Pharmacology of Gemcitabine in the Asian Population

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Abbreviations

AIC: Akaike Information Criterion;

ANC: absolute neutrophil count;

AUC: area under the concentration-time curve;

BSA: body surface area;

CBC: complete blood count;

CDA: cytidine deaminase;

CNT: concentrative nucleoside transporters;

CR: complete response;

CT: computed tomography;

DCTD: deoxycytidylate deaminase;

dCK: deoxycytidine kinase;

dFdC : 2'-deoxy-2', 2'-difluorocytidine;

dFdCDP: gemcitabine diphosphate;

dFdCMP: gemcitabine monophosphate;

dFdCTP: gemcitabine triphosphate;

dFdU: 2'-deoxy-2', 2'-difluorouridine;

dFdUMP: difluorodeoxyuridine monophosphate;

ENT: equilibrative nucleoside transporters;

ITT: intent-to-treat;

KPS: Karnofsky performance status;

LC-MSMS: liquid chromatography tandem mass spectrometry;

LOD: limit of detection;

LLOQ: low limit of quantitation;

NCA: non-compartmental analysis;

NPC: nasopharyngeal carcinoma;

NSCLC: non-small cell lung cancer;

NTs: Nucleoside Transporters;

PBMCs: peripheral blood mononuclear cells;

PCR: polymerase chain reaction;

PD: pharmacodynamics;

PD (tumor response): progressive disease;

PK: pharmacokinetics;

PLT: Platelet Count Nadir;

PR: partial response;

QC: quality control;

RDI: relative dose intensity;

RRM1: ribonucleotide reductase M1;

RRM2: ribonucleotide reductase M2;

SCCHN: squamous cell carcinoma of the head and neck;

SD: stable disease;

SNP: single nucleotide polymorphism;

TTP: time to progression;

TYMS: thymidylate synthase;

ULN: upper limit of normal;

WBC: white blood cell;

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Conference Abstracts:

1. **Ling-Zhi Wang,**[1,2] Wei-Peng Yong,[1] Lai-San Tham,[1] Theresa-May-Chin Tan,[3] Ross-A. Soo,[1] Soo-Chin Lee[1], Boon-Cher Goh,[1,2] How-Sung Lee[2*] Micro Protein Precipitation with Negligible Matrix Effect for Rapid Determination of Gemcitabine and Its Metabolite in Human Plasma by LC/MS/MS. AAPS Annual Meeting 2008.

AAPS Award Winner (AAPS is abbreviation of American Association of Pharmaceutical Scientists).

2. **LZ Wang**[1, 2], RA Soo[1], WP Yong[1], LS Tham[1], SC Lee[1], HS Lee[2], BC Goh[1, 2] Correlation between Plasma Metabolite vs Gemcitabine Ratios and NSCLC Non-responders to Gemcitabine based Therapy. NHG Annual Meeting 2008.

Summary

Gemcitabine (dFdC) is a broad spectrum antimetabolite effective for treating non-small cell lung cancer (NSCLC), breast cancer and nasopharyngeal cancer (NPC). Its complex disposition pathway and treatment schedule dependence provide a unique opportunity to investigate pharmacokinetic and pharmacodynamic interactions, including their genetic determinants in order to optimise clinical use.

Firstly, the progress in gemcitabine research was reviewed with respect to its chemical structure, formulation and clinical application. This is followed by a discussion on the current status and the recent development in pharmacokinetics, pharmacodynamics and pharmacogenetics of gemcitabine. The possible drug resistance mechanisms were analyzed including the important aspects of gemcitabine intracellular transporters and metabolic enzyme activities. A novel potential combination chemotherapy was proposed based on the significant synergistic effect between gemcitabine and PXD101, a HDAC inhibitor.

Validated analytical methods were developed to provide an important research platform for clinical study of gemcitabine. These included 1) a 16-fold improved sensitivity LC-MSMS methodology which was validated and applied to Phase II clinical sample quantification of gemcitabine and its deaminated metabolite; 2) a more efficient quantitation of intracellular dFdCTP (gemcitabine triphosphate) which is the main active form of gemcitabine inside the cells.

