accumulation of DTX was 3-fold higher than that of paclitaxel with the same initial extracellular concentration (Riou et al., 1994). Conversely, the efflux rate of DTX from P388 cells was 3-fold lower than that of paclitaxel.

The use of DTX is higher than other taxanes due to its enhanced efficacy in most type of cancers. The enhanced efficacy of DTX is due to its increased potency to stabilize the microtubular assembly and inhibit cell replication. Microtubules are hollow cylindrical macromolecular structures which are built out of 13 longitudinal protofilaments composed of tubulin, a dimeric protein containing an α- and a β-subunit (Figure 2.4A) (Arnal and Wade, 1995). Microtubules are important building blocks of the cytoskeleton and play pivotal roles in various dynamic processes including cell migration, organelle movement and spindle formation during mitosis. Taxanes bind to the inner surface of microtubules, specifically through interaction with the β-tubulin subunit and thereby promote both their formation and their stabilization (Figure 2.4B) (Rao et al., 1999). DTX has stronger binding to tubulin with about 2–4 times more cytotoxicity effects on tumour cells than paclitaxel (Saremi et al., 2013). The latter partly explains the lower dose of DTX required for obtaining similar *in vitro* and *in vivo* anti cancer effects. This drug has also been shown to inhibit angiogenesis, induce signalling aberrations, and induce mitotic catastrophe or apoptosis (Herbst and Khuri, 2003).