Effects of FUX on gemcitabine sensitivity in lung cancer cells

by

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Abstract

Lung cancer has accounted for the most deaths from cancer (19.2% of all cancer deaths) in registered cancer cases in New Zealand. At present lung cancer treatment is inadequate, as patients treated with the front-line drugs, such as gemcitabine, rapidly develop drug resistance by decreasing cellular accumulation and/or avoiding apoptosis. Fucoxanthin (FUX), extracted from edible seaweed such as *Undaria pinnatifida*, has recently been reported to inhibit membrane drug efflux transporters (ABC transporters) and induce apoptosis in various cancer cell lines. Previous studies in AUT have defined FUX extracted from New Zealand *Undaria pinnitifida* with anti-cancer properties by using in vitro cell models. FUX has been reported to have few adverse effects in some animal models. We hypothesize that FUX may be a safe sensitizer to reverse gemcitabine resistance in lung cancer cells by increasing cellular accumulation of gemcitabine. The primary objective of this study was to assess the potential effects of FUX to reverse gemcitabine resistance in human lung cancer cell lines. The secondary objective of current study is to investigate the mechanisms of FUX actions if FUX may potentiate gemcitabine sensitivity. The third objective of this study is to evaluate the effects of FUX on modifying gemcitabine toxicity in two typical normal human cell lines.

Several types of human cell lines were used in this study including a lung carcinoma cell line A549, and two typical normal human cell lines embryonic kidney cell HEK293 and adult dermal fibroblasts (HDFa). Anti-proliferative effects were determined by 48-hr and 72-hr MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assays. As a single agent, either gemcitabine or FUX showed concentration-dependant inhibition of lung cancer proliferation in 72-hr MTT assays, with IC_{50} values of 9nM and 13μM, respectively. FUX increases gemcitabine sensitivity in an NSCLC cell line, A549 cell in a time and concentration dependant manner. Indeed, the 72-hr IC_{50} value for gemcitabine was only 3.9nM in the presence of 8μM FUX, which was decreased by 59% when comparing with control ($P< 0.05$). More importantly, FUX has no apparent effects on gemcitabine toxicity in two typical cell lines representing normal human tissues. It would be expected that FUX may represent a unique sensitizer, which may turn a less effective anti-cancer drug into an exceptional one. To elucidate the mechanisms of action of FUX, it is necessary to

carry out a mechanistic study to investigate if FUX changes the intracellular gemcitabine accumulation in A549 cells.

To determine gemcitabine in A549 cellular homogenates, an HPLC method has been developed and validated. In this study, while gemcitabine cannot be separated sufficiently from the cellular interferences using a conventional C18 column, aphenyl-hexyl column was found to be efficient to achieve better separation for quantitation of gemcitabine. This is because that separation using the phenyl column is conducted via the π electron, which in this case utilizes the π-π interaction between the phenyl group π electron and the analyte's π electron. Validation data indicates that the method is sensitive and reliable, with acceptable accuracy (85-115% of true values) and precision (CV < 15%). The assay specificity was indicated by the absence of interfering chromatographic peaks in cellular homogenates, and the LOQ of the assay was 0.5 μM. Calibration curves for gemcitabine were linear with the mean correlation coefficients > 0.987. This method has the advantage of being relatively rapid and efficient, with the retention time of gemcitabine separated from the substances in cellular homogenates. Therefore, this HPLC method is suitable for gemcitabine measurement in A549 cellular homogenates studies.

Cellular accumulation studies suggest uptake of gemcitabine may reach equilibrium after 4-hr in the presence or absence of FUX. FUX (10 μM) shows the potentials to increase the steady-state accumulation of gemcitabine in A549 cells. However, it does not affect the initial cellular uptake of gemcitabine in A549 cells. While this mechanistic research provides some clues to elucidate the effects of FUX on gemcitabine accumulation, more details about the exact mechanisms of its action, are warranted for further studies in the future.

However, a major limitation of this HPLC method is a lack of detection of gemcitabine metabolites. The cytotoxic action of gemcitabine has been attributed to inhibition of DNA synthesis by dFdCDP and dFdCTP. The HPLC method described in this study may not be suitable to simultaneously measure these active metabolites. Thus it is worthwhile to determine the cellular pharmacokinetics of gemcitabine and its metabolites in A549 cells and other NSCLC cells simultaneously by using an LC-MS/MS system.

In conclusion, fucoxanthin increases gemcitabine sensitivity to A549 cancer cell lines, and more importantly, it has no apparent effects on gemcitabine toxicity in two typical cell lines representing normal human tissues. It would be expected that FUX may represent a unique sensitizer, which may turn a less effective anti-cancer drug into an exceptional one.

Abbreviations

ACN: Acetonitrile

BSA: Bovine serum albumin

CV: Coefficient Variation

DNA: Deoxyribonucleic acid

EDTA: Ethylenediaminetetraacetic acid

EGFR: epidermal growth factor receptor

FBS: Fetal bovine serum

FUX: Fucoxanthin

Gem: Gemcitabine

HPLC: High performance liquid chromatograph

hr: Hour

IC_{50}: the concentration (μM) of the experimental compounds generating a 50% inhibition in cell growth

LC-MS/MS: Liquid chromatography-Mass spectrometry/Mass spectrometry

Log: Logarithm

LOQ: Limit of quantification

LSGS: Low Serum Growth Supplement

mg/mL: milligram per milliliter

mg/m^2: milligram per square meter

mM: milli-mole per liter

MTT: 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide

nm: nanometer

nM: nano-mole per litre

NSCLC: Non-small-cell lung carcinoma/cancer

PBS: Preparation of Phosphate Buffered Saline

QC: Quality control

RNA: Ribonucleic acid

SCLC: Small-cell lung carcinoma

SD: Standard deviation

SEM: Standard error of the mean

µM: micro-mole per liter

µg/mL: micro-gram per milliliter

µL: micro-liter

***U. pinnatifida*:** *Undaria pinnatifida*

UV: Ultraviolet

VIS: Visible

Table of Contents

List of Figures

List of Tables

1 Chapter 1 Introduction

1.1 Overview of cancer

1.1.1 Definition of Cancer

Accumulating clinical evidence suggests cancer is a major health burden in New Zealand as well as many parts of the world (American Cancer Society, 2012; Garattini & La Vecchia, 2001; Jemal et al., 2011; Van oosterom, 1997). Each year globally, about 14 million people learn they have cancer and 8 million people die from the disease (http://www.cdc.gov/cancer/dcpc/resources/features/worldcancerday/). In 2012, the most common causes of cancer death worldwide were lung cancer (19% of all cancer deaths, 1.6 million people), liver cancer (9% of all cancer deaths, 800,000 people), stomach cancer (9% of all cancer deaths, 700,000 people). This suggests that more than twice as many people die from cancer than from AIDS, malaria, and tuberculosis combined. Accordingly cancer has been suggested to be "the leading cause of death in economically developed countries and the second leading cause of death in economically developing countries."

Cancer, which is named medically as malignantneoplasia, is a group of diseases due to uncontrollable cell growth. Usually unregulated growth will lead to invasion into adjacent or even distal parts of the body through the lymphatic system or bloodstream. In order to spread, some cells from the primary cancer must break away, travel to another part of the body and start growing there. Cancer cells do not stick together as well as normal cells do. They may also produce substances that stimulate them to move. However, benign tumours do not invade neighbouring tissues and do not spread throughout the body. Cancer is not just one disease but many diseases. There are over 200 different known cancers that affect humans (http://www.cancerresearchuk.org/cancer-help/type/). Most cancers are named for the organ or type of cell in which they start - for example, cancer that begins in the lung or colon is called lung or colon cancer, respectively. In 2012, the most commonly diagnosed cancers worldwide were lung cancer (13% of all cancers diagnosed, 1.8 million people), breast cancer (12% of all cancers diagnosed, 1.7 million people) and colorectal cancer (10% of all cancers diagnosed, 1.4 million people).

According to National Cancer Institute (NCI), cancer types can be classified into five groups including carcinoma (cancer that begins in the skin or in tissues that line or cover internal organs. There are a number of subtypes of carcinoma, including adenocarcinoma, basal cell carcinoma, squamous cell carcinoma, and transitional cell carcinoma), sarcoma (cancer that begins in bone, cartilage, fat, muscle, blood vessels, or other connective or supportive tissue), leukaemia (cancer that starts in blood-forming tissue such as the bone marrow and causes large numbers of abnormal blood cells to be produced and enter the blood), lymphoma and myeloma (cancers that begin in the cells of the immune system) and central nervous system cancers (cancers that begin in the tissues of the brain and spinal cord).

The causes of cancer are diverse, complex, and remain the major research topics in biomedical regimen. Approximately 5–10% of cancers can be traced directly to inherited genetic defects (Kinzler & Vogelstein, 2002). However, it seems that environmental factors may play more important roles as many things are known to increase the risk of cancer, including tobacco use, dietary factors, certain infections, drug abuse, exposure to radiation, lack of physical activity, obesity, and environmental pollutants (American Cancer Society, 2012). Cancerous mutations can be triggered by directly damage genes or combine with existing genetic faults within cells (Kinzler & Vogelstein, 2002). Many cancers could be prevented by avoiding/reducing these risk factors, such as quitting smoking, eating more vegetables, fruits and whole grains, eating less meat and refined carbohydrates, maintaining a healthy weight, exercising, minimizing sunlight exposure, and being vaccinated against some infectious diseases (Anand et al., 2008).

Cancer can be detected in a number of ways, including the presence of certain signs and symptoms, screening tests, or medical imaging. Once a possible cancer is detected it is diagnosed by microscopic examination of a tissue sample. Cancer is usually treated with chemotherapy, radiation therapy and surgery. The chances of surviving the disease vary greatly by the type and location of the cancer and the extent of disease at the start of treatment. While cancer can affect people of all ages, and a few types of cancer are more common in children, the risk of developing cancer generally increases with age. In 2007, cancer caused about 13% of all human deaths worldwide

(7.9 million). Rates are rising as more people live to an old age and as mass lifestyle changes occur in the developing world (Jemal et al., 2011).

1.1.2 A Serious Health Problem in the World & in New Zealand

Cancer is one of the major growing health problems around the world, particularly with the steady rise in life expectancy, increasing urbanisation and the subsequent changes in environmental conditions, including lifestyle (Surh, 2003). It accounts for approximately 20% of current fatality statistics in the world, making it one of the leading causes of death. According to the statistic report by the World Health Organization (WHO) in 2011, more than 10 million cancer cases per year were reported worldwide with increasing rate at 3% per annum over the last 20 years. There were approximately 20 million people alive with cancer in 2002, also the number of cancer patients will be expected to increase to 30 million by 2020. In 2003, about 1,300,000 new cancer cases were diagnosed, and approximately 550,000 people died from cancer in USA (Beyer & Rushton, 2009). In 2009, it was estimated that more than 1,500,00 new cancer cases were diagnosed, and approximately 600,000 people died from cancer in USA (American Cancer Society, 2012). Therefore, based on these reports, cancer was globally identified as one of the most common health problem to cause of death in the world.

Currently, the main clinical strategies for cancer treatment are surgery, radiation, chemotherapy and immunotherapy (Garattini & La Vecchia, 2001; Gibbs, 2000). Surgery and radiation are generally only successful if the tumour is found at an early stage. For treatment of advanced cancers, chemotherapy plays an important role, killing tumour cells by applying direct cytotoxic effect, or by activating the host's immune response, inhibiting the proliferation of tumours cells, and inducing apoptosis (Gibbs, 2000; Makin, 2002). Despite advances in cancer chemotherapy, drug resistance and dose-limiting toxicity are the main obstacles for effective cancer therapy (Ratain, 1997). Only 2–3% of advanced cancers can be cured with current therapies (Garattini & La Vecchia, 2001) (American Cancer Society, 2012).

1.1.3 Cancer incidence in New Zealand

In 2010, 21,235 new registrations of primary cancer were reported to the New Zealand Cancer Registry. Males accounted for 11,068 of these registrations (52.1%)

and females accounted for 10,167 (47.9%). The number of people who suffered in cancer has gradually increased throughout the years. Between 2000 and 2010 the number of registrations has been increased by 18.7%. During the same period registration rates decreased 6.9% from 368.2 per 100,000 population in 2000 to 342.9 in 2010. In 2010, 8593 people had cancer recorded as their underlying cause of death; of these deaths, 52.5% were male (Ministry of Health, 2010). The NZ Ministry of Health reported in 2010 that lung cancer accounted for the most deaths from cancer (19.2% of all cancer deaths) in registered cancer cases in NZ (Ministry of Health, 2010). For males, the most common cause of death from cancer was lung cancer (19.8%), followed by colorectal cancer and then prostate cancer. For females, the most common cause of death from cancer was also lung cancer (18.5%), followed by breast cancer and then colorectal cancer.

1.2 Lung Cancer

The lungs are a pair of cone-shaped breathing organs in the chest. Each lung has sections called lobes. The left lung has two lobes. The right lung is slightly larger and has three lobes. Two tubes called bronchi lead from the trachea (windpipe) to the right and left lungs. The lungs have many tiny air sacs called alveoli, where oxygen is exchanged with the waste carbon dioxide generated in body. Both the alveoli and bronchi epithelial cells can be out of control of growth and are involved in lung cancer development.

1.2.1 Definition and classification

Lung cancer is the most common cause of cancer mortality in New Zealand and other parts of the world. In 2012, the most commonly diagnosed cancer and the most common cause of cancer death worldwide was lung cancer (19% of all cancer deaths, 1.6 million people). It is the most commonly diagnosed and most fatal cancers in males, while having the fourth highest incidence rate and second highest mortality rate in females (American Cancer Society, 2012; Jemal et al., 2011).

Lung cancers are classified according to histological type (Herbst, Heymach, & Lippman, 2008). This classification is important for determining management and predicting outcomes of the disease. Lung cancers are carcinomas as they arise from

epithelial cells. Lung carcinomas are categorized by the size and appearance of the malignant cells seen by a histopathologist under a microscope. For therapeutic purpose, two broad classes are distinguished: non-small cell lung carcinoma and small cell lung carcinoma (Ihde, 1992).

About 85% to 90% of lung cancers are non-small-cell lung cancer (NSCLC). Non-small-cell lung carcinoma (NSCLC) can be categorised as three subtypes including adenocarcinoma, squamous-cell carcinoma and large-cell carcinoma (Longo et al., 2011). But they are grouped together because the approach to treatment and prognosis (outlook) are often very similar. Adenocarcinoma is the most common form of lung cancer and it accounts for about 40% of lung cancers (Subramanian & Govindan, 2007). These cancers start in early versions of the cells that would normally secrete substances such as mucus. Most cases of adenocarcinoma are associated with smoking; however, it also occurs among people who have smoked fewer than 100 cigarettes in their lifetimes ("never-smokers") (Longo et al., 2011). It is more common in women than in men, and it is more likely to occur in younger people than other types of lung cancer. Adenocarcinoma is usually originates in peripheral lung tissue. It tends to grow slower than other types of lung cancer, and is more likely to be found before it has spread outside of the lung. A subtype of adenocarcinoma, the bronchioloalveolar carcinoma, is more common in female never-smokers, and may have a better long term survival (Raz, He, Rosell, & Jablons, 2006). In addition, people with a type of adenocarcinoma called adenocarcinoma in situ (previously called bronchioloalveolar carcinoma) tend to have a better outlook (prognosis) than those with other types of lung cancer (Donker et al., 2000).

About 25% to 30% of all lung cancers are squamous cell carcinomas. They typically occur close to large airways. A hollow cavity and associated cell death are commonly found at the centre of the tumour (Longo et al., 2011). These cancers start in early versions of 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 middle of the lungs, near a bronchus. Large cell (undifferentiated) carcinoma accounts for about 10% to 15% of lung cancers. It is so named because the cancer cells are large, with excess cytoplasm, large nuclei and conspicuous nucleoli (Longo et al., 2011). It can appear in any part of the lung. It tends to grow and spread quickly,

which can make it harder to treat. A subtype of large cell carcinoma, known as large cell neuroendocrine carcinoma, is a fast-growing cancer that is very similar to small cell lung cancer (Nguyen, Mirejovsky, Melinova, & Mandys, 2000).

In small-cell lung carcinoma (SCLC), the cells contain dense neurosecretory granules (vesicles containing neuroendocrine hormones), which give this tumour an endocrine/paraneoplastic syndrome association (Rosti et al., 2006). Most cases arise in the larger airways (primary and secondary bronchi) (Collins, Haines, Perkel, & Enck, 2007). These cancers grow quickly and spread early in the course of the disease. Sixty to seventy percent have metastatic disease at presentation. This type of lung cancer is strongly associated with smoking (Longo et al., 2011).

Four other histological subtypes are recognized, although some cancers may contain a combination of different subtypes (Maitra & Kumar, 2007). Rare subtypes include glandular tumours, carcinoid tumours, and undifferentiated carcinomas (Longo et al., 2011).

1.2.2 Causes and pathogenesis

Cancers are primarily due to an environmental changes and genetic damages. These affect the normal functions of the cell, including cell proliferation, programmed cell death (apoptosis) and DNA repair. As more damage accumulates, the risk of cancer increases. There are several contributors to lung cancer, such as smoking, air pollution, radon gas, asbestos, genetics and so on. At least 80% of lung cancer deaths are thought to result from smoking (Biesalski et al., 1998). The risk for lung cancer among smokers is many times higher than among non-smokers. Over 60 known carcinogens were found from cigarette smoke (Hecht, 2003), including radioisotopes from the radon decay sequence, nitrosamine, and benzopyrene. Additionally, nicotine appears to depress the immune response to cancerous growths in exposed tissue (Sopori, 2002). Across the developed world, 90% of lung cancer deaths in men during the year 2000 were attributed to smoking (70% for women) (Peto, Fund, & Organization, 1994).