Sensitivity of NPC and NSCLC tumour cell lines to gemcitabine and the novel combination of gemcitabine with PXD101 were tested. *In vitro* experiments suggested that the duration of incubation would be the primary determinant of intracellular dFdCTP

accumulation when the real time concentration of dFdC was ≥ 2 μM. A plateau concentration of intracellular dFdCTP was achieved after 8 h incubation with initial concentration above 10 μM dFdC. On the other hand, the cell viability was of the same magnitude with 48 h incubation when the initial exposure concentration of dFdC was ≥ 10 μM. The resultant viability was consistent with the combined effect of dFdCTP accumulation level and retention duration (incubation time). Potent synergistic cytotoxicity was obtained even with different cell models especially with p53-null cell line (H1299) (Combination Index = 0.5001) when PXD101 was added to gemcitabine.

Pharmacokinetics and pharmacodynamics of a fixed dose rate infusion of 10 mg/m^2/min of gemcitabine was studied in human subjects. The result suggested that the target plasma gemcitabine concentration above 10 μM could be achieved after 75 min infusion of gemcitabine at a constant rate of 10 mg/m^2/min. Pharmacokinetic comparison between a fixed dose rate infusion of 10 mg/m^2/min of gemcitabine and standard 30-min infusion of 1000 mg/m^2 was conducted. Despite a 25% lower total dose of gemcitabine at an infusion rate of 10 mg/m^2/min in combination with carboplatin in NSCLC, a similar clinical efficacy and safety profile was achieved compared to the standard 30-min infusion regimen. Pharmacokinetic analyses of gemcitabine and dFdCTP suggest that the 30-min infusion is a pharmacologically less efficient compared to a fixed dose rate of 10 mg/m^2/min. In addition, we found that intracellular dFdCTP exposure predicts myelosuppression in the 30-min infusion regimen but it is not useful for tumour response. Plasma metabolite/parent ratios at 120 min were found to be associated with the early phase (after 2nd cycle) tumor shrinkage. Briefly, the ratios of dFdU/gemcitabine for nonresponders were significantly higher than those of the responders according to

RECIST criteria. There would be as high as 95% probability in predicting non-responders to infusion gemcitabine in combination with carboplatin as long as the ratios were ≥ 500 due to fast deamination of gemcitabine. This finding has provided a useful marker in evaluating the efficacy of gemcitabine at an early phase of chemotherapy.

Genetic variants in transporter hCNT2 (SLC28A2+65 C>T and SLC28A2+225 C>A) were identified as a potential determinant of neutropenia and patient survival in the gemcitabine-carboplatin combination treatment. These genotypic variants were significantly associated with increased hematological toxicity, response and survival in Asian patients with advanced non-small cell lung cancer (NSCLC) receiving gemcitabine based chemotherapy.

CHAPTER ONE

Literature Review

1.1 Introduction of Gemcitabine

Gemcitabine hydrochloride (Gemzar®) was approved by FDA in 1996 as a novel anticancer agent in advanced or metastatic pancreatic cancer. Initially, gemcitabine, 2'-deoxy-2', 2'-difluorocytidine (dFdC), was investigated for its antiviral effects. However, this novel deoxycytidine analogue showed a high potential in cancer management, especially in solid tumors.[1] The gemcitabine chemical structure, formulation, pharmacokinetics, pharmacodynamics and pharmacogenetics will first be reviewed**.**

1.2 Chemistry and Formulation of Gemcitabine

The anti-metabolite gemcitabine is a nucleoside pyrimidine analogue that has been used clinically as an anticancer drug for more than ten years. The chemical structure of gemcitabine is shown in Figure 1.1 in which the hydrogens on the 2' carbon of deoxycytidine are replaced by fluorides. Its molecular weight is 263.1 and its pKa is 3.6. Gemcitabine is water soluble.