Passive smoking (the inhalation of smoke from another's smoking) is a cause of lung cancer in non-smokers. A passive smoker can be classified as someone living or working with a smoker. Studies from the US (Collins et al., 2007) (Centers for

Disease Control and Prevention, 2001; "Health effects of exposure to environmental tobacco smoke. California Environmental Protection Agency," 1997), Europe (Hecht, 2003), the UK (Peto et al., 1994), and Australia (Council, 1994) have consistently shown a significantly increased risk among those exposed to passive smoke (Centers for Disease Control and Prevention, 2001). Investigations of second-hand smoke suggest it is more dangerous than direct smoke (Schick & Glantz, 2005). This is because inhaled fresh sidestream cigarette smoke is approximately four times more toxic per gram total particulate matter than mainstream cigarette smoke.

Outdoor air pollution is estimated to account for 1–2% of lung cancers (Waun et al., 2010). Industries, households, cars and trucks emit complex mixtures containing fine particulate matter. Most fine particulate matter comes from fuel combustion. Fine particulate matter is associated with a lung cancer and cardiovascular diseases (Waun et al., 2010). Worldwide, it is estimated to cause about 16% of lung cancer deaths and more than 20% of ischaemic heart disease and stroke. Particulate matter pollution is an environmental health problem that affects people worldwide, but low- and middle-income countries disproportionately experience this burden. Fine particulates (PM2.5) and sulfate aerosols are associated with slightly increased risk (EPA, 2006; Waun et al., 2010). For nitrogen dioxide, an incremental increase of 10parts per billion increases the risk of lung cancer by 14% (S. Chapman, Robinson, Stradling, & West, 2009).

On the other hand, indoor air pollution affects about 2.4 billion people globally (Warrell, Cox, & Firth, 2010), and is believed to account for 1.5% of lung cancer deaths (Dudley & Karczewski, 2013). Women who are exposed to indoor coal smoke have about twice the risk and a number of the by-products of burning biomass are known or suspected carcinogens (Dudley & Karczewski, 2013). Indoor radon gas is the second-most common cause of lung cancer in the USA, after smoking (Collins et al., 2007). The risk increases 8–16% for every 100 Bq/m^3 increase in the radon concentration in some hot spots worldwide (Organization). There appears to be no such hot spots in New Zealand (http://www.health.govt.nz/your-health/healthy-living/environmental-health/radiation-environment/radon-radioactive-gas) but the United States Environmental Protection Agency (EPA) estimates one in 15 homes in the US has radon levels above the recommended concentration(EPA, 2006).

Accumulating evidence suggests that 8 to 14% of lung cancer is due to genetic factors (Dudley & Karczewski, 2013). Similar to many other cancers, lung cancer is an endpoint of activation of oncogenes or inactivation of tumour suppressor genes (Fong, Sekido, Gazdar, & Minna, 2003). Oncogenes are believed to make people more susceptible to cancer. The epidermal growth factor receptor (EGFR) regulates cell proliferation, apoptosis, angiogenesis, and tumour invasion (Herbst et al., 2008). Mutations and amplification of EGFR are relatively common in non-small-cell lung cancer, which is a therapeutic target for EGFR-inhibitors. The *p53* tumour suppressor gene, located on chromosome 17p, is suppressed in 60–75% of cases (Devereux, Taylor, & Barrett, 1996).

1.2.3 Overview of lung cancer management

Currently, the main clinical strategies for lung cancer treatment are surgery, radiation, chemotherapy and immunotherapy (Garattini & La Vecchia, 2001; Gibbs, 2000; Ihde, 1992). Surgery and radiation are generally only successful if the tumor is found at an early stage. For example, in most cases of early-stage NSCLC, removal of a lobe of lung (lobectomy) is the surgical treatment of choice. For treatment of advanced cancers, chemotherapy plays an important role, killing tumour cells by applying direct cytotoxic effect, or by activating the host's immune response, inhibiting the proliferation of tumours cells, and inducing apoptosis (Gibbs, 2000; Herbst et al., 2008; Makin, 2002).

Chemotherapy started in the treatment of cancer by chemicals since 1940s (Chabner, Myers, Coleman, & Johns, 1975; Ihde, 1992). The most common chemotherapy aims to destroy cells by targeting uncontrolled tumour cells with rapid dividing property, so that tumour cell can be a higher possibility to be harmed by chemotherapy drugs than normal cell or tissue. The conventional cytotoxic chemotherapy agents include alkylating agents, platinum drugs, anti-metabolites, topoiosmerase inhibitors, and anti-microtubule drugs. Accumulating evidence suggests most cytotoxic drugs (except anti-microtubule drugs) may inhibit directly DNA synthesis, retard synthesis (S) phase of cell cycle and induce cell apoptosis. The anti-microtubule drugs, however, can promote its cytotoxic effect in mitotic (M) phase of the cell cycle.

Recently, with the development of molecular genetics, the identification of several oncogenes such as EGFR genes and tyrosine kinase genes, has led to the development of new chemotherapeutic inhibitors against EGFR or tyrosine kinase, including gefitinib, erlotinib for lung cancer, and cetuximab for colon cancer, and imatinib for Philadelphia chromosome-positive (Ph+) chronic myelogenous leukemia (Cataldo, Gibbons, Perez-Soler, & Quintas-Cardama, 2011; Leisewitz, Zimmerman, Jones, Yang, & Graves, 2008). Cetuximab is an example of monoclonal antibody inhibitor against EGFR. This IgG_1 type of monoclonal antibody blocks the extracellular ligand binding domain and prevents signal molecules binding, leading to inactivation of the tyrosine kinase. This result in cellular cytotoxicity against cancer cells and it has been used in clinic for treatment of lung cancer and colon cancer. Other monoclonals in clinical development are zalutumumab, nimotuzumab, and matuzumab.

Since EGFR mutation exists only in cancer cells and not in healthy cells, many therapeutic approaches are aimed at the small molecule EGFR inhibitors. New drugs such as gefitinib or erlotinib works as a type of targeted therapy and thus only cancer cells are killed through the drug's action. Patients have been divided into EGFR-positive and EGFR-negative, based upon whether a tissue test shows a mutation. EGFR-positive patients have shown a 60% response rate, which exceeds the response rate for conventional chemotherapy (Jackman et al., 2009). Patients harbouring sensitizing EGFR mutations should be considered for first-line erlotinib or gefitinib. However, EGFR-negative patients have shown limited response to these drugs, which is logical as these “magic bullets” is useless if the target is missing. In addition, many patients develop resistance possibly due to amplification or mutation of oncogenes and poor cellular delivery of drugs to targeted cancer tissues (Olive et al., 2009) . So far there was no consensus of an accepted approach to reverse resistance.

Despite advances in cancer chemotherapy, drug resistance and dose-limiting toxicity are the main obstacles for effective cancer therapy (Ratain, 1997; Szakács, Paterson, Ludwig, Booth-Genthe, & Gottesman, 2006). Only 2–3% of lung cancers can be cured with today's therapies.

1.2.4 Anti-cancer drug resistance and ABC transporters

One main mechanism, by which cancer cells become resistant, is up-regulation of various ATP-binding cassette transporters (ABC transporters), which efficiently remove the drug from the cancer cell, thus causing insufficient accumulation of anti-cancer drugs and thus loss of effects. On the other hand, ABC transporters are present in all tissues and play pivotal roles in the defense of the body against amphipathic carcinogens and toxins. The human family of ABC transporters contains 49 members with 7 subfamilies including several important drug transporters such as P-glycoprotein (P-gp, ABCB1), multidrug resistance associated protein 1-9 (MRP 1-9, ABCC1-6 and ABCC10-12, respectively) and breast cancer resistance protein (BCRP, ABCG2) (Borst and Elferink, 2002). Their substrates include amino acids, lipids, inorganic ions, peptides, saccharides, metals, xenobiotics, and proteins. Efflux of the substrates against their concentration and chemical potential gradients was driven by the hydrolysis of ATP in most cases of primary active transport that have been observed (Higgins, 1992). Many mammalian ABC transporters are under tight transcriptional regulation by nuclear receptors, suggesting their functions are subject to environmental and dietary influences (P. Borst & Elferink, 2002).

P-glycoprotein (P-gp) is the product of the multidrug resistance gene (MDR1/ABCB1). It is an efflux transporter located on the plasma membrane of many cancer cells, as well as in many normal tissues such as intestine, liver, kidney and brain. P-gp plays a pivot role in the tissue distribution of, and exposure to lipophilic and amphipathic drugs, carcinogens, toxins, cytokines and other xenobiotics (Drach et al., 1996; Litman, Druley, Stein, & Bates, 2001). Various structurally diverse drugs have been shown to be P-gp substrates including vinca alkaloids (vinblastine, vinorelbine) (Tamai & Safa, 1991), camptothecin derivates (topotecan) (van Veen et al., 1996), tubulin polymerizing drugs (paclitaxel, docetaxel), anthracyclines (doxorubicin, idarubicin) (G. Chen et al., 1997), epipodophyllotoxins (etoposide) (Sikic et al., 1997), calcium channel blockers (e.g., nicardipine) (Pascaud & Garrigos, 1998), β-antagonists (e.g., talinolol) (Spahn-Langguth et al., 1998), digitalis glycosides (e.g., digoxin) (Cavet, West, & Simmons, 1996), immunosuppressive agents (e.g., cyclosporine) (Fricker, Gutmann, Droulle, Drewe, & Miller, 1999), steroids (e.g., cortisol, aldosterone, and dexamethasone) and HIV protease inhibitors (e.g., saquinavir, ritonavir, and nelfinavir) (Washington, Duran, Man, Sikic, &

Blaschke, 1998). Typical P-gp inhibitors include verapamil, cyclosporine A, tamoxifen, quinidine, erythromycin, elacridar (GF120918), zosuquidar (LY335979) and tariquidar (XR9576) (Fox & Bates, 2007; Litman et al., 2001; McDevitt & Callaghan, 2007; Schwarz, Gramatte, Krappweis, Oertel, & Kirch, 2000; Washington et al., 1998). Several polyoxyethylene ester surfactants such as Tween 40, Cremophor EL and Solutol-HS 15 have the capacity to reverse P-gp efflux but are not inhibitors of P-gp. They are believed to affect the transporter proteins indirectly by inducing alterations in the physical state of the lipids in the plasma membrane (Dudeja, Anderson, Harris, Buckingham, & Coon, 1995). In most of these MDR reversal agents, the C-C-N-C-C sequence plus the presence of a carboxylic acid or a quaternary ammonium group was required for reversal activity (Klopman, Shi, & Ramu, 1997).

As the function of P-gp appears to be nonessential for cellular homeostasis, it has been suggested that complete inhibition of P-gp activity by a non-toxic inhibitor or a group of inhibitors may effectively reverse multidrug resistance. Several studies in experimental animals have indicated that co-administration of flavonoids increased the bioavailability of several P-gp substrates (Choi, Choi, & Shin, 2004; Choi & Shin, 2005). However, since most *in vivo* studies used compounds which were substrates of both P-gp and the metabolizing enzyme cytochrome P450 3A4 (CYP3A4), and many flavonoids also inhibit CYP3A4 activity, it is difficult to distinguish the contribution of each mechanism to the increased oral bioavailability. Accumulating *in vitro* studies have indicated that P-gp-mediated efflux can be diminished by many natural phytochemicals such as flavonoid aglycones (e.g. genistein, biochanin A, quercetin, morin, phloretin, silymarin, chrysin, hesperetin, naringenin), polyphenols, epicatechin gallate, catechin gallate, lignans, alkaloids and carotenoids (Castro & Altenberg, 1997; De Castro, Mertens-Talcott, Derendorf, & Butterweck, 2007; Jodoin, Demeule, & Beliveau, 2002; Taur & Rodriguez-Proteau, 2008; Wang, Barecki-Roach, & Johnson, 2002; Zhang & Morris, 2003) (Deli, Molnar, Matus, & Toth, 2001).

More interestingly, multidrug resistance-associated protein (MRP, ABCC) was initially cloned from a multidrug-resistant lung cancer cell line (Cole et al., 1992). To date, nine (MRP1-9, ABCC1-6 and ABCC10-12, respectively) have been cloned and functionally identified (P. Borst, Evers, Kool, & Wijnholds, 1999). MRP1, MRP4

and MRP5 proteins are widely distributed in the body, whereas MRP2, MRP3 and MRP6 appear to be mainly expressed in the liver, kidney and gut (M. Kool et al., 1997; M. Kool, van der Linden, de Haas, & Baas, 1999). MRP8/ABCC11 mRNA transcript is highly expressed in breast cancer but also has shown a low to moderate level of expression in a variety of human tissues including breast, testes, liver, brain, and placenta (Bera, Lee, Salvatore, Lee, & Pastan, 2001). In vitro, the MRP transporters can collectively confer resistance to anticancer drugs and their conjugated metabolites, platinum compounds, folate antimetabolites, nucleoside and nucleotide analogues, arsenical and antimonial oxyanions, peptide-based agents, and in concert with alterations in phase II conjugating or biosynthetic enzymes, classical alkylating agents, alkylating agents. Accumulating evidence also suggests MRP members caused an increased efflux and decreased intracellular accumulation of anticancer drugs and other anticancer agents, leading to association with tumour resistance.

The primary role of MRP appears as an efflux pump for endogenous amphiphilic anions such as leukotriene C4, as well as glutathione (GSH), glucuronic acid, and sulfate conjugates, cyclic guanosine monophosphate (cGMP) and cyclic adenosine monophosphate (cAMP) (Z. S. Chen, Lee, & Kruh, 2001; Jedlitschky, Burchell, & Keppler, 2000) (Kavallaris, 1997). Some anti-cancer drugs such as cisplatin or arsenite (H_3AsO_3) appear to be transported as conjugates of GSH, glucuronate, or sulfate, while others such as vinca alkaloids and anthracyclines, seem to be co-transported with GSH (P. Borst et al., 1999; P. Borst, Evers, Kool, & Wijnholds, 2000; Zhou et al., 2008). Both transport patterns lead to decreased cellular accumulation of anti-cancer drugs or their active metabolites and thus loss of cytotoxic effects in resistant cancer cells. For example, MRP4, 5 and 8 confer resistance to a purine derivatives 9'-(2'-phosphonylmethoxyethyl)-adenine (PMEA)efficiently by efficiently pump it out cancer cells (Wijnholds et al., 2000). MRP4 and 5 also cause the resistance to 6-mercaptopurine and 6-thioguanine by effluxing their monophosphates (Wielinga et al., 2002), and MRP5 and 8 confer resistance to 5-FU by extrusion of 5-fluorodeoxyuridine monophosphate (Kruh, Guo, Hopper-Borge, Belinsky, & Chen, 2007; Pratt et al., 2005). One antimetabolite, methotrexate, can be transported by MRP1-5 (P. Borst et al., 2000; Wielinga et al., 2002), but not by MRP6 (Z. S. Chen et al., 2001). MRP1-6 can be inhibited by typical organic anion transport inhibitors, such as probenecid and sulfinpyrazone (P. Borst et

al., 2000; Zhou et al., 2008). Drug substrates for active transport by MRPs cover various pharmacological agents, including non-steroidal anti-inflammatory drugs (e.g., indomethacin), tuberculostatics (rifampicin), nucleotide-based antiviral drugs (e.g., azidothymidine (AZT)), stavudine (d4T)), cytotoxic agents (e.g., cisplatin, anthracyclines, methotrexate (MTX), vincristine, etoposide).

Drug targeting of these transporters to overcome MRP-mediated multidrug resistance may change an ineffective anti-cancer drug into an exceptional one. Most MRP transporters are subject to inhibition by a variety of compounds including various phtochemicals. Accumulating evidence indicates that many flavonoid aglycones (e.g. quercetin, genistein, biochanin A, apigenin, morin, chalcone, silymarin, phloretin, chrysin, kaempferol, naringenin, myricetin), polyphenols and some carotenoids can inhibit MRP1-mediated transport (Versantvoort, Broxterman, Lankelma, Feller, & Pinedo, 1994; Versantvoort, Rhodes, & Twentyman, 1996; Versantvoort et al., 1993). Quercetin and silymarin have also been reported to inhibit MRP4- and 5-mediated transport with high affinity (low μM level) (Wu, Calcagno, Hladky, Ambudkar, & Barrand, 2005). A better understanding of their substrates and inhibitors has important implications in drug development and lead compound identification.

1.2.5 Chemotherapy in lung cancer treatment

Small-cell lung carcinoma (SCLC), even relatively early stage disease, is treated primarily with chemotherapy and radiation. Around 70% of NSCLC patients are diagnosed with advanced disease at diagnosis. In advanced NSCLC, chemotherapy improves survival and is used as first-line treatment, provided the person is well enough for the treatment (Group, 2008). In addition, randomized studies comparing chemotherapy with the “best supportive care” have shown that chemotherapy reduces symptoms and improves the quality of life (Cullen et al., 1999). Cisplatin-based chemotherapy plays a vital role in NSCLC treatment but concerns exist about the toxic effects associated with cisplatin-based chemotherapy.

With the discovery of the aetiological effects of EGFR over expression in NSCLC development, EGFR inhibitors such as erlotinib or gefitinib have been developed to improve clinical response and limit off-target toxicity (Ciardiello & Tortora, 2008). It has been suggested that patients with sensitizing EGFR mutations should be

considered for first-line erlotinib or gefitinib (Jackman et al., 2009). A study combines patient data from five trials in predominantly Western populations to assess the impact of EGFR mutations on first-line therapy with an EGFR-tyrosine kinase inhibitor (TKI) and compare clinical versus molecular predictors of sensitivity. Chemotherapy-naive patients with advanced non-small cell lung cancer and known EGFR mutation status treated with erlotinib or gefitinib monotherapy until disease progression or unacceptable toxicity. This study has shown that EGFR mutation status is associated with sensitivity to treatment with an EGFR-TKI in patients with advanced non-small cell lung cancer. However, the major disadvantages of EGFR inhibitors include high cost, development of drug resistance, the lack of effects in EGFR-negative patients.

Recently a number of new agents have become available for the treatment of metastatic NSCLC cancer, including the taxanes, gemcitabine, and vinorelbine. Among these new drugs, gemcitabine is an active and effective therapy for patients with non-resectable, locally advanced or metastatic NSCLC and it has been reported that gemcitabine was better tolerated than the cisplatin-based chemotherapy (Crinò et al., 1999).

1.3 Gemcitabine

Gemcitabine is an important anti-cancer drug that is used for the treatment of non-small cell lung cancer (Crinò et al., 1999), pancreatic cancer (Burris et al., 1997), metastatic breast cancer (Morandi, 2006), and ovarian cancer (Lorusso, Di Stefano, Fanfani, & Scambia, 2006) in clinic. It has also shown promising efficacy for the treatment of other solid tumours and hematological malignancies (Wong, Soo, Yong, & Innocenti, 2009).

1.3.1 Mechanisms of action

Gemcitabine (dFdC) is sort of a prodrug as it is metabolised intracellularly by nucleoside kinases to the active diphosphate (dFdCDP) and triphosphate (dFdCTP) nucleosides (Heinemann, Hertel, Grindey, & Plunkett, 1988; Plunkett, Huang, Searcy, & Gandhi, 1996). The cytotoxic action of gemcitabine has been attributed to

inhibition of DNA synthesis by dFdCDP and dFdCTP. The 5′-triphosphate of dFdC (dFdCTP) has been reported to be the major cellular metabolite (85–90%) and dFdCTP is accumulated and retained in solid tumours and cell lines (Heinemann et al., 1988; Van Haperen et al., 1994). dFdCTP competes with dCTP for incorporation into DNA to inhibit the cell proliferation. dFdCDP is a potent inhibitor of ribonucleotide reductase, which is uniquely responsible for catalysing the reactions that generate the deoxynucleoside triphosphates for DNA synthesis (Baker et al., 1991). Inhibition of this enzyme by dFdCDP causes a reduction in the concentrations of deoxynucleosides in general, and especially in that of dCTP. Thus, dFdCDP potentiates the incorporation of dFdCTP into DNA by decreasing the intracellular dCTP. After gemcitabine is incorporated into DNA, one additional nucleotide is added to the growing DNA strands. After this addition there is essentially a complete inhibition in further DNA synthesis (masked chain termination) (Plunkett, Huang, & Gandhi, 1995). This is due to the fact that DNA polymerase epsilon is essentially unable to remove gemcitabine and repair the growing DNA strands. After incorporation into DNA, gemcitabine then appears to induce apoptosis (the programmed cellular death process) by modulating protein kinase C signalling events (Cartee & Kucera, 1998).

Incorporation of gemcitabine into RNA and inhibition of RNA synthesis represent an alternative and important mechanism of action of this drug (Ruiz van Haperen, Veerman, Vermorken, & Peters, 1993). dFdCTP is not only important as a DNA precursor, but also appears to interfere with normal ribonucleotide metabolism (Van Haperen et al., 1994). In several solid tumours and cell lines, the most predominant in vitro cell line dependent changes were a decrease in CTP concentrations, accompanied by an increase in UTP and GTP concentrations.

Some direct evidence also suggests that gemcitabine is a potent inhibitor on the activity of human mitochondrial DNA polymerase (Fowler, Brown, Johnson, & Suo, 2008). This may, at least partially, explain some peripheral neuropathy, hematological dysfunction, and pulmonary toxicity caused by gemcitabine in cancer patients. The frequency and severity of the adverse effects are affected by the dose, infusion rate and intervals between doses. The most commonly reported adverse medicine effects associated with gemcitabine treatment include: nausea with or without vomiting; raised liver transaminases (AST/ALT) and alkaline phosphatase, reported in

approximately 60% of patients; proteinuria and haematuria reported in approximately 50% patients; dyspnoea reported in 10–40% of patients (highest incidence in lung cancer patients); and allergic skin rashes, which occur in approximately 25% of patients and are associated with itching in 10% of patients.

1.3.2 Indications of gemcitabine in lung cancer treatment

Gemcitabine is indicated for treatment of patients with locally advanced or metastatic non-small cell lung cancer (NSCLC) (Crinò et al., 1999; Gridelli et al., 1999; Mitchell, 2000; ten Bokkel Huinink et al., 1999). Four phase 2 single agent studies were conducted with the primary endpoint being tumour response and a secondary measure of symptomatic improvement. The studies were conducted using gemcitabine doses from 800–1250 mg/m^2 as a single agent. Three major studies provides evidence that single-agent gemcitabine is an effective therapy for patients with non-resectable, locally advanced or metastatic NSCLC and good performance status. The three major studies conducted suggested uniform response rates from 19.7–22.5% of evaluable patients and from 17.9–20.5% on an intent to treat based analysis after assessment by external peer review boards. The median response duration was 7.6 to 12.7 months, while the overall median survival (for responders and non responders) was from 8.1 to 9.2 months. The major study conducted had 3 patients (2%) achieve complete response and 30 patients (20%) experience partial response out of 151 patients. The fourth trial was much smaller, with only a total of 34 patients (Crino et al., 1999). The mean effective patient dose in this smaller trial was 741mg/m^2 which was lower than that in the 3 major studies ($\geq$ 960mg/m^2). A response rate of 1 patient (3.2%) out of 31 evaluable patients was observed. The following data is retrieved from New Zealand Medsafe (http://www.medsafe.govt.nz/profs/datasheet/g/GemcitabineEbeweinj.pdf), which shows an integrated summary of adverse events (events that occurred in $\geq$ 2 % of patients without causality assessment) for the 4 pivotal trials (n=360): dyspnoea = 7.5% (27), anaemia = 6.9% (25), fever = 4.2% (15), nausea = 3.9% (14), vomiting = 3.3% (12), carcinoma of lung = 3.1% (11), pain = 2.5% (9), pneumonia = 2.5% (9), dehydration = 2.2% (8), pleural effusion = 2.2% (8) and discontinuation due to progressive disease = 53.6% (193). In summary, single-agent gemcitabine is an active and effective therapy for patients with non-resectable, locally advanced or metastatic NSCLC and good performance status, and that it is better tolerated than the

combination cisplatin/etoposide.

Gemcitabine is also an effective agent for NSCLC when combining with cisplatin. In a phase 3 randomised trial, a total of 522 patients were enrolled to receive gemcitabine plus cisplatin (GC) (260) or single agent cisplatin (262) over a 4-week schedule (Sandler et al., 2000). The median survival was 9.1 months (95% CI 8.3 to 10.6 months) for the GC-treated patients, which was significantly superior to cisplatin-treated patients [7.6 months (95% CI 6.5 to 8.2 months)]. The estimate of median time to disease progression was 5.6 months (95% CI of 4.6 to 6.1 months) for GC-treated patients, which was significantly superior to cisplatin-treated patients [3.7 months (95% CI 3.3 to 4.2 months)]. The overall response rate was 30.4% for GC-treated patients and 11.1% for patients treated with single agent cisplatin ($p < 0.0001$). In another phase 3 randomised trial, A total of 135 patients were enrolled to receive GC (69) or cisplatin plus etoposide (66) over a 3-week schedule. The median survival was 8.7 months (95% CI 7.7 to 10.2 months) for the GC arm and 7.2 months (95% CI 6.1 to 9.8 months) for the patients treated with cisplatin plus etoposide, which was not significantly different. The estimate of median time to disease progression was 6.9 months (95%CI of 5.0 to 8.1 months) for GC-treated patients, which was significantly superior to cisplatin plus etoposide treated patients [4.3 months (95% CI 3.5 to 4.7 months)] ($p = 0.0147$). The overall response rate (intent-to-treat) was 40.6% for GC-treated patients and 21.2% for patients treated with cisplatin plus etoposide ($p = 0.0167$).

1.3.3 Pharmacokinetics

Pharmacokinetics describes absorption, distribution, metabolism and excretion of a drug after administration into human body. As a cancer chemotherapeutic agent, gemcitabine is generally administered intravenously and thus its absorption is considered to be complete. The dispersal of gemcitabine around the body and into various tissue compartments such as the muscle, fat, CNS etc. This is normally how a drug reaches its site of action unless drug is administered directly into site of action. Gemcitabine is reported to be taken up into cells by equilibrative and concentrative nucleoside transporters (ENTs and CNTs) (Bhutia, Hung, Patel, Lovin, & Govindarajan, 2011; Kim et al., 2011). It is initially phosphorylated by deoxycytidine kinase (DCK) to 2',2'-difluorodeoxycytidine monophosphate and then is further phosphorylated to its active diphosphorylated and triphosphorylated forms, dFdCDP

and dFdCTP (Heinemann et al., 1988; Sugiyama et al., 2010), which have not been detected in plasma or urine. dFdCTP can be found in peripheral blood mononuclear cells with a terminal elimination half-life of 0.7 - 12 hours. Intracellular dFdCTP concentrations increase in proportion to gemcitabine doses of 35 - 350mg/m^2/30 min, which give steady state concentrations of 0.4 - 5mg/mL. At gemcitabine plasma concentrations above 5mg/mL, dFdCTP levels do not increase, suggesting that the formation is saturable in these cells. It has been reported that P-glycoprotein and MRP1 overexpression possibly caused a cellular stress resulting in increased gemcitabine metabolism and sensitivity (Bergman et al., 2003).

The pharmacokinetics of gemcitabine have been carried out in patients and in healthy populations examined in several studies (Bhargava et al., 2001; Blackstein et al., 2002; Kuenen et al., 2002; Patel et al., 2001). The ratio of women to men is 1:2 and the age ranged from 29 to 79 years. The pharmacokinetic parameters were obtained for doses ranging from 500 to 2592 mg/m^2 that were infused from 0.4 to 1.2-hr. Peak plasma concentrations (obtained within 5 minutes of the end of the infusion) ranged from 3.2 to 45.5 µg/mL. Plasma protein binding was negligible and gemcitabine is distributed well as volume of distribution of the peripheral compartment is 47.4 L/m^2. There was gender difference on the volume of distribution of the central compartment: 12.4 L/m^2 for women and 17.5 L/m^2 for men (inter-individual variability was 91.9%). Systemic clearance is quick depending on gender and age (inter-individual variability was 52.2%), which may contribute to inter-patient differences in the plasma concentration of gemcitabine and its rate of elimination from the systemic circulation (reflected by differences in half life). Clearance for women is approximately 25% lower than the values for men. Although rapid, clearance for both men and women appears to decrease with age. Gemcitabine is mainly excreted via urine as metabolites and less than 10% excreted as unchanged medicine. Half life ranged from 42 to 94 minutes depending on age and gender. For the recommended dosing schedule, gemcitabine elimination should be virtually complete within 5 to 11 hours of the start of the infusion. There is no apparent accumulate of gemcitabine in patient's body when administered once weekly.

In the liver, kidney, blood and other tissues, the majority of gemcitabine is rapidly metabolized by cytidine deaminase (CDA) to an inactive metabolite, 2',2'-

difluorodeoxyuridine (dFdU), which is completely excreted into the urine (Wong et al., 2009). Gemcitabine dose of 1,000mg/m^2/30 minute infusion gives dFdU peak plasma concentrations (3–15 minutes after infusion) of 28–52 mg/mL. Trough concentration following once weekly dosing: 0.07–1.12mg/mL, with no apparent accumulation. Tissue distribution of dFdU is extensive with a mean volume of distribution of 18 L/m^2. The mean terminal phase half life of dFdU is 65 hours (range 33–84 hr) and mean steady state volume of distribution (Vss) is 150 L/m^2 (range 96–228 L/m^2).

1.3.4 Resistance to gemcitabine

At present lung cancer treatment is inadequate; as patients treated with the front-line drug, gemcitabine, rapidly develops drug resistance. The over-expression of ABC efflux pumps in tumour cells is one of the main mechanisms responsible for multidrug resistance (MDR) in cancer. The ABC transporters appear to limit drug accumulation in the cancer cells, leading to insufficient intracellular concentrations to kill the cell, and consequently clinical multidrug resistance. Most inhibitors developed to date target only P-gp, but many cytotoxic compounds are effluxed by MRPs. For example, there is accumulating evidence that another ABC transporter, multidrug resistance protein 5 (MRP5), is over-expressed in lung cancer cells and confers resistance to gemcitabine (J. Davidson, Ma, & Iverson, 2002 Apr 6–10; Oguri et al., 2006b). The expression levels of MRP5 were inversely correlated with gemcitabine sensitivity significantly in 17 NSCLC cells, whereas the expression of MRP5 in the gemcitabine-resistant NSCLC cell line H23/GEM-R was the same as that in parental NCI-H23 cells. Treatment with the ABCC5 inhibitor zaprinast altered the sensitivity to gemcitabine in MRP5-expressing NSCLC cells. In addition, decreasing the expression of MRP5 by small interfering RNA altered the cytotoxicity to gemcitabine. These results indicate that modulation of MRP5 activity could be used to reverse the gemcitabine resistance in NSCLC.

Another important mechanism of gemcitaibine resistance in NSCLC is by avoiding apoptosis (Davidson et al., 2004; Ikeda et al., 2011). Thus patients harbouring malignancies may initially display a response, but most frequently end up with cancer progression. It has been suggested that gemcitabine resistance in NSCLC cell lines is associated with ribonucleotide reductase, the rate-limiting step in DNA synthesis, because it is the only known enzyme that converts ribonucleotides to deoxyribonucleotides, which are required for DNA polymerization and repair. Ribonucleotide reductase holoenzyme

consists of dimerized large and small subunits ribonucleotide reductase subunits 1 and 2 (RRM1 and RRM2), respectively. Specifically, the increased expression of RRM1 is associated with the gemcitabine-resistant phenotype in two independently generated NSCLC models. It has been suggested that RRM1 may be acting as a "molecular sink" for gemcitabine, whereby the drug binds to RRM1 and irreversibly inactivates that subunit. There appeared to be direct binding of gemcitabine diphosphate to RRM1, and this interaction may be irreversible. As such, both drug and protein may be effectively inactivated by such an interaction. Results from one clinical study indicate RRM1 levels may influence both response and survival of NSCLC patients to gemcitabine/cisplatin combination therapy (Resell et al., 2003).

1.4 Anti-cancer phytochemicals derived from U. pinnatifida

Currently there are more than 45 new and ongoing clinical trials to investigate the potential values of phytochemicals in cancer clinical (ClinicalTrials.gov.). Phytochemicals are being investigated because of 1) their potent effects on ABC transporter modulation (Zhang et al., 2004c; Wu et al., 2005a; Chearwae et al., 2006; Shukla et al., 2008); 2) their potential therapeutic effects attributable to inactivation of survival signalling cascade within tumours (Banerjee et al., 2005; Kunnumakkara et al., 2007; Banerjee et al., 2009) and 3) fewer associated toxicities (Sarkar and Li, 2007; Sarkar and Li, 2009). For example, CBT-1 is in phase III clinical trials as a P-gp inhibitor and curcumin in phase II clinical trials as an ABC transporter modulator (ClinicalTrials.gov.). In phase I clinical trial, CBT-1 is safe and had no effects on the disposition and toxicity of paclitaxel (Oldham et al., 2000). Similarly, orally-administered curcumin was non-toxic to humans up to 8,000 mg/day for 3 months and has shown biological effects in phase II clinical trials for pancreatic cancer (Dhillon et al., 2008). More recently, The U.S. Food and Drug Administration (FDA) approved eribulin mesylate (E7389), a sea sponge-derived nontaxane microtubule dynamics inhibitor, for the treatment of patients with metastatic breast cancer, who have previously received an anthracycline and a taxane in either the adjuvant or metastatic setting and at least 2 chemotherapeutic regimens for the treatment of metastatic disease (Jain & Vahdat, 2011). Therefore, marine products may represent an attractive

resource for effective treatments in cancer, with the goal of palliating symptoms and improving survival while minimizing toxicity and maintaining a good quality of life.

1.4.1 *U. pinnatifida* as a source of anti-cancer agents

The ocean is rich in resources, especially in the ocean fringe. A large number of drugs are derived from terrestrial organism. Indeed, (Glaser & Mayer, 2009) recently suggest that the marine pharmacology has become a popular research field in recent years as mainstay pharmacology. Since the past decade, several anticancer drugs have been successfully developed from ocean, which constitutes 70% of the world's surface. New Zealand is an island country with good weather and good geographic location, which is also rich of ocean resources.

The brown marine algae *U. pinnatifida* has been introduced as an invasive species to New Zealand since 1988 (Williams & West, 2000). *U. pinnatifida* is native to Japan, Korea, and China but can be spread widely as it was ranked as the top 100 invasive species in the world (Wallentinus, 2007). Indeed it has been found in France, Australia, Spain, North and South American, and NZ (Ministry of Fisheries (MFish), 2001). The first discovery of *U. pinnatifida* in NZ was in the Wellington Harbour in 1987. It was probably arrived accidentally in the late 1980s, via shipping from Asia contained in ballast water. (Williams & West, 2000). Its further spread within NZ can be partially attributed to natural dispersal as each fertile plant can generate millions of zoospores, and these can be transplanted by the wave and currents to other parts of the oceans (Ministry of Fisheries (MFish), 2001). The spore of *U. pinnatifida* can be translocated over hundreds of metres, while the whole sporophytes can be spread up to a few kilometres(Russell, Hepburn, Hurd, & Stuart, 2008). Furthermore, spores of *U. pinnatifida* can attach itself on to hulls of ships or boats to be transferred to other location of the coast. Therefore, *U. pinnatifida was* distributed through vessel fouling, and this distribution was already certainly occurred. However, in recent years, the *Undaria* gametophytes were transported through vessel fouling in the introduction of *Undaria* to ports and harbours at Gisborne, Wellington, Porirua, Marlborough Sounds, Nelson, Lyttelton, Akaroa, Timaru, Oamaru, Bluff and Halfmoon Bay (Stuart, 2004). Recently, harvesting and culturing *U. pinnatifida* domestically has been allowed by New Zealand government.