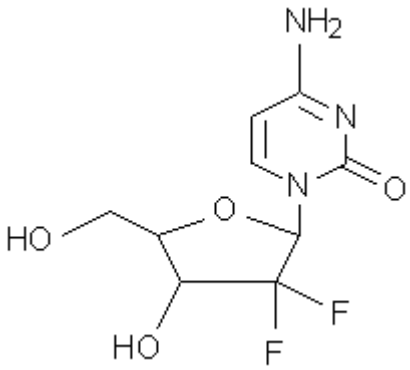


Figure 1.1 The Chemical Structure of Gemcitabine

It is marketed as Gemzar® by Eli Lilly. The nonpropietary name is gemcitabine hydrochloride and the Lilly compound number is LY188011 HCl. The chemical nomenclature is 2'-deoxy-2', 2'-difluorocytidine monohydrochloride. The drug is a lyophilized product comprising of the equivalent of 200 or 1000 mg of gemcitabine free base and the inactive ingredients mannitol, sodium acetate, and water for injection. The drug is stable at room temperature.

As a prodrug, gemcitabine exerts its anticancer activity after a rate limiting phosphorylation to gemcitabine diphosphate (dFdCDP) and gemcitabine triphosphate (dFdCTP) intracellularly by deoxycytidine kinase (dCK). Only 10% of gemcitabine is converted into its active dFdCDP and dFdCTP due to a fast and extensive deamination by cytidine deaminase (CDA) in blood, liver, kidney and other tissues to the inactive metabolite 2'-deoxy-2', 2'-difluorouridine (dFdU) which will be excreted mainly in the urine. This rapid deamination also resulted in a very short half life (about 15 min) of gemcitabine in human blood. In order to overcome this, biopharmaceutical scientists have attempted to increase the efficacy of gemcitabine through chemical modification, formulation optimization as well as targeting delivery system. [2-5]

Several series of gemcitabine derivatives have been synthesized. Among these compounds, esters or amides of gemcitabine derivatized by conjugating saturated and monounsaturated 18–20 carbon atom chains to the 3- and/or 5-OH and/or the 4-amino group showed improved cytotoxic activity than the parent drug.[6] Peripheral benzodiazepine receptors (PBRs) were selected as targets to selectively enhance gemcitabine delivery through a PBR ligand-drug conjugates due to the overexpression of PBRs in brain tumors compared to normal brain tissues.[7] Recently, a series of

increasingly lipophilic prodrugs of gemictabine were synthesized by linking the 4-amino group with valeroyl, heptanoyl, lauroyl and stearoyl linear acyl derivatives. These compounds were further developed into liposomes, prolonging their plasma half-life and increasing intracellular release of the free drug.[8] Gemcitabine-loaded liposomes were tested in human anaplastic thyroid carcinoma cells.[9] The results showed that liposome encapsulated gemcitabine has improved cytotoxicity at a lower concentration and shorter exposure time when compared to free gemcitabine. Liposome encapsulated gemcitabine promises to be an exciting alternative to clinicians considering lower doses and reduced toxicity.

1.3 Bio-analyses of Gemcitabine and its Metabolites

Gemcitabine is used in combination with cisplatin for the treatment of advanced non-small cell lung cancer (NSCLC) in the first-line setting.[10, 11] Gemcitabine inhibits DNA synthesis through its intracellular phosphorylated metabolites, dFdCDP and dFdCTP.[12, 13] Many new gemcitabine combinations are being tested in clinical trials to find the relationship between response rates, toxicities and pharmacokinetic profiles as well as genetic variants, including Asian patients.[14, 15] Even though gemcitabine is a prodrug, its plasma concentrations have been reported to be closely related to accumulation rate of its intracellular therapeutically active phosphate metabolites.[16] Hence, monitoring of gemcitabine and its intracellular metabolite concentrations is important for pharmacokinetic and pharmacodynamic study of gemcitabine and will result in pharmacologically guided individualized treatment in the clinical setting.