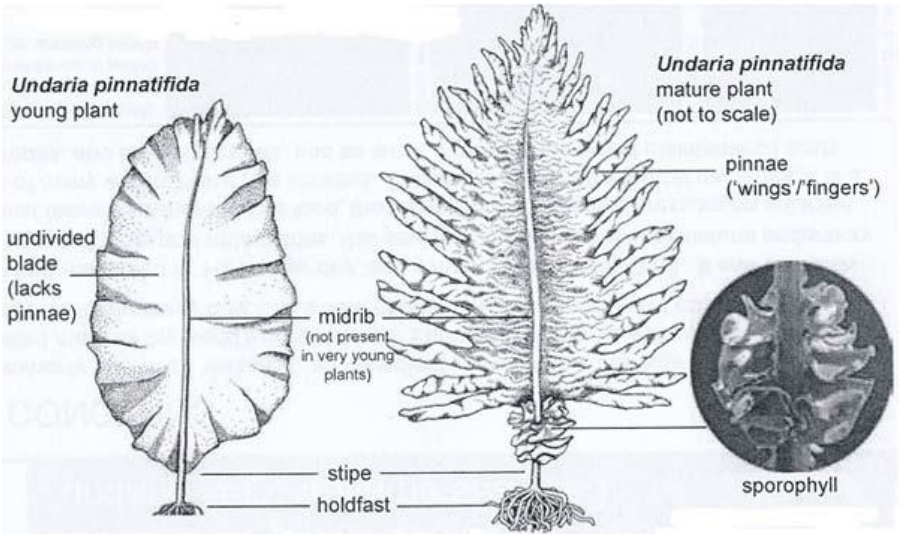

Figure 1 Physical description of mature *U*. pinnatifida. Adapted from"Guid to marine invaders in the gulf of maine" by S. Lonhart (2011, July 22). Retrieved from http://www.mass.gov/czm/invasives/docs/potentialinvaders/u_pinnatifida.pdf

The structure of *U. pinnatifida* sporophyte (Figure 1) includes blade (undivided blade lacks pinnae in young plant; pinnae or wings/fingers in mature plants), sporophyll (only present in mature plants), stipe and holdfast.

Since ancient times, many seaweeds have been used in most part of human life such as the dietary foods, medical and medicinal treatment for human (Ye et al., 2005). *U. pinnatifida* is one of the traditional food stuffs in East Asia, and has been cultured for hundreds of years. It also has been shown pharmacological benefits since the ancient period, and used as a traditional Chinese medicine for hundreds of years (Ye et al., 2005). *U. pinnatifida* is one of the most important species of brown seaweed. *U. pinnatifida* is appears to be very safe as it is a particularly important dietary compound in Japan, Korea (Helen Fitton, 2003) and in China for hundreds of years (Ye et al., 2005). In medical and medicinal treatment, *U. pinnatifida* is source of biologically active phytochemicals, and it contains a wide range of components including carotenoids, phycobilins, fatty acids, polysaccharides, vitamins, sterols, tocopherol, and phycocyanins (Plaza & Cifuentes, 2008). The earliest record showed that *U. pinnatifida* was used in 2700BC in the compilation of "Chinese Herbs" by Emperor ShenNun, which are indicated to "eliminate phlegm and more water" and are also recognized source of iodine g (X. Chen et al., 2006; Helen Fitton, 2003). In traditional Chinese medicine, the brown seaweed such as *Undaria* was also used for treatment of cancer and it has also been

recommended in ancient Ayurvedic texts, despite that the anticarcinogenic properties of brown seaweeds are not completely understood. It has also been recorded in Chinese medicine literatures that *U. pinnatifida* could control of hyperlipidaemia, thrombosis, tumours, and obesity (Helen Fitton, 2003). It was well-known in Chinese and Japanese medicine that dried thallus (stem and spore areas) area of brown seaweeds (*Laminaria, Undaria, or Ecklonia* species) were of medicinal values (Helen Fitton, 2003). In addition, crude brown seaweed such as *U. pinnatifida* was used as an important detoxifying agent, possibly because iodine and other elements that are present in brown seaweed can strongly inhibit absorption of similar radioactive elements by the body.

In the 1960s, *U. pinnatifida* extract called Algasol T331 in Italy was used to treat cancer for the first time in Western Medicine. Intramuscular injections of Algasol T331 were used by Claudio & Standardo to help 68% of 162 cancer patients achieve "good recovery" following administration (V. Chapman, 1970). Good recovery has been attributed to less serious adverse side effects, increasing appetite and hair regrowth in patients who was receiving chemotherapy. This interesting discovery suggested that some potential compounds present in the *U. pinnatifida* might be used as an important adjunction therapy in concert with conventional cancer chemotherapy, leading to increased life quality in these patients.

Epidemiological studies have been undertaken to assess *U. pinnatifida* consumption against the cancer incidence. There were several *in vitro* and *in vivo* studies to determine the relationship between incidence of cancer and brown seaweed *U. pinnatifida* consumption. In a large prospective dietary study 21,852 Japanese nurses in Japan with a 9 year follow-up, the data suggested high intakes of miso soup was highly associated with the lowest breast cancer risk (Yamamoto et al., 2003). Since miso soup is a hot water extract of seaweed with a tablespoon or less of miso and usually a few vegetables added it is very suggestive that seaweed and seaweed soup consumption may help explain the lower breast cancer rates of women in Japan. Therefore, the evidence was approved a significant reductions in cancer risk associated with increasing seaweed consumption. Moreover, compared to conventional chemotherapies, it was suggested some of compounds in *U. pinnatifida* may be present to decrease the risk of cancer in 1980s. In the further studies, Hoshiyama and Takafuni, *et al.* 1992 reported consumption of *U. pinnatifida* two or more times in a week could strongly reduce the incidence of both single and multiple stomach cancer. In addition, the consumption of *U. Pinnatifida* also

related to significantly decrease the risk of rectal cancer and colon cancer (Hosokawa et al., 1999).

Given the ability of *U. pinnatifida* to reproduce through the whole year in New Zealand's suitable temperature, it is of huge economic or ecological concerns for such a non-indigenous species on New Zealand environment. It may be as detrimental to native species and ecosystems worldwide as loss and degradation of habitats (Vitousek, D'Antonio, Loope, & Westbrooks, 1996). The impact of this invasive species in NZ could include: 1) possible changes of benthic and biological assemblages and reduced biodiversity; 2) serious impacts on an ecosystem-level over a broad geographic range; 3) possible changes with the availability of light, change of the nutritive food cycling to ecosystem and food availability to herbivore; and 4) the development of monocultures due to displacement of native species (Piazzi & Cinelli, 2003; Scheibling & Gagnon, 2006), (Forrest & Taylor, 2002). On the other hand, large-scale collection of *U. pinnatifida* may turn this waste into valuable medicinal resources, which will support New Zealand's efforts to build on its strengths through the delivery of value-added natural products and ingredients to the market.

Dietary seaweed is not without its potential problems. Considering that WHO has set an upper limit of 1,000 μg/d for iodine, it could be difficult to approximate the typical dietary consumption of 4-7 g/d of seaweed without jeopardizing thyroid health. Alternatively the major components of *U. pinnatifida* including major pigment compound fucoxanthin and sulfate polysaccharide fucoidan have been found to induce apoptotic damage to various tumour cells. These phytochemicals may represent a new direction for modern use of dietary seaweed as health promoting and even therapeutic agents.

1.4.2 Fucoxanthin

Fucoxanthin (FUX) (Figure 2), a natural marine carotenoid with the formula $C_{42}H_{58}O_6$, is mainly extracted from edible seaweed such as *U. pinnatifida* (wakame) and hijikiafusiformis.

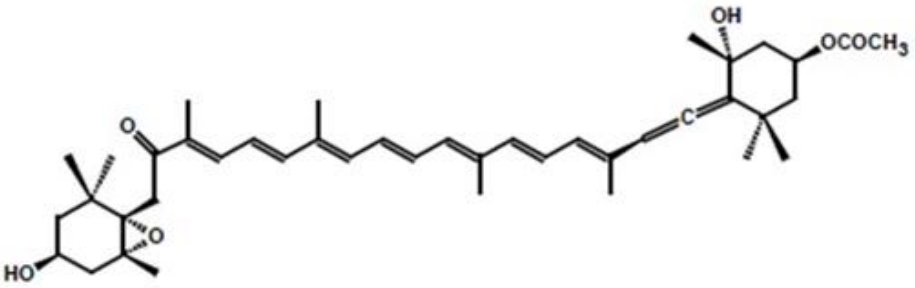

Figure 2 Structure of fucoxanthin

FUX is the most important pigment compound in brown seaweed (D'Orazio et al., 2012). It has a unique structure which contains an unusual allelic bond and some oxygenic functional groups such as epoxy, hydroxyl, carbonyl and carbonyl moieties. Based on this structural characteristic, there are two configurations including *trans* and *cis*-formation of FUX. All-*trans*-FUX is the major isomer present in fresh *U. pinnatifida* with approximate 88% (Holdt & Kraan, 2011). 9'-*cis* and 13'-*cis* can be other types of isomer for FUX with about 9% when it stores in dark conditions (Nakazawa, Sashima, Hosokawa, & Miyashita, 2009). Fucoxanthin absorbs light primarily in the blue-green to yellow-green part of the visible spectrum, peaking at around 510-525 nm by various estimates and absorbing significantly in the range of 450 to 540 nm.

In mice, FUX has been shown to be totally digested and deacetylated into fucoxanthinol in the intestinal tract by lipase and esterase from the pancreas or intestinal cells (Sugawara, Baskaran, Tsuzuki, & Nagao, 2002). Furthermore, fucoxanthinol was converted into amarouciaxanthin A through dehydrogenation /isomerisation in liver microsomes (Asai, Sugawara, Ono, & Nagao, 2004). So, the dietary FUX was suggested to be metabolised into fucoxanthinol in the gastrointestinoal tract, with the later achieving further biotranformation to amarouciaxanthin A in the liver.

Intestinal absorption of carotenoids has been found to be increased with their lipophilicity and phospholipids has been shown to increase the absorption of carotenoid (E. Kotake-Nara & Nagao, 2012). Actually, the absorption of carotenoids in intestinal cells highly depends on the solubility of the carotenoid in mixed micelles.

Depending on this characteristic, the absorption of carotenoid can be increased after metabolism of pancreatic phosphlipase A2 and lysophosphatidylcholine. A simple diffusion mechanism has been suggested for the absorption of FUX from the intestinal epithelium through to blood stream, however, other active transport process may not completely ruled out. Nevertheless, there is no FUX which can be detected in plasma or liver tissue after a single intubated dose of FUX of 0.83 micromol in rats (Sangeetha, Bhaskar, & Baskaran, 2009), while fucoxanthinol and amarouciaxanthin A can be readily detected in plasma, erythrocytes, liver, lung, kidney, heart, spleen, and adipose tissue in mice (Sugawara et al., 2002). In *in vivo* multiple-dosing studies in mice, the daily oral administration of FUX for 1 week showed that a small amount of parental FUX was detectable in the liver, lung, kidney, heart, spleen, and adipose tissue of the mice. In human studies, after oral administration of *U. pinnatifida* for one week, the plasma concentration of fucoxanthinol was detectable but quite low, and no FUX or amarouciaxanthin A was detected in plasma. Thus dietary FUX is suggested to be certainly hydrolyzed to fucoxanthinol in the intestinal tract by lipase and esterase from the pancreas or in intestinal cells, and this hydrolyzed product is taken up by the intestinal cells and secreted into the lymph *in vivo* (Sugawara et al., 2002).

Accumulating evidence suggests anti-proliferative effects of FUX on several types of cancer cell lines including human lung cancer (A549, NSCLC-N6 and SRA 01/04) (Moreau et al., 2006), leukemia HL-60 (Peng, Yuan, Wu, & Wang, 2011), colon cancer (Caco-2, WiDr, HT-29 and DLD-1)(Das et al., 2005; Hosokawa et al., 2004; Sugawara et al., 2002), prostate cancer (PC-3, DU 145, and LNCaP) (Eiichi Kotake-Nara, Asai, & Nagao, 2005; Satomi, 2012; Yoshiko & Hoyoko, 2007), liver cancer (Hep G2)(Das, Hashimoto, & Kanazawa, 2008), gastric adenocarcinoma (MGC-803) (Yu, Hu, Xu, Jiang, & Yang, 2011), and neuroblastoma cancer (GOTO) (Okuzumi et al., 1990). Das et al. reported in 2005 that people who had high consumption of carotenoids reduces the risk of colon cancer, suggesting carotenoids are of potential anticancer properties. More recently, FUX was found to suppress metastasis of highly metastatic B16-F10 melanoma cells *in vitro* and *in vivo* in mice models (Chung et al., 2013). A recent study suggests FUX can also efficiently inhibit the growth of MiaPaCa-2 human pancreatic cancer cells (Unpublished data, personal communication with Dr Yan Li, AUT).

The anticancer activities of FUX have been attributed to its proapoptotic effects in various cancer cells and antiangiogenic effects in tumour growth (J. Peng, J. P. Yuan, C. F. Wu, & J. H. Wang, 2011). FUX induces apoptosis in various cancer cells, resulting in inhibitory growth of human lung cancer A549 cells (Boo et al., 2011), hepatic carcinoma HepG2 cells (Das et al., 2008; Yoshiko & Hoyoku, 2007) and SK-Hep-1 cells (C.-L. Liu, Y.-S. Huang, M. Hosokawa, K. Miyashita, & M.-L. Hu, 2009), colon cancer cells (Caco-2, HT-29, DLD-1 cells) (Hosokawa et al., 2004), gastric adenocarcinoma MGC-803 cells (Yu et al., 2011), prostate cancer PC-3 cells (Kotake-Nara et al., 2005), and primary effusion lymphomas cell lines (Yamamoto, Ishikawa, Katano, Yasumoto, & Mori, 2011). It has recently been reported that FUX causes cell cycle arrest at G_0/G_1 phase in human hepatic carcinoma and prostate cancer cells (S. K. Das et al., 2005; C. L. Liu, Y. S. Huang, M. Hosokawa, K. Miyashita, & M. L. Hu, 2009; Satomi, 2012; Satomi & Nishino, 2009). Das et al. (2008) reported that growth inhibition of human hepatic carcinoma cells by FUX is associated with down-regulation of cyclin D1 and D3, and FUX induces G_0/G_1 arrest. The induction of GADD45A gene seems to be associated with the G_1 arrest by FUX in human hepatic carcinoma cells and prostate cancer cells. (Satomi, 2012; Yoshiko & Hoyoku, 2007).

Interestingly, FUX can be biotransformed by human cells into several bioactive metabolites, such as fucoxanthinol , amarouciaxanthin A, and halocynthiaxanthin. As these metabolites are similar in chemical structures, containing a common ground of the allelic bond, which is found mainly in carotenoids and is responsible for their biological effects. It has been suggested that fucoxanthinol rather than FUX itself should therefore be considered in mechanistic studies of the biological actions of FUX. For example, fucoxanthinol have been reported to induce apoptosis in human HL-60 leukemia cells, human MCF-7 breast cancer cells, PC-3 human prostate cancer cells and human Caco-2 colon cancer cells (Konishi, Hosokawa, Sashima, Kobayashi, & Miyashita, 2006) (Akira, Tatsuya, Hiroshi, & Akihiko, 2004). Compared with FUX, the antiproliferative and apoptosis-inducing effects of halocynthiaxanthin and fucoxanthinol on cancer cells were reported to be significantly more potent.

More recently, FUX has been recently reported to be an efficient inhibitor of several ABC transporters (Eid et al., 2012; Liu et al., 2012). Proteins of the ATP-binding

cassette superfamily, mainly P-glycoprotein (P-gp; MDR1), play an important role in the development of multidrug resistance (MDR) in cancer cells and thus in the potential failure of chemotherapy. A selection of carotenoids (beta-carotene, crocin, retinoic acid, canthaxanthin, and fucoxanthin) was investigated whether they are substrates of P-gp, and if they can reverse MDR in resistant Caco-2 and CEM/ADR5000 cells as compared to the sensitive parent cell line CCRF-CEM. Fucoxanthin at 50-100 μM produced a 3-5-fold higher retention of the fluorescent probes than the known competitive inhibitor verapamil. The carotenoids increased accumulation of these P-gp substrates in a dose-dependent manner indicating that they themselves also function as substrates. FUX synergistically enhanced the cytotoxicity of 5-FU 53.37-fold, of vinblastine 51.01-fold, and of etoposide 12.47-fold. FUX significantly decreased P-gp levels to 12% as compared to untreated control level. Thus FUX may be further developed as a chemosensitiser.

1.4.3 Fucoidan

Fucoidan, a sulfated polysaccharide, can be largely found in *U. pinnatifida* as another major bioactive compound. Since 1913, there were a large amount of published research articles related to fucoidan. These research papers found that fucoidan, a bioactive compound from *U. pinnatifida* polysaccharides possess several pharmacological properties including anti-tumour, anti-coagulant, anti-thrombotic, anti-virus, immunomodulatory, anti-oxidant, and anti-inflammatory effects (Li & Lu, 2008). The anticancer effects of fucoidan from *U. pinnatifida* on A549 human lung carcinoma cells were recently assessed (Boo et al., 2011). Treatment of A549 cells with fucoidan resulted in potent antiproliferative activity. Also, some typical apoptotic characteristics, such as chromatin condensation and an increase in the population of sub-G_1 hypodiploid cells, were observed. Further studies indicate that fucoidan induces apoptosis of A549 human lung cancer cells through down-regulation of p38, PI3K/Akt, and the activation of the ERK1/2 MAPK pathway, reduced Bcl-2 expression, stimulation of caspase-9 activation.