1.3.1 Quantification of dFdC and dFdU in Human Plasma

After i.v. administration, gemcitabine is converted rapidly in the plasma to the inactive product dFdU by CDA. Hence, plasma quantification of dFdC is difficult because this prodrug has an extremely short half-life. [17] Metabolism and elimination of the drug is rapid and highly variable. Like most other anti-cancer drugs, gemcitabine has a narrow therapeutic index. The principle dose-limiting toxicity of gemcitabine therapy is myelosuppression. It is therefore critical to develop a simple and sensitive quantitative method to quantify dFdC for evaluation of the pharmacokinetic and pharmacodynamic profiles of gemcitabine in clinical trials. This method can be utilized for therapeutic drug monitoring as well. Furthermore, simultaneous quantitation of dFdU is necessary for us to understand the pharmacokinetic profile of the parent drug even though dFdU is regarded as inactive metabolite but may contribute to gemcitabine toxicity. [18] Several assays have been described for determination of gemcitabine and dFdU in plasma, urine and tissue using reversed-phase HPLC with or without ion-pair reagents. [19-26] Currently, the most sensitive assay using HPLC-UV is a normal-phase HPLC system.[27] A 0.05 μg/ml limit of quantitation for both dFdC and dFdU was achieved in the assay. However, its tedious sample preparation limits its application in monitoring clinical samples.

So far, several simultaneously analytical methods have been published for quantification of dFdC and dFdU in plasma, urine and tissues using high-performance liquid chromatography coupled with UV detection. However, it is very difficult to monitor the very low parent drug concentrations at later sampling points during the elimination phase because of, firstly, rapid decline of dFdC plasma concentration after the end of infusion and secondly the limitation of UV detection sensitivity. In order to overcome this

limitation of UV detection, a sensitive LC-MS method was developed for measurement of the anticancer agent gemcitabine and its deaminated metabolite at low concentrations in human plasma. [28] This method provided a ten-fold improvement on the detection sensitivity (5 ng/mL) compared to that of the most sensitive UV assay. In addition, a better specificity was also achieved by using mass spectrometry. A more sensitive and more specific HPLC-MSMS was also developed for simultaneous low concentration determination of gemcitabine and its metabolite in human urine. [29]

1.3.2 Quantification of dFdCTP in White Blood Cells

Since gemcitabine is a prodrug, it can be activated only after entering the cells. The activation is a multi-phosphoration process limited by dCK. The resultant nucleotides are gemcitabine monophosphate (dFdCMP), dFdCDP and dFdCTP. Among them, dFdCTP is the main active metabolite proposed to incorporate into DNA, resulting in inhibition of DNA synthesis and finally cell death. In addition, pre-clinical models have demonstrated a good correlation between intra-cellular dFdCTP accumulation and cytotoxic activity of gemcitabine. Thus, dFdCTP can be considered pharmacologically the most important metabolite of gemcitabine. [30, 31]

Due to the importance of dFdCTP concentrations in interpreting pharmacodynamic effect, the quantification of intracellular dFdCTP content is crucial for gemcitabine clinical evaluation. In recent years, several analytical methods on determination of dFdCTP have been published including the latest one by using tandem mass spectrometry.[32-35] However, all of them are derived from a pioneer publication on analysis of 9-beta-D-arabinofuranosyladenine 5’-triphosphate levels in murine leukemia cells by high-pressure liquid chromatography as early as 1977. [36]

1.4 Pharmacokinetics of Gemcitabine

1.4.1 Distribution, Metabolism and Excretion

Due to its short half life, gemcitabine is usually administered by continuous infusion so as to reach the targeting blood concentration (10-15 μM). After i.v. infusion, gemcitabine is rapidly distributed into total body water with half life ranging from 2 to 42 minutes by using non-compartmental analysis. [18, 20] In modelling pharmacokinetic analysis, gemcitabine shows linear kinetics between doses of 53 to 1000 mg/m^2. Gemcitabine shows biphasic elimination kinetics, with a t½ α and t½ β of 3.5 min and 8 min respectively. The drug can be rapidly deaminated by cytidine deaminase, likely in the liver and the kidney, to dFdU which exerts only minimal antitumor activity. Peak dFdU concentrations were observed 5-15 minutes after the end of gemcitabine infusion. [18] Unchanged parent drug accounts for only 5% of the dose and the rest of the gemcitabine dose is excreted as dFdU. Elimination of dFdU is biphasic with an initial t½ of 23.5-27 minutes and a terminal t½ of 14-22.4 hours. About 98% of the gemcitabine dose is eliminated in the urine within one week. In addition, gemcitabine can be metabolized intracellularly by nucleoside kinases to active metabolites dFdCDP and dFdCTP; also metabolized intracellularly and extracellularly by cytidine deaminase to inactive metabolite difluorodeoxyuridine (dFdU). [37] The plasma protein binding is less than 10% due to its high polarity. [38] The proximal tubule of the kidney is known to be capable of nucleoside reabsorption. It is not clear if there are multiple different active nucleoside transporters. However, high concentrations of deaminase in kidney may contribute to the fast deactivation of gemcitabine to dFdU. [39] Gemcitabine Triphosphate in peripheral mononuclear cells appears to be saturated at a dosage of 350 mg/m^2 through the 30-

minute infusion of gemcitabine. This corresponds to the saturation of the rate-limiting enzyme deoxycytidine kinase in the cell. [18] In another study to determine if the saturation of dFdCTP was infusion rate dependent, a similar dose of 790 mg/m^2 to 800 mg/m^2 with different infusion rates resulted in a 4-fold higher dFdCTP accumulation with a longer infusion time (60 min) than that with a shorter infusion time (30-minute) [40]

1.4.2 Pharmacokinetic Parameters of Gemcitabine

Gemcitabine shows linear kinetics between doses of 53 to 1000 mg/m2 and can be described by a 2-compartment model. The volume of distribution of gemcitabine is influenced by many factors such as infusion scheduling, age and sex. [41] This study showed that the volume of distribution is increased with longer infusions suggesting slowly equilibrating body compartments. However, clearance of gemcitabine is independent of the dose and the duration of infusion. But clearance of gemcitabine is quite variable with sex and age.

A phase I study designed to evaluate the clinical feasibility of this pharmacologically-based strategy showed that high weekly doses of gemcitabine administered at a fixed dose rate of 10 mg/m^2/min was effective for patients with refractory malignancies with 9.7% response rate and toxicity was tolerable. [42] The fixed infusion rate of 10 mg/m^2/min has been shown to achieve plasma gemcitabine concentrations of 15 to 20 µM, resulting in maximizing the intracellular rate of accumulation of the active dFdCTP. Similar maximum concentrations (18.0 µM[43] and 18.6 µM[44]) were also achieved in other two clinical studies for fixed rate infusion of gemcitabine at 10 mg/m^2/min for 80 min or 120 min respectively. However, there were also some exceptional cases reported

such as a clinical trial conducted in The University of Texas MD Anderson Cancer Centre showed a nearly doubled Cmax (35.3 μM) [20] was achieved after the fixed rate infusion of gemcitabine at 10 mg/m^2/min for 120 min (Table 1.1).

Table 1.1 Reported Pharmacokinetic Parameters of Gemcitabine [Mean (SD)]

Study Sites subjects(n)	Dose (mg/m2) Infusion Time(min)	AUC (μM*h)	Vd (L/m^2)	Cl (L/h/m^2)	T1/2 (min)	Cmax (μM)
1 (13)	800 80	22.32† -	52.2 (28.6)	136.3 (40.8)	17.0 (11.6)	18.0 (5.5)
2 (5)	1000 30	41.3 (31.5)	- -	408.4 (501.4)	8.2 (2.6)	56.4 (35.7)
3 (6)	1200 120	28.9 (5.7)	- -	- -	10.7 (3.4)	18.6 (6.8)
4 (5)	1200 120	81.2 (25.4)	-	107.5 (33.1)	-	35.3 (11.1)
5 (3)	1000 30	42.25† -	9.7 (6..8)	90.0 (17.6)	- -	- -
6 (6)	1000 30	32.2 -	22.0 -	124.7 -		64.6 -

†: The value of AUC was calculated according to corresponding Clearance and Dose.