1.5 Objectives of Study

Lung cancer is the most common cause of cancer mortality in the world. At present lung cancer treatment is inadequate, as patients treated with the front-line drug, gemcitabine,

rapidly develop drug resistance by decreasing cellular accumulation and/or avoiding apoptosis (Davidson et al., 2004; Ikeda et al., 2011). FUX has been recently reported to be an efficient inhibitor of several ABC transporters, which can increase cellular accumulation of various anti-cancer drugs by inhibiting ABC transporter-mediated efflux (Eid, El-Readi, & Wink, 2012; Liu, Lim, & Hu, 2012). Also because FUX can induce apoptosis in various cancer cells (Das et al., 2005; Liu, Huang, Hosokawa, Miyashita, & Hu, 2009; Peng et al., 2011; Satomi & Nishino, 2009), it may reverse gemcitabine resistance in lung cancer cells as a potential sensitizer. The pharmacology of most anticancer agents presently in clinical use can be well defined based on *in vitro* cell culture models (Thurston, 2006). Thus it is noteworthy to investigate the anticancer effects of FUX using cultured cancer cells. It is also desirable to identify a potential sensitizer to reverse gemcitabine resistance in lung cancer. Previous studies have defined FUX extracted from New Zealand *Undariapinnitifida* with anti-cancer properties by using in vitro cell models. FUX has few adverse effects on normal cells and may be developed as an efficient chemosensitiser for cancer chemotherapy. However, it remains unclear 1) if FUX may potentiate gemcitabine sensitivity in lung cancer cells; and 2) if FUX may modify gemcitabine toxicity in normal cells.

The primary objective of this study was to assess the potential effects of FUX to reverse gemcitabine resistance in human lung cancer cell lines. The secondary objective of current study is to investigate the mechanisms of FUX actions if FUX may potentiate gemcitabine sensitivity. The third objective of this study is to evaluate the effects of FUX on modifying gemcitabine toxicity in two typical normal cell lines.

2 Chapter 2 Effects of FUX on gemcitabine sensitivity on human lung cancer cells and human normal cells

2.1 Introduction

Lung cancer is the most common cause of cancer mortality in the world. At present lung cancer especially NSCLC treatment is inadequate, as patients treated with the front-line drug, gemcitabine, rapidly develop drug resistance by avoiding apoptosis (J. D. Davidson et al., 2004; Ikeda et al., 2011). It is desirable to identify a safe and potent sensitizer to reverse gemcitabine resistance in lung cancer. However, most sensitizers have Janus faces, reversing anti-cancer drug resistance in cancer cells but potentiating cytotoxicity in normal tissues. FUX seems to be a unique compound as it has been reported to be safe to normal cells even at extremely high dose/concentrations.

To identify the concentration- and time-dependence of the effects of FUX on an NSCLC cell line A549 and two normal cell lines, a specific and robust cytotoxic assay is desired. While optimal conditions for each cell line (cell number plated and doubling time) must be established, using characterized tumour cell lines, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylformazan bromide assay (MTT assay) could be automated and thus be of great value in identifying the effects of FUX on cytotoxicity of three different human cell lines.

2.2 Materials

The main materials involved in this study were listed in Table 1.

Table 1 Main materials involved in this study

Material	**Provider**
Cell lines	ATCC (Cryosite Ltd, NSW, AU)

RPMI 1640 Cell Culture Medium	Sigma-Aldrich
Trypsin-EDTA	Invitrogen
Foetal Bovine Serum (FBS)	Invitrogen
Culture flasks	Sigma-Aldrich
Culture plates ×100	Sigma-Aldrich
Phosphate Buffered Saline (PBS)	Invitrogen
Dimethyl Sulfoxide	Sigma-Aldrich
MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MW = 414)	Sigma-Aldrich

2.3 Methods

2.3.1 Cell line information

Three human cell lines (Table 2) were stored in -80℃ freezer and in liquid nitrogen dewell. After thawing the cell lines, they were maintained in tissue culture incubator at 37°C in 5% carbon dioxide humidified air.

Table 2 Three human cell lines

Cancer Cell Line Description	*Source*	*Catalogue Number*	*Cell Line Designation*
Lung Carcinoma; Human	ATCC	CCL-185	A549
Embryonic kidney cells; Human	ATCC	CRL-1573	HEK293
Adult Dermal Fibroblasts, Human	Invitrogen	C-013-5C	HDFa

2.3.2 Preparation of complete medium

To culture A549 or HEK293 cells in completed culture medium, RPMI or DMEM

base medium was supplemented with 1% of Penicillin-Streptomycin, 1% of L-glutamine and 10% of foetal bovine serum. For HDFa cells, the complete medium consists of Medium 106 supplemented with LSGS.

2.3.3 Basic cell culture

2.3.3.1 Starting cell cultures from frozen stocks

Cells were thawed rapidly by briefly immersing the vial in a 37°C water bath for 2–3 min with constant agitation. Upon thawing, immediately wipe the outside of the vial with 70% ethanol, then transfer the contents of the vial to a T25 flask containing 1mL of prewarmed complete medium. An additional 3-6 mL of medium was added to the flask. After gently swirling the flask to distribute cells evenly over the growth surface, the culture was placed in a 37°C, 5% CO_2, humidified incubator. The next day, the cells were examined under a microscope. Healthy cells display a flat morphology and adhere well to the plate. The medium was aspirated and replaced with fresh, warm growth medium. Cell cultures should be split when they each about 70–80% confluency.

2.3.3.2 Split the cells

To split the cells, the medium was removed and the cells were washed once with prewarmed sterile PBS (containing no Ca^{2+} or Mg^{2+}). An aliquot of 1–2 mL of TrypLE™ Express solution were added and the cell monolayer was treated for 1–2 min, or longer, until cells detach. To stop trypsinization, 5–10 mL of growth medium was added and then the cells were resuspended gently but thoroughly. After counting cells using a haemocytometer, the viable cells (1-2 $\times 10^4/cm^2$) were then transferred to a new culture flask containing an appropriate volume of growth medium. The flask was gently swirled to evenly distribute the cells.

2.3.3.3 Preparing frozen stocks of cells

Once the cells have been established in culture, the frozen stock should be prepared from an early passage to ensure a renewable source of cells. To trypsinize the cells, the medium was removed and the cells from the desired number of flasks were washed once with prewarmed sterile PBS (containing no Ca^{2+} or Mg^{2+}). An aliquot of 1–2 mL of TrypLE™ Express solution were added into each flask and the cell

monolayer was treated for 1–2 min, or longer, until cells detach. To stop trypsinization, 5–10 mL of growth medium was added into each flask and then the cells were resuspended gently but thoroughly. Pooled cell suspensions were counted and total viable cell number calculated. After centrifuging cells at 125–180 x g for 10 min, the supernatant was aspirated. The cell pellet was resuspended at a density of at least 1–2 · 10^6 cells/mL in freezing medium (Invitrogen). Aliquots of cell suspension (0.5–1 mL) were then dispensed into sterile cryovials. Freeze slowly (1°C per min) by placing vials in a thick-walled styrofoam container at –20°C for 1 hr before storage at a –80°C freezer overnight. Cryovials were removed from styrofoam container the following day and placed in liquid nitrogen for storage. Two or more weeks later, confirm the viability of the frozen stocks by starting a fresh culture from frozen cells as described above (Folkman, 1995).

2.3.4 Determination of doubling time of A549 and HEK293 cells

Doubling time refers to the time taken, in the middle of the exponential phase, for the cell population to double. It can be calculated using the following equation:

$$\frac{N1}{N0} = 2^{\frac{t}{DT}}$$

Where N_0 stands for the initial concentration of cells; N_1, the final concentration of cells; t, the duration of culture; DT, the doubling time.

For A549 cells, the initial concentration (N_0) is 5 ×10^4 cells/mL and the final concentration of the cells (N_1) 187.5 ×10^4 cells/mL after 75-hr. Accordingly the doubling time (DT) of A549 is calculated to be 18.16 hr. For HEK293 cells, N_0 is 5 × 10^4 cells/mL and N_1 111 · 10^4 cells/mL after 72-hr. Thus the DT of HEK293 is 16.11 hr.

2.3.5 Pure FUX Standard Preparation

FUX were purchased from Sigma NZ. An aliquot of 750 µL pure ethanol were added into FUX original vials (containing 10 mg FUX) to make a stock solution of 20 mM. Then aliquots of 50µL per tube was stored in a -80^{o}C freezer.

2.3.6 Preparation of MTT solution

An MTT stock solution (12 mM) was prepared by adding 1 mL of sterile PBS to one vial containing 5 mg MTT powder. Mix by vortexing or sonication until dissolved. Occasionally there may be some particulate material that will not dissolve; this can be removed by filtration. Once prepared, the MTT solution can be stored for four weeks at 4°C protected from light.

2.3.7 Standard procedures of MTT Assay and its application

Briefly, 0.1 mL of cells with a density of 3-5,000 cells/mL in complete medium was seeded onto each well of 96-well microtiter plates. After 24-hour incubation at 37ºC, the medium in each well was removed, and the cells treated with 0.1 mL fresh medium containing various drugs. After 24-, 48-, 72-, 96-hour incubation at 37ºC, the entire medium was carefully removed and replaced with 100 µL of fresh culture medium. An aliquot of 10 µL of MTT stock solution was added to each well. A negative control of 10 µL of the MTT stock solution was added to 100 µL of medium alone. After incubation at 37°C for 4 hours, all but 25 µL of medium was removed from the wells. An aliquot of 150 µL of DMSO was added to each well and mixed thoroughly using an orbit plate shaker. After incubating at 37°C for 30 minutes, the plate was shaken briefly and absorbance was measured at 540 nm and 630 nm (for background).

To determine MTT assay linearity range for each cell line in this study, 0.1 mL fresh medium containing cells were plated into 96-well microtiter plates at various densities from 1953 to 500,000 cells/mL. Each concentration had at least three trials. For blank wells containing no cells, 0.1 mL fresh medium was added. After 5-hour incubation at 37ºC, an aliquot of 10 µL of MTT stock solution was added to each well. A negative control of 10 µL of the MTT stock solution was added to 100 µL of medium alone. After incubation at 37°C for 4 hours, all but 25 µL of medium was removed from the wells. An aliquot of 150 µL of DMSO was added to each well and mixed thoroughly using an orbit plate shaker. After incubating at 37°C for 30 minutes, absorbance was measured at 540 nm and 630 nm.

To determine the cytotoxicity of FUX only to each cell line, 0.1 mL of FUX in culture medium at various concentrations was added into the 96 well plates. The final

concentration of ethanol is 1%, which has no apparent effects on MTT assay results according to pilot studies. As there were 100 μL cells in medium already, the final concentration of FUX was half of the original concentration. Each test had three trials. The details of solution preparation were detailed in Table 3.

Table 3 The inhibition of FUX only on cell lines plan

Stock sol in ethanol (mM)	Stock	Medium	Ori conc. (μM)	Final conc. (μM)
20	8μL	392μL	400	200
10	8μL	392μL	200	100
5	8μL	392μL	100	50
2.5	8μL	392μL	50	25
1.25	8μL	392μL	25	12.5
0.625	8μL	392μL	12.5	6.25
0.3125	8μL	392μL	6.25	3.125
0.15625	8μL	392μL	3.125	1.5625

To determine the effects of FUX on gemcitabine sensitivities in various cell lines, the 96 well plates were divided into two parts (as shown in Table 4). The gemcitabine only part tested nine different concentrations of 0, 1, 2.5, 5, 10, 25, 50, 100 and 500 nM. Each concentration had three trials. The drug combined parts were planned as follows: Blank group was added nothing except the cell culture medium which was the same with gemcitabine only part, while the control group was adding FUX only without gemcitabine. Unknown groups were added the nine concentrations as the same as the plan of gemcitabine only part but with different concentrations of FUX in each drug combined part: 2, 4, 8 μM. Each group had three trials as well.

Table 4 Plates plan for cell culture

Plate 1	**Plate 2**
Gemcitabine (nM) only (Blank | Control | 1 | 2.5 | 5 | 10 | 25 | 50 | 100 | 500)	Gemcitabine (nM) combined with 2 μM FUX (Blank | Control | 1 | 2.5 | 5 | 10 | 25 | 50 | 100 | 500)
Gemcitabine (nM) combined with 8 μM FUX (Blank | Control | 1 | 2.5 | 5 | 10 | 25 | 50 | 100 | 500)	Gemcitabine (nM) combined with 4 μM FUX (Blank | Control | 1 | 2.5 | 5 | 10 | 25 | 50 | 100 | 500)

2.3.8 Effects of FUX on specificity of MTT assay

To eliminate the possibility of FUX's colour impact on MTT assay, it is important to do a test of high concentration of FUX. As shown in Figure 3, the absorbance values are very minor even at FUX 200µM. Also there is no significant absorbance difference in the presence and absence of FUX of 200µM, which is much higher than the concentration used in the combination study.

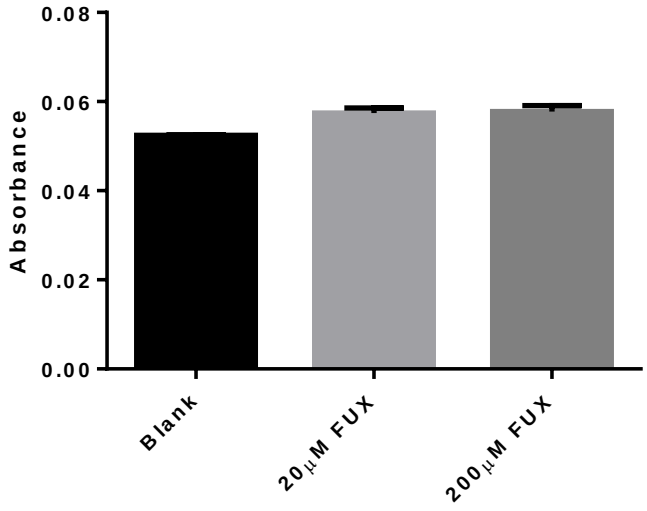

Figure 3 Effects of FUX on specificity of MTT assay. Absorbance was determined in wells without any cells.

2.3.9 Data analysis

The half maximal inhibitory concentration (IC_{50}) is a measure of the effectiveness of a substance in inhibiting cell proliferation in this study. This quantitative measure indicates how much of the drugs are needed to inhibit the cell lines by half. It is also calculated by PRISM® software (Graphpad, Version 6.0).

2.3.10 Statistical Analysis

The initial statistical analysis to evaluate the differences in IC_{50} values among the different groups was performed by a one-way analysis of variance (ANOVA) with a post-hoc test (Dunnett's multiple comparison test) by using PRISM® software (Graphpad, Version 6.0). All data analysed using ANOVAs met the assumptions of

equal variance and homogeneity. Student's unpaired *t* test was conducted for comparisons between 2 groups with a significance level of $P < 0.05$.

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2.4 Results and Discussion

2.4.1 Effects of FUX on gemcitabine sensitivity in A549 cells

To make sure all the cell culture experiments would be taken under a good cell condition, the concentration of seeding cells should not be over-crowded or too scarce. As shown in Figure 4, for the A549 cancer cells, the absorbance determined at 540nm wavelength was linear (R square = 0.9617) within the cell density range. It is reasonable to use the range of cell density to do the experiment and easily to measure the cell cytotoxicity.

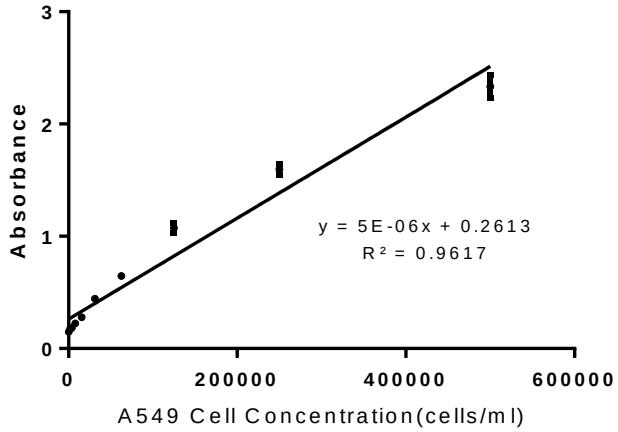

Figure 4 Linearity between A549 cell numbers and absorbance values. Data are means ± SD (n=3).

A549 cancer cells were then treated with FUX at 1.56, 3.13, 6.25, 12.5, 25, 50, 100 and 200 μM for 72-hr. The same experiment was repeated in another day, and the results are shown in Figure 5 and Figure 6, respectively. Both concentration-cell viability curves fit well with sigmoid model of negative exponential distribution, showing slightly decreased cell viability in the low concentration range (1.5625 to 6.25 μM) followed by relatively steep drop of cell viability and then a plateau. IC_{50}

values were 13.45 µM and 12.98 µM, respectively, indicating reasonable between-day assay reproducibility.

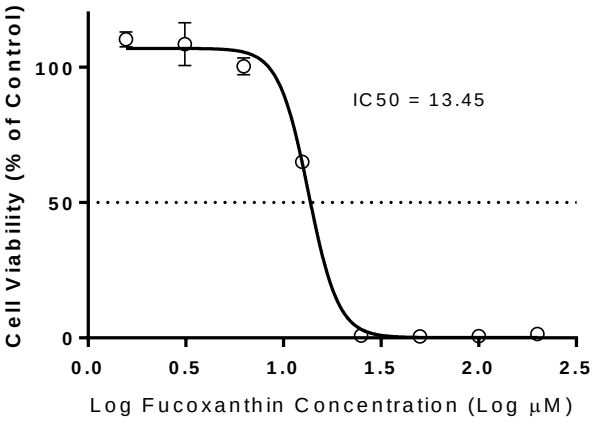

Figure 5 Effects of FUX at various concentrations (at 1.56, 3.13, 6.25, 12.5, 25, 50, 100 and 200 µM) on growth of A549 cells treated for 72-hr (experiment 1). Data are means ± SD (n=3).

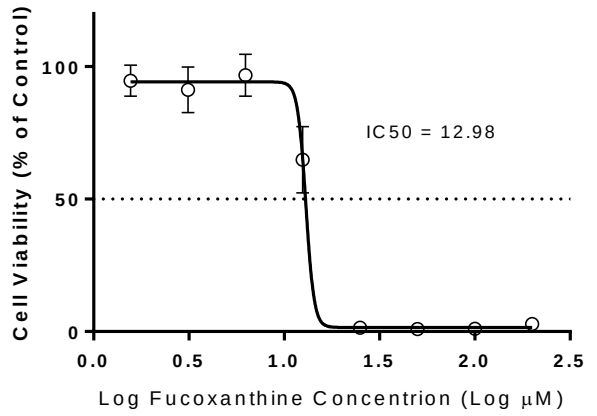

Figure 6 Effects of FUX at various concentrations (at 1.56, 3.13, 6.25, 12.5, 25, 50, 100 and 200 µM) on growth of A549 cells treated for 72-hr (experiment 2). Data are means ± SD (n=3).

Representative concentration-viability curves for A549 cells treated with gemcitabine in the presence or absence of FUX at 2, 4 and 8 µM for 48-hr and 72-hr were shown in Figure 7 and Figure 8, respectively. As expected, gemcitabine shows concentration and time-dependant cytotoxicity effects on A549 cells, with IC_{50} values of 14.0 and 9.4 nM for 48-hr and 72-hr, respectively. All concentration-cell viability curves generally fit well with sigmoid model of negative exponential distribution, showing slightly decreased cell viability in the low concentration range followed by relatively

steep drop of cell viability and then a plateau. From the figures, FUX showed concentration-dependant effects on increasing gemcitabine cytotoxicity to A549 cells as it caused concentration-dependant shift of cell growth curves toward to the left-hand. This translates into an FUX concentration-dependant effects on decrease of gemcitabine IC_{50} values. For more accurate results and standard explanation, it is important to use One-way ANOVA analysis test, and the results are organized and shown in Table 5. 48-hr MTT assay results indicate apparent concentration-dependant effects of FUX on gemcitabine cytotoxicity to A549 cells, despite that the effects is not statistically significant. Nevertheless, in the presence of 8μM FUX significantly ($P < 0.05$) increased gemcitabine sensitivity in A549 cells after 72-hr treatment. Indeed, the IC_{50} value was only 3.9 ± 1.4 nM for gemcitabine in the presence of 8μM FUX, which is decreased by 59% when comparing with control.

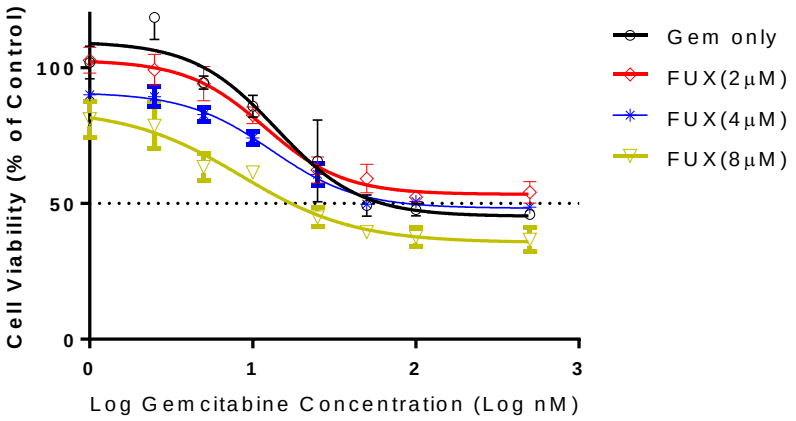

Figure 7 A representative viability curve for A549 cells treated with gemcitabine (at 1.56, 3.13, 6.25, 12.5, 25, 50, 100 and 500 nM) in the presence or absence of FUX for 48-hr. Data are means ± SD (n=3).

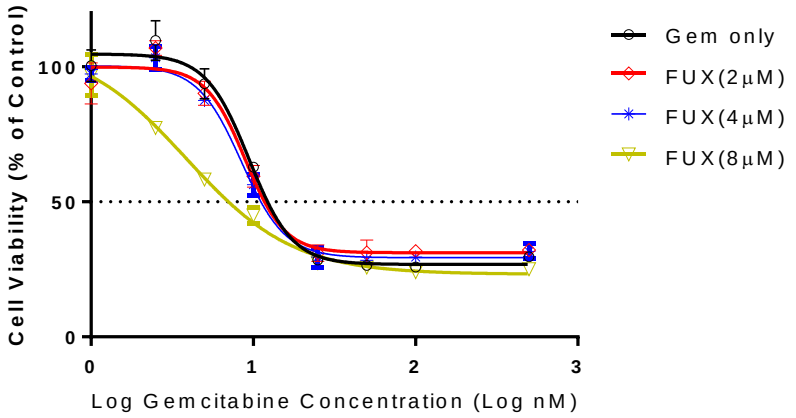

Figure 8 A representative viability curve for A549 cells treated with gemcitabine (at 1.56, 3.13, 6.25, 12.5, 25, 50, 100 and 500 nM) in the presence or absence of FUX for 72-hr. Data are means ± SD (n=3).

Table 5 Effects of FUX on drug sensitivity of A549 cells to Gemcitabine (Gem). IC50 values were determined from 3 experiments each performed in triplicate and expressed as mean ± SEM

	IC_{50} (nM)	
	48-hr	72-hr
Control (Gem only)	14.0 ± 5.3	9.4 ± 0.4
Gem + FUX (2 μM)	11.9 ± 2.2	8.9 ± 0.5
Gem + FUX (4 μM)	13.0 ± 2.4	8.5 ± 0.6
Gem + FUX (8 μM)	8.5 ± 2.3	3.9 ± 1.4*

*P< 0.05, compared with control

2.4.2 Effects of FUX on gemcitabine sensitivity in HDFa cells

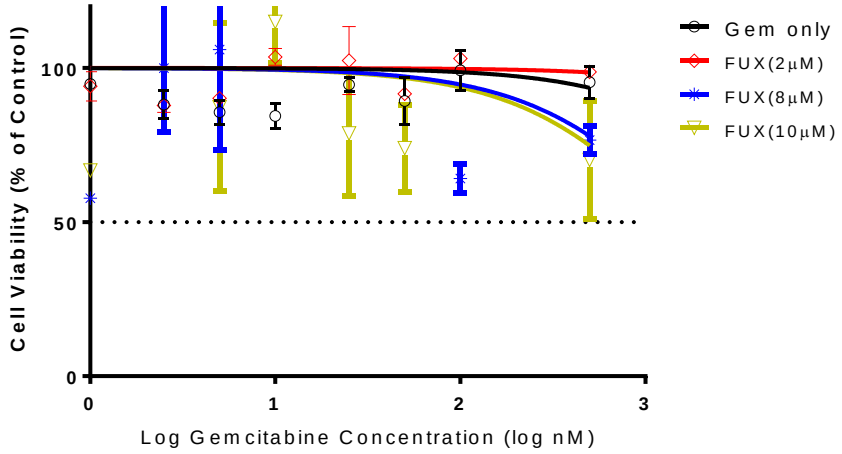

Figure 9 A representative viability curve for HDFa cells treated with gemcitabine (at 1.56, 3.13, 6.25, 12.5, 25, 50, 100 and 500 nM) in the presence or absence of FUX for 72-hr. Data are means ± SD (n=3).

Figure 9 shows a representative concentration-viability curve for HDFa cells treated with gemcitabine in the presence or absence of FUX at 2, 4 and 8 μM for 72-hr. Gemcitabine at 500 nM has no apparent toxic effects in HDFa cells. However, FUX appears to increase gemcitabine (100 nM) sensitivity to HDFa cells at 8 μM but not at 2 or 10 μM. At other gemcitabine concentrations, FUX has no apparent effects on gemcitabine cytotoxicity in HDFa cells.

2.4.3 Effects of FUX on gemcitabine sensitivity in HEK293 cells

The HEK293 cell lines are considered to be normal cells and they are quite different with the cancer cell lines. For better results, it is reasonable to compare the effects between normal cells and cancer cells. From Figure 10, the absorbance under 540nm wavelength was linear with the cell density (R square which was 0.9936), suggesting a wide linear range for MTT assay for this cell line. It was reasonable to use the range of cell density to do the experiment and easily to know the cell cytotoxicity.

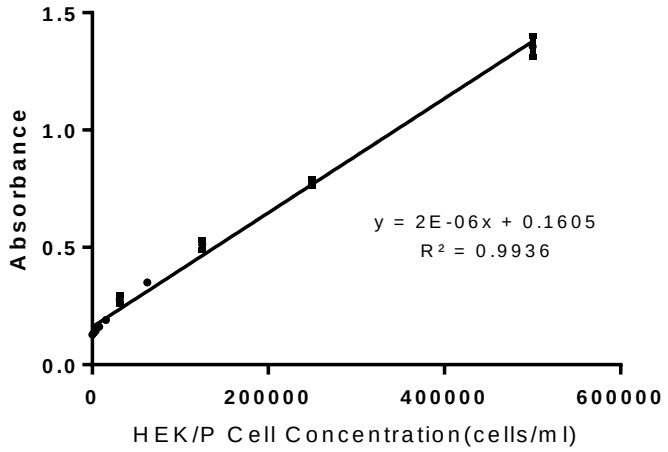

Figure 10 HEK293 Growing curve

Representative growth curves for HEK293 treated with gemcitabine in the presence or absence of FUX for 48-hr and 72-hr were presented in Figure 11 and Figure 12, respectively. From the figures, it seems that all the treatments show similar effect on cell cytotoxicity. For more accurate results and standard explanation, it is important to use One-way ANOVA test, and the results are organized and shown in Table 6. No significant difference displayed. The IC_{50} values were all above 26 nM for gemcitabine, comparing with the IC_{50} values on A549 cancer cell lines, the gemcitabine and FUX concentrations used as anticancer drugs are quite safe for normal cells.

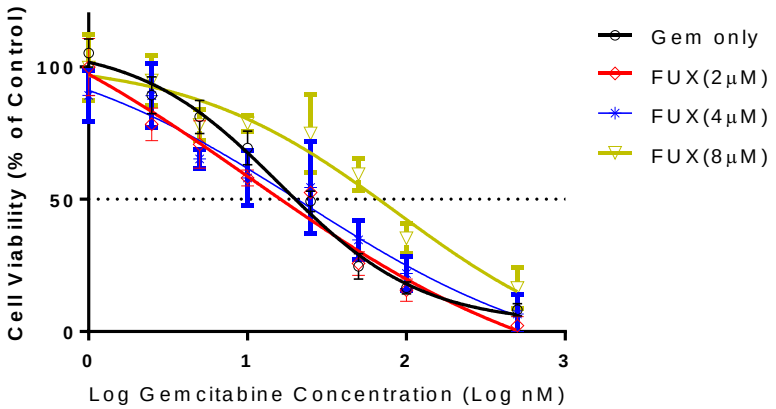

Figure 11 Representative growth curve for HEK293 treated with gemcitabine (at 1.56, 3.13, 6.25, 12.5, 25, 50, 100 and 500 nM) in the presence or absence of FUX for 48-hr. Data are means ± SD (n=3).

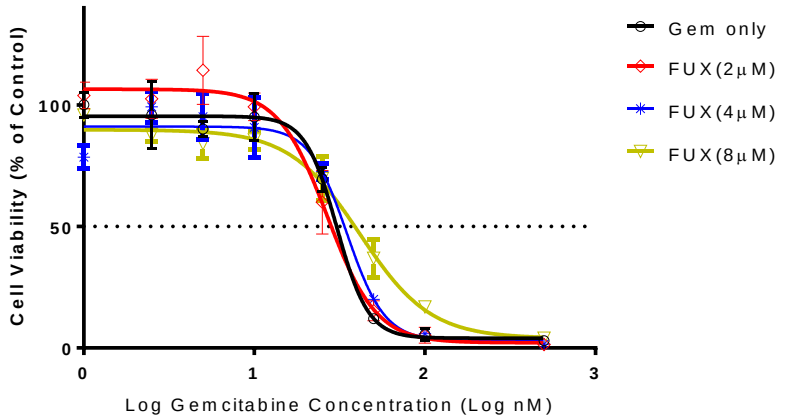

Figure 12 Representative growth curve for HEK293 treated with gemcitabine (at 1.56, 3.13, 6.25, 12.5, 25, 50, 100 and 500 nM) in the presence or absence of FUX for 72-hr. Data are means ± SD (n=3).

Table 6 Effects of FUX on drug sensitivity of HEK293 to Gem. IC50 values were determined from 3 experiments each performed in triplicate and expressed as means ± SEM

	IC_{50} (nM)	
	48-hr	72-hr
Control (Gem only)	38.85 ± 6.0	30.44 ± 2.1
Gem + FUX (2 μM)	47.90 ± 3.3	26.74 ± 3.2
Gem + FUX (4 μM)	68.85 ± 20.1	34.95 ± 0.5
Gem + FUX (8 μM)	63.05 ± 18.9	41.59 ± 4.9

2.5 Summary

FUX(8μM) increases gemcitabine sensitivity in an NSCLC cell line, A549 cell in a time and concentration dependant manner. More importantly, it has no apparent effects on gemcitabine toxicity in two typical cell lines representing normal human tissues. It would be expected that FUX may represent a unique sensitizer, which may turn a less effective anti-cancer drug into an exceptional one. To elucidate the mechanisms of action of FUX, it is necessary to carry out a mechanistic study to investigate if FUX changes the intracellular gemcitabine accumulation in A549 cells.

3 Chapter 3 Development of an HPLC method to determine the cellular accumulation of gemcitabine in A549 cells

3.1 Introduction

Chapter 2 shows FUX potentiated gemcitabine sensitivity in A549 cells and one plausible mechanism may be due to its effects on cellular accumulation of gemcitabine. Cellular accumulation of a drug may be determined by an analytical method after extracting it from cell homogenates. High-performance liquid chromatography (HPLC) is a technique in analytic chemistry used to separate the components in a mixture, to identify each component, and to quantify each component. Therefore HPLC may be useful to separate gemcitabine from cellular matrix components and to quantify gemcitabine specifically. The acceptability of the performance of an analytical method to measure the concentrations of analytes in a specified biological matrix (e.g. cell homogenates) must be evaluated before use (Shah et al., 1991; Green, 1996). The important parameters used to assess and monitor the performance of an HPLC method include the specificity (selectivity), the linearity of calibration curve, the accuracy and the precision of the drug in the matrix under experimental conditions (Robert, 1994). In this chapter, the HPLC method used to determine the concentrations of gemcitabine in the cellular homogenates, and its validation are described.

3.2 Terminology

3.2.1 Specificity (Selectivity)

The specificity is the ability of a method to measure a unique component which it is intended to measure. By using an appropriate mobile phase and solid phase, interference components can be separated from gemcitabine and eluted at different time points. Consequently no interference occurs in distinguishing the analyte from other drugs or components in the biological matrix (Shah et al., 1991).

3.2.2 Linearity

The detection response must be determined mathematically to be linearly proportional to the concentration of the analyte (Shah et al., 1991). This can be assessed by

preparing a calibration curve using 5 to 8 increasing known concentrations of analyte in the biological matrix, and all are taken through the method (Robert, 1994). In HPLC, peak areas or peak-area ratios of analyte to internal standard are then plotted against the known concentrations of analyte. Linear regression analysis is then used to draw the best-fit line through the points and generate a correlation coefficient. A correlation coefficient of ≥ 0.990 is generally acceptable for assay linearity (Shah et al., 1991).

3.2.3 Accuracy (Recovery)

Accuracy is defined as the closeness of the value measured by an analytical method to the actual value of the analyte. It is determined by replicate measurement of samples containing known amount of analyte over an appropriate range of concentrations (Shah et al., 1991). Accuracy is normally expressed as the percentage of deviation of the measured value from the actual value (Hanahan & Folkman, 1996), which should not exceed 15% deviation except at LOQ, here it should not exceed 20%.

3.2.4 Precision

Precision refers to the closeness of replicate determinations of an anlayte obtained from multiple analysis of a homogeneous samples. Mathematically precision can be determined by calculating a coefficient of variation (CV), which is calculated by dividing standard deviation (SD) by the mean and expressing as a percentage. The acceptance criterion for precision is a CV < 15%, except at the limit of quantitation (LOQ), where a CV < 20% is acceptable (Karnes et al., 1991; Green, 1996). Precision can be classified as intra-assay (within-day) precision and inter-assay (between-day) precision. Intra-assay precision is determined by multiple measurements of a homogenous sample on the same day or within an assay run; whereas inter-assay precision is determined by performing a series of repeated measurements on homogenous samples on different days or assay runs (Karnes et al., 1991; Green, 1996). In this thesis, all the samples were run on the same day, and precision would described as intra-assay precision.

3.3 Method development

3.3.1 Prepare stock solution of gemcitabine

Dissolve gemcitabine in Milli-Q water which had been autoclaved, and make the concentration as 10 mM for stock solution. Aliquots of stock solution were stored in a -80˚C freezer.

3.3.2 Prepare cell homogenates

The cells which were grown in T75 flasks were detached with TrypLE™ Express solution. The cells were centrifuged for 5 minutes. The cells were then resuspended in Milli-Q water. The cells were frozen and thawed for three circles. The cells were then passed through a 29-gauge needle to obtain cell homogenates. The cells homogenates were stored in a -80°C freezer.

3.3.3 Prepare standard samples and quality control samples

Quality control (QC) samples containing gemcitabine were prepared from weighing independent of those used for preparing stock solution and standard samples in cell homogenates. Final concentrations of low, medium and high QC samples were 0.1, 1 and 10 μM, and the concentrations of standard samples were 0.25, 0.5, 1, 2.5, 5, 10 μM respectively. QC samples were prepared on the day of analysis in the same way as standard samples. The performance of the HPLC method was assessed by analysis of 12 QC samples (four each of low, medium, and high concentrations) on a single assay day to determine accuracy and precision.

3.3.4 Effects of different mobile phases on gemcitabine separation

Different conditions for HPLC get different retention time for the same chemical substances, meanwhile, the same condition get different retention time for different chemical substances. One important factor is mobile phase. It depends on the pH value, the polarity of the mobile phase. At the very beginning, a mobile phase consisting of 4% acetonitrile (ACN) and 96% Milli-Q water was used in the HPLC method development, which is based on a recently published method (Bansal et al., 2013). This, however, generates an extremely short retention time for gemcitabine thus insufficient separation from cellular matrix. Another mobile phase consisting of

4% methanol with 96% Milli-Q water showed similarly poor separation by using the same HPLC column. Thereafter changing the mobile phase to 100% Milli-Q water extended the gemcitabine retention time but the peak shape was compromised. It was well-know that buffer based mobile phase may improve analyte peak shape. Small changes in the mobile phase pH can also have a dramatic effect on the selectivity of weakly ionizable compounds. Thus either 10mM phosphate buffer (pH = 2.7) or citric acid buffer (pH = 2.6) was tested as the mobile phase but neither can obtain sufficient retention of gemcitabine. When changing the mobile phase to 50mM citric acid (pH = 4.7), a good retention time for gemcitabine (around 14.4 min) was recorded, but was interfered by the matrix in A549 cellular homogenates. The last trial was 50mM phosphate buffer (pH = 6.5~7.5), and the results were better.

3.3.5 Effects of different stationary phases on gemcitabine separation

However, in the process of investigating the mobile phase conditions, there are cases in which the separation conditions cannot be improved. Although mobile phase is an important factor, the stationary phase which also plays an important role because HPLC column is as important as the mobile phase. The column which was used to separate gemcitabine in this project at the very beginning was a C18 column (Prodigy 5μm ODS-3V, 250 × 4.6mm), but the separation was shown to be insufficient. The C18 column is an ODS type of column that is marketed by instrument and column manufacturers, in which octadecyl groups are bonded to a silica base to provide wide separation applicability. An appropriate ODS column is typically the first type selected from the various types available at the start of a reverse-phase analysis (Lesellier & West, 2007). However, if a C18 column cannot provide sufficient separation, one possible solution in such a case is to use a phenyl column. In this study, a phenyl-hexyl column (Luna 5u Phenyl-Hexyl 10, 250 × 4.6mm, 5u micron) was approved to achieve baseline separation of gemcitabine from other cellular interferences, without changing the mobile phase or sample preparation procedures. Other columns such as a HILIC column (Luna 5u HILIC 200A, 250 × 4.6mm, 5 micron) and an amine column (Luna 3u NH_2 100A, 50 × 3.00mm, 3 micron) were tested to achieve better separation. However, the mobile phase and sample preparation procedures are not compatible with these stationary phases. Thus the

phenyl-hexyl column was selected for HPLC analysis of gemcitabine in cellular homogenates.

3.3.6 Effects of sample preparation on gemcitabine separation

Sample preparation also plays a pivotal role in the results. If chemical substances got bad release from cell homogenates or too much interference by the sample preparation, the results could not be received even using the right mobile phase and column. First a mixture of 50% Acetonitrile (ACN) and 50% methanol containing 0.8% perchloric acid was used to extract gemcitabine into the solvent from cell homogenates. There was an interference peak around the gemcitabine peak. It was thought that the perchloric acid modified the pH value of mobile phase. Then the mixture of 50% ACN and 50% methanol was tested for releasing gemcitabine, followed by the removal of organic solvent inside a hood overnight. This step was to avoid the interference from the high concentration of organic solvent injection into the 100% buffer. In the end, the sample preparation was decided as follows: 100μl cell homogenate samples plus 200μl organic solvent mixture (50% ACN with 50% methanol) into a 1.5mL PCR tubes, and then put the tubes in the hood overnight. After centrifugation at 15000 rpm for 10 min at 4°C, the supernatant was injected for analysis.

3.4 HPLC method validation

3.4.1 HPLC chromatograms

Representative chromatograms of gemcitabine in Milli-Q water or cellular homogenates are shown in Figure 13–17. The chromatographic conditions were as following: HPLC system consists of a LC-20AT pump system (Shimadzu), a SIL-10A auto injector (Shimadzu), a UV-Vis SPD-20A (Shimadzu) absorbance detector and online analysis software (LC Solution version 1.25 SP2). The column for gemcitabine quantification is a Luna 5u Phenyl-hexyl (250 × 4.6 mm, 5u micron). The mobile phase was 100% phosphate buffer (50 mM, pH = 6.5~7.5) at flow rate of 2 mL/min. The volume of sample injection was 50μL. The wavelength for gemcitabine detection was set at 272nm. Under the conditions above, the retention time for gemcitabine was around 7.5 min. The total chromatography run time was 10 min.

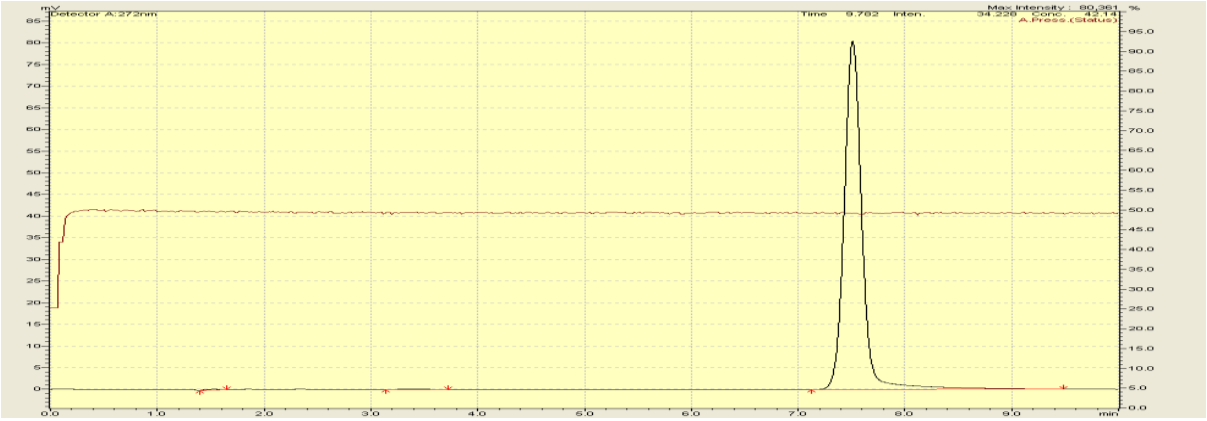

Figure 13 A representative HPLC chromatogram for gemcitabine in Milli-Q water

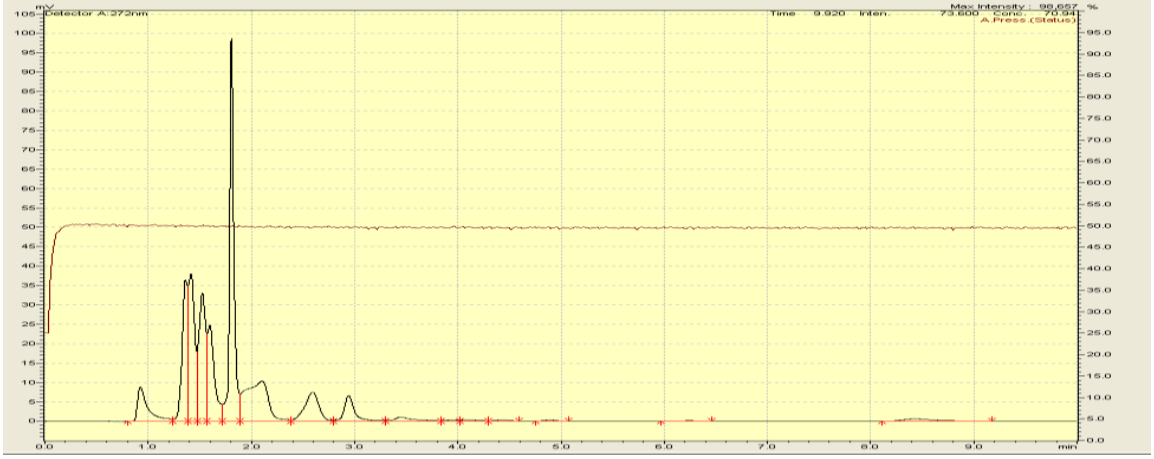

Figure 14 A representative HPLC chromatogram for blank cell homogenates only

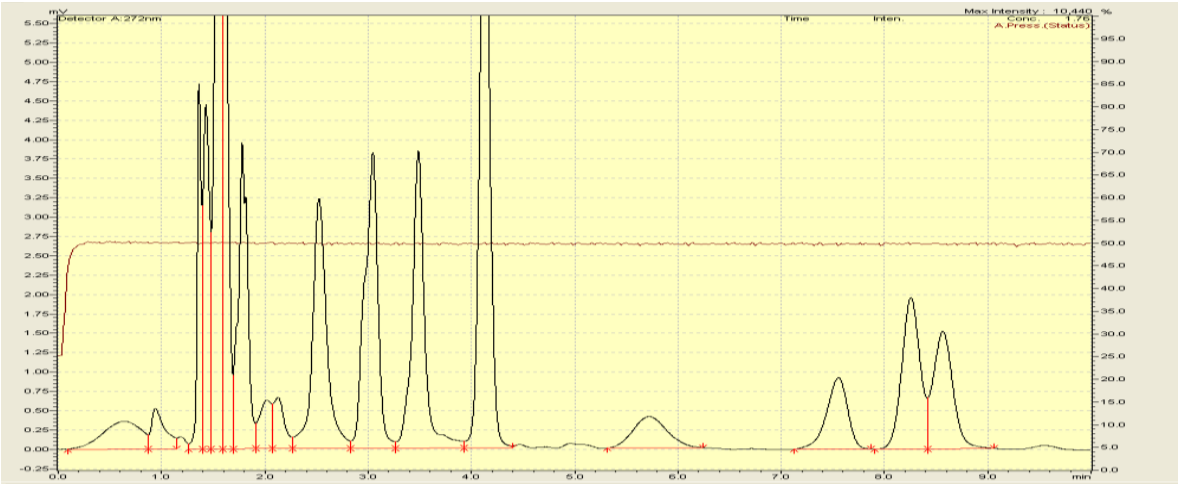

Figure 15 A representative HPLC chromatogram for gemcitabine extracted from A549 cell homogenates

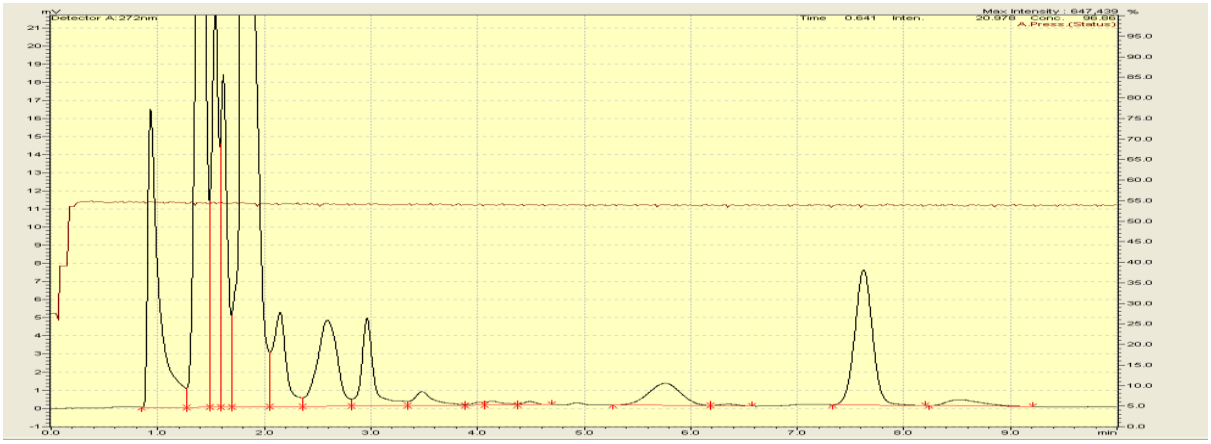

Figure 16 A representative HPLC chromatogram for gemcitabine extracted from standard sample. The gemcitabine standard sample was prepared by spiking stock solution into blank A549 cell homogenates.

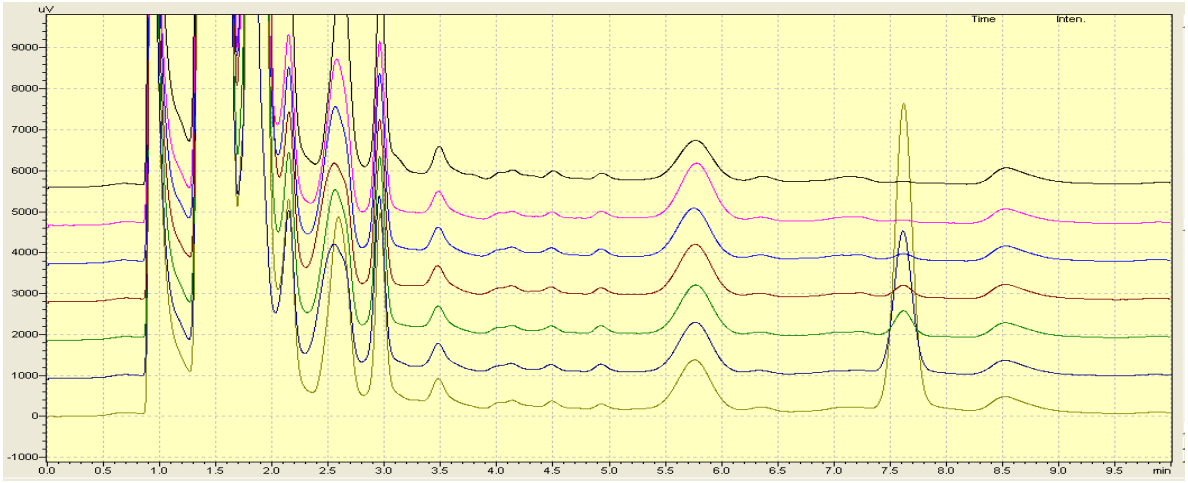

Figure 17 Combined HPLC chromatograms for gemcitabine standard samples at 0.25, 0.5, 1, 5 and 10 μM.

3.4.2 Linearity

Calibration curves were linear over the concentration range of 0.25–10 μM with the mean correlation coefficients > 0.987 (n = 6). The differences between the theoretical and the actual concentration and the relative standard deviations were all less than 15% at any QC concentrations. Typical calibration curves for gemcitabine are shown in Figure 18 and Figure 19.

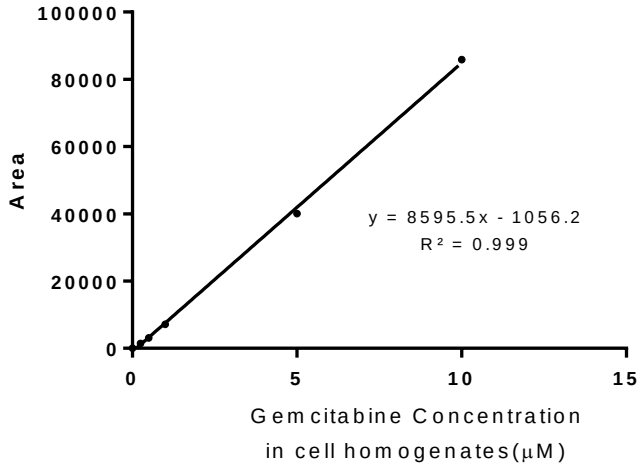

Figure 18 Calibration curve for Gemcitabine in cellular homogenates using HPLC (experiment 1)

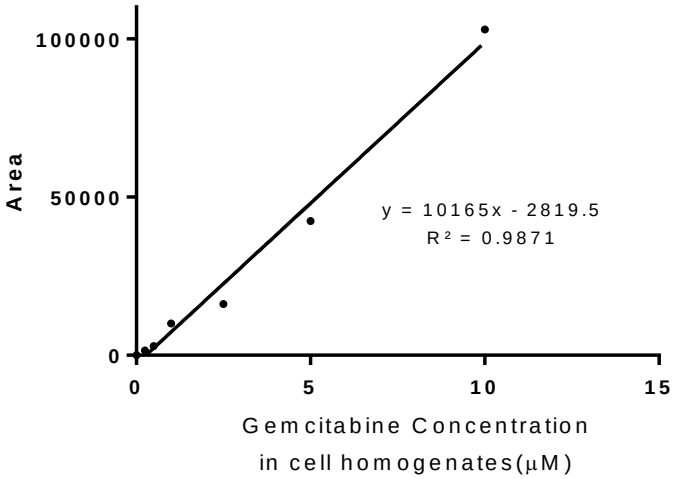

Figure 19 Calibration curve for Gemcitabine in cellular homogenates using HPLC (experiment 2)

3.4.3 Accuracy and precision

The differences between the theoretical and the actual concentration and the relative standard deviation were less than 15% at medium and high QC concentrations. The results of the accuracy and precision were shown in Table 7.

Table 7 Accuracy and precision of the HPLC methods for the analysis of gemcitabine in A549 cellular homogenate.

Theoretical concentration (μM)	Measured concentration (μM) (mean ± SD)	% deviation from the actual concentration	CV (%)	No. of samples (n)
0.5	0.525 ± 0.055	+5.0	10.5	2
1	1.069 ± 0.032	+6.9	3.02	3
10	9.348 ± 0.338	-6.5	3.61	4

3.4.4 Specificity and sensitivity

The specificity of the method was examined by determining if interfering chromatographic peaks were present in Milli-Q water or in the presence of cellular homogenates. The LOQ was evaluated based on the precision and accuracy of the assay performed. Below 0.5 μM for gemcitabine in cell homogenates, the accuracy and precision of the HPLC methods were not acceptable. The LOQ of the assay was 0.5 μM for a 50μl aliquot of gemcitabine in cell homogenates.

3.5 Summary

A HPLC method to determine gemcitabine in A549 cellular homogenates has been developed and validated. In this study, while gemcitabine cannot be separated sufficiently from the cellular interferences using a C18 column, a phenyl-hexyl column was found to be efficient to achieve better separation for quantitation of gemcitabine. This is because that separation using the phenyl column is conducted via the π electron, which in this case utilizes the π-π interaction between the phenyl group π electron and the analyte's π electron.

Validation data indicates that the method is sensitive and reliable, with acceptable accuracy (85–115% of true values) and precision (CV < 15%). The assay specificity was indicated by the absence of interfering chromatographic peaks in cellular homogenates, and the LOQ of the assay was 0.5 μM. Calibration curves for gemcitabine were linear with the mean correlation coefficients > 0.987. This method has the advantage of being relatively rapid and efficient, with the retention time of gemcitabine separated from the substances in cellular homogenates. Therefore, this

HPLC method is suitable for gemcitabine measurement in A549 cellular homogenates studies.

However, a major limitation of this HLPC method is a lack of detection of gemcitabine metabolites. The cytotoxic action of gemcitabine has been attributed to inhibition of DNA synthesis by dFdCDP and dFdCTP (Heinemann et al., 1988; Plunkett et al., 1996). The HPLC method described in this chapter may not be suitable to simultaneously measure these active metabolites. A more robust and specific assay such as an LC-MS/MS assay is thus desired to profile the cellular pharmacokinetics of gemcitabine and its active metabolites.

4 Chapter 4 Determination of effects of FUX on cellular accumulation of gemcitabine in A549 cells

4.1 Introduction

Gemcitabine belongs to the pyrimidine anti-metabolites, which are related to the prolonged inhibition of DNA synthesis after removal of exogenous nucleoside. It is indicated as front-line treatment for a number of solid tumour types including NSCL, pancreatic, ovary, bladder, and breast cancer. A couple of *in vitro* cell culture models have demonstrated a good correlation between intracellular gemcitabine accumulation and its cytotoxic activity (Heinemann et al., 1988; Metharom, Galettis, Manners, & Links, 2010). Generally, the increased cellular accumulation of gemcitabine is associated with more sensitive anti-cancer effects. Some ABC transporters (e.g. MRP5) appear to play vital roles in determining gemcitabine accumulation and thus its sensitivity in various cancer cells including NSCLC cells (J. Davidson et al., 2002 Apr 6–10; Hagmann, Faissner, Schnolzer, Lohr, & Jesnowski, 2010; Oguri et al., 2006a). Thus modulation of these ABC transporters could be useful to reverse gemcitabine resistance.

In Chapter 2, FUX appears to potentiate gemcitabine cytotoxicity in a concentration-dependent manner. Indeed, FUX has been recently reported to be an efficient inhibitor of several ABC transporters (Eid et al., 2012; Liu et al., 2012). We put up a hypothesis that FUX may increase intracelleular accumulation of gemcitabine. To test this hypothesis, an *in vitro* uptake study was undertaken to generate cellular pharmacokinetic profile of gemcitabine in the presence and absence of FUX. Gemcitabine content was extracted from A549 cell homogenates and then quantified by a validated HPLC method described in Chapter 3.

4.2 Materials

For uptake study, 60-mm plastic culture dishes were purchased from Corning Costar Corp. (Cambridge, MA). A549 cancer cells were from ATCC. Gemcitabine and FUX were obtained from Sigma NZ and all cell culture medium and supplements from Invitrogen (Auckland, NZ). The cellular protein concentration was measured by using a DC protein assay kit (Bio-Rad, USA).

4.3 Methods

4.3.1 Uptake of gemcitabine by A549 cells

The uptake of gemcitabine was measured in confluent A549 monolayer cultures grown in 60 mm plastic culture dishes as described (Mizuuchi, Katsura, Saito, Hashimoto, & Inui, 1999; Walgren, Lin, Kinne, & Walle, 2000). The A549 cells were inoculated with 5×10^4 cells in 5 mL of the complete culture medium, and culture medium was replaced three times a week and cells were used 7–9 days post seeding. After removal of the culture medium, each dish was washed once with 5 mL of incubation medium (pH 7.4) and further incubated with 2 mL of the same medium for 10 min at 37°C. The cells were then incubated with 2 mL of incubation medium containing gemcitabine in the presence or absence of FUX for specific periods at 37 °C. Thereafter, the medium was aspirated off, and the dishes were rapidly rinsed 3 times with 5 mL of ice-cold incubation medium (pH 7.4). HPLC analysis of final washes ensured that there was no residual gemcitabine. The cells were scraped off with a rubber policeman into 1 mL of Milli-Q water and the cells were frozen and thawed for three circles. The cells were then passed through a 29-gauge needle to obtain cell homogenates. The cells homogenates were stored in a -80 °C freezer before HPLC analysis of gemcitabine.

4.3.2 Determination of protein concentrations of A549 cell homogenates

Cellular protein concentration was determined using the well-documented Lowry's method (Lowry, Rosebrough, Farr, & Randall, 1951). An aliquot of 100 µl NaOH (0.2N) was first added into 100µl cellular homogenates. After a brief mix, the mixture was stored overnight at 4 °C. An aliquot of 20 µl HCl (1N) was then added to neutralize the solution. 5 µl of every 220 µl protein solutions above was added into a clean, dry microtiter plate for analysis. Standard samples were prepared by a serial dilution of a BSA stock solution (10 mg/mL, stored in -20°C) into 0.2, 0.5, 0.75, 1.0, 1.5 mg/mL protein in cell lysis buffer (0.2N NaOH 500 µl / 1N HCL 100 µl). 5 µl of every standard sample was added in the plate above for analysis as well. Both unknown samples and standard samples were measured in duplicates. An aliquot of 25 µl of reagent A was added into each well, followed by addition of 200 µl reagent B

into each well. Before reading the results, the plate was mixed well for 5 seconds in a plate reader, and read at 750 nm after 15 min. The substances were stable for about 1 hour.

4.3.3 Data analysis

The uptake rate of gemcitabine was expressed as nmol/mg cellular protein. Student's unpaired *t* test was conducted for comparisons between 2 groups with a significance level of $P < 0.05$.

4.4 Results and discussion

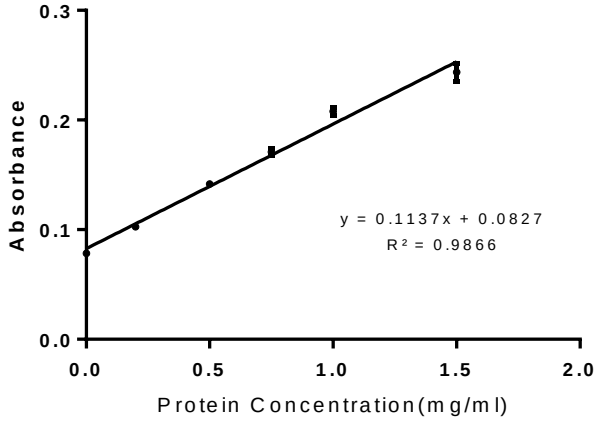

Figure 20 Standard curve of protein concentration by using BSA as a standard. Data are means ± SD (n=3).

The standard curve of protein concentration (BSA as standards) is shown in Figure 20. It appears to be linear over 0.2–1.5 mg/mL. The cell lysis buffer has no apparent effects on the final reading. The CV determined is generally less than 4% for triplicates. Thus the cellular protein concentration can be readily measured by using this method. The uptake rate of gemcitabine was normalized by using cellular protein concentration determined.

The time course of cellular accumulation of gemcitabine by A549 cells in the presence and absence of FUX is shown in Figure 21. Cellular accumulation of gemcitabine appears to reach equilibrium after 4-hr, no matter in the presence or absence of FUX. At 24-hr, cellular accumulation of gemcitabine in the presence of

FUX 10 μM was increased by 88% when compared with control (gemcitabine only), despite the *P* value is 0.07. However, there was no apparent effect of FUX 10 μM on cellular accumulation of gemcitabine at other time points.

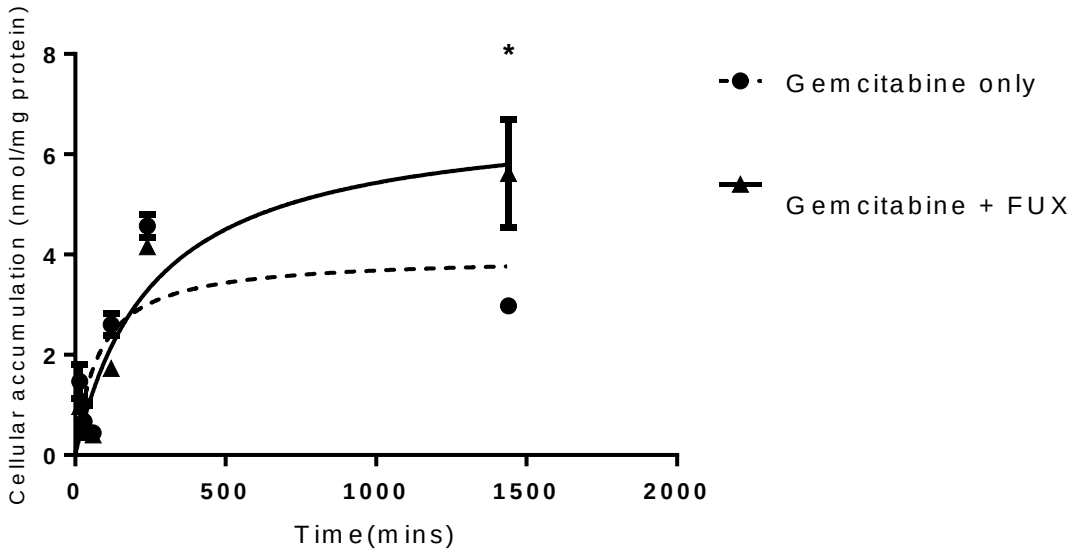

Figure 21 Effects of FUX (10 μM) on cellular accumulation of gemcitabine (10 μM) in A549 cell at 0.25, 0.5, 1, 2, 4 and 24 hours. Data are means ± SEM (n=2). *, P =0.07

4.5 Summary

Cellular accumulation studies suggest uptake of gemcitabine may reach equilibrium after 4-hr either in the presence or absence of FUX. FUX (10 μM) shows the potentials to increase the steady-state accumulation of gemcitabine in A549 cells. However, it does not affect the initial cellular uptake of gemcitabine in A549 cells. While this mechanistic research provides some clues to elucidate the effects of FUX on gemcitabine accumulation, more details about the exact mechanisms of its action, are warranted for more studies in the future. For example, it is worthwhile to determine the cellular pharmacokinetics of gemcitabine and its metabolites in A549 cells and other NSCLC cells simultaneously by using an LC-MS/MS system.

5 Chapter 5 General Discussion

In our gemcitabine cytotoxicity studies, FUX reversed gemcitabine sensitivity in NSCLC A549 cells in a time- and concentration-dependant manner. Indeed, the 72-hr IC_{50} value was only 3.9 ± 1.4 nM for gemcitabine after co-treatment with FUX (8 μM), which is decreased by 59% when comparing with control (gemcitabine treatment only). Accumulating evidence suggests concentration-dependant anti-proliferative effects of FUX on human lung cancer cell lines (A549 and NSCLC-N6) (Moreau et al., 2006). However, the increased gemcitabine sensitivity cannot be simply explained by the additive effects of FUX and gemcitabine, because FUX alone at the concentrations tested in this study show no apparent toxicity to A549 cells. To the best of our knowledge, this is the first observation that FUX may synergistically increase gemcitabine sensitivity in a NSCLC cell line. Similarly, FUX synergistically enhanced the cytotoxicity of 5-fluouracil 53.37-fold, of vinblastine 51.01-fold, and of etoposide 12.47-fold (Eid et al., 2012). Patients treated with gemcitabine may be sensitive to the first-round chemotherapy but can rapidly develop drug resistance. Because gemcitabine is also one of key agents in NSCLC chemotherapy, it is important to better understand the mechanisms of action of FUX so that the determinants of sensitivity and/or resistance to gemcitabine in NSCLC can be further elucidated.

More importantly, this study suggests FUX has no apparent effects on gemcitabine toxicity in two typical cell lines representing normal human tissues. It would be expected that FUX may represent a unique sensitizer, which may turn a less effective anti-cancer drug into an exceptional one. FUX has few adverse effects on normal cells, and reverses gemcitabine resistance in human lung cancer cell lines. FUX may be developed as an efficient chemosensitiser for cancer chemotherapy.

It has been reported that FUX alone induces apoptosis in lung cancer cells, resulting in inhibitory growth of human lung cancer A549 cells (Boo et al., 2011). Previous studies have defined FUX extracted from New Zealand *Undaria pinnitifida* with anti-cancer properties by using in vitro cell models. In addition, multidrug resistance in cancer cells is often attributed to ABC transporters via efflux of anticancer drugs. Indeed, FUX has been recently reported to be an efficient inhibitor of several ABC transporters (Eid et al., 2012; Liu et al., 2012). Thus FUX may inhibit ABC

transporters (e.g. MRP5) in A549 cells, leading to increased cellular accumulation of gemcitabine. Thus the effects of FUX on gemcitabine cellular accumulation were assessed in A549 cells.

Before the cellular gemcitabine concentration can be measured readily, a HPLC method to determine gemcitabine in A549 cellular homogenates has been developed and validated. In this study, while gemcitabine cannot be separated sufficiently from the cellular interferences using a "popular" C18 column, a phenyl-hexyl column was found to be efficient to achieve better separation for quantitation of gemcitabine. This is because that separation using the phenyl column is conducted via the π electron, which in this case utilizes the π-π interaction between the phenyl group π electron and gemcitabine's π electron.

Validation data indicates that the method is sensitive and reliable, with acceptable accuracy (85–115% of true values) and precision (CV < 15%). The assay specificity was indicated by the absence of interfering chromatographic peaks in cellular homogenates, and the LOQ of the assay was 0.5 μM. Calibration curves for gemcitabine were linear with the mean correlation coefficients > 0.987. This method has the advantage of being relatively rapid and efficient, with the retention time of gemcitabine separated from the substances in cellular homogenates. Therefore, this HPLC method is suitable for gemcitabine measurement in A549 cellular homogenates studies.

Our studies on cellular accumulation of gemcitabine suggest FUX almost doubled steady-state accumulation of gemcitaibine in A549 cells within 24-hr, despite the results were not statistically significant (P = 0.07) possibly due to limited sample numbers. However, this strongly points out that FUX may increase cellular accumulation in A549 cells when cellular influx and efflux of gemcitabine achieved equilibrium. Interestingly, FUX did not change initial uptake of gemcitabine in A549 cells, suggesting it plays minor role on the activities of SLC transporters responsible for initial uptake of gemcitaibine, such as equilibrative and concentrative nucleoside transporters (ENTs and CNTs). Indeed FUX has been reported to significantly decrease cellular expression of some ABC transporters and increase cellaular accumulation of standard substrates (Eid et al., 2012).

However, a major limitation of this study is a lack of detection of gemcitabine metabolites. The cytotoxic action of gemcitabine has been attributed to inhibition of DNA synthesis by dFdCDP and dFdCTP (Heinemann et al., 1988; Plunkett et al., 1996). The HPLC method developed in this study may not be specific and sensitive enough to simultaneously measure these active metabolites. Further investigations are needed to answer these questions.

Another area of interest for future research involves the apoptosis induction by FUX in A549 cells and other lung cancer cells. FUX induces apoptosis in various cancer cells, resulting in inhibitory growth of human lung cancer A549 cells (Boo et al., 2011), hepatic carcinoma HepG2 cells (Das et al., 2008; Yoshiko & Hoyoku, 2007) and SK-Hep-1 cells (C.-L. Liu, Y.-S. Huang, M. Hosokawa, K. Miyashita, & M.-L. Hu, 2009), colon cancer cells (Caco-2, HT-29, DLD-1 cells) (Hosokawa et al., 2004), gastric adenocarcinoma MGC-803 cells (Yu et al., 2011), prostate cancer PC-3 cells (Kotake-Nara et al., 2005), and primary effusion lymphomas (Yamamoto, Ishikawa, Katano, Yasumoto, & Mori, 2011). FUX also show concentration-dependent anti-proliferative effects on two human lung cancer cell lines (A549 and NSCLC-N6) (Moreau et al., 2006). The studies focusing on proapoptotic effects of FUX may further elucidate the mechanisms of drug resistance in NSCLC cells and help develop new therapeutic regimen for lung cancer treatment.

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