1: University of North Carolina. [43]

2. The University of Texas MD Anderson Cancer Centre. [18]

3. Zhejiang University, China. [44]

4. The University of Texas MD Anderson Cancer Centre. [20]

5. City of Hope Comprehensive Cancer Center. [45]

6. University of Southern California Norries Cancer Center. [46]

1.5 Pharmacodynamics of Gemcitabine

Gemcitabine displays potent anticancer effects on several cancers, especially for solid tumors. Hematological toxicity is the major adverse effect of gemcitabine even though this generally used anticancer agent has been thought to be tolerable in most cases. The mechanisms of action for gemcitabine have been explored intensively in last decade. Its main mechanisms of action and pharmacodynamics will be briefed as follows.

1.5.1 Mechanism of Action

Like other prodrugs, gemcitabine is also needed to be activated by dCK through intracellular phosphorylation for its anticancer activity. It enters the cell through the sodium-dependent nucleoside transporter on the cell membrane and then undergoes phosphorylation to the active dFdCDP and dFdCTP. (Figure 1.2) Both dFdCDP and dFdCTP inhibit processes required for DNA synthesis even though they target different sites. The main mechanisms include inhibition of DNA synthesis, ribonucleotide reductase inhibition, poisoning Topoisomerase I and self-potentiation. Preclinical and clinical data suggest that many factors such as enzymes, transporters and tumour type may affect the intracellular gemcitabine phosphorylation activation. [47]

1.5.1.1 Reduction of DNA Synthesis

Biochemical studies demonstrated that the ultimate intracellular fate of gemcitabine is to become incorporated into DNA, causing cell death. [47] DNA synthesis decreased in an inverse relationship with the cellular accumulation of gemcitabine nucleotides. [12] A strong correlation was found between incorporation of gemcitabine into DNA and the

loss of viability which provided evidence for a mechanistic relationship between the mechanism of gemcitabine and its biologic actions. Incorporation of dFdCTP into DNA chain is most likely the major mechanism by which gemcitabine causes cell death. After incorporation of gemcitabine nucleotide on the end of the elongating DNA strand, one more deoxynucleotide is added, resulting in inhibition of further DNA synthesis. DNA polymerase epsilon is unable to remove the gemcitabine nucleotide and repair the growing DNA strands which resulted in masked chain termination.

1.5.1.2 Ribonucleotide Reductase Inhibition

The ribonucleotide reductase is the major source of deoxynucleotides, which are necessary components for DNA replication and for repair. The effect of gemcitabine on ribonucleotide reductase activity is closely correlated to a decrease in the concentration of deoxynucleotides in cells shortly after being exposed to the drug.[13] This is because nucleotides of dFdC may be viewed as potential alternative substrates or inhibitors of ribonucleotide reductase, causing a decrease of deoxynucleotide pools. Surprisingly, the analogue of gemcitabine, cytarabine, lacks this effect due to minor difference in their chemical structure. Studies with partially purified human enzyme indicated that dFdCDP is the inhibitory metabolite. [48]

1.5.1.3 Poisoning Topoisomerase I

The effects of gemcitabine incorporation on topoisomerase I (top1) activity and the role of top1 poisoning in gemcitabine-induced cytotoxicity in cancer cells were tested by purified oligodeoxynucleotides. It was found that top1-mediated DNA cleavage was

enhanced when gemcitabine was incorporated immediately 3' from a top1 cleavage site on the nonscissile strand. This position-specific enhancement was attributable to an increased DNA cleavage by top1 and was likely to have resulted from a combination of gemcitabine-induced conformational and electrostatic effects. [49]

1.5.1.4 Self-Potentiation

Furthermore, the unique actions that gemcitabine metabolites exert on cellular regulatory processes serve to enhance the overall inhibitory activities on cell growth. This interaction is termed "self-potentiation" and is evidenced for very few other anticancer drugs. [50] The reduction in the intracellular concentration of natural dCTP pool by the action of gemcitabine diphosphate enhances the incorporation of gemcitabine triphosphate into DNA through competitive mechanism.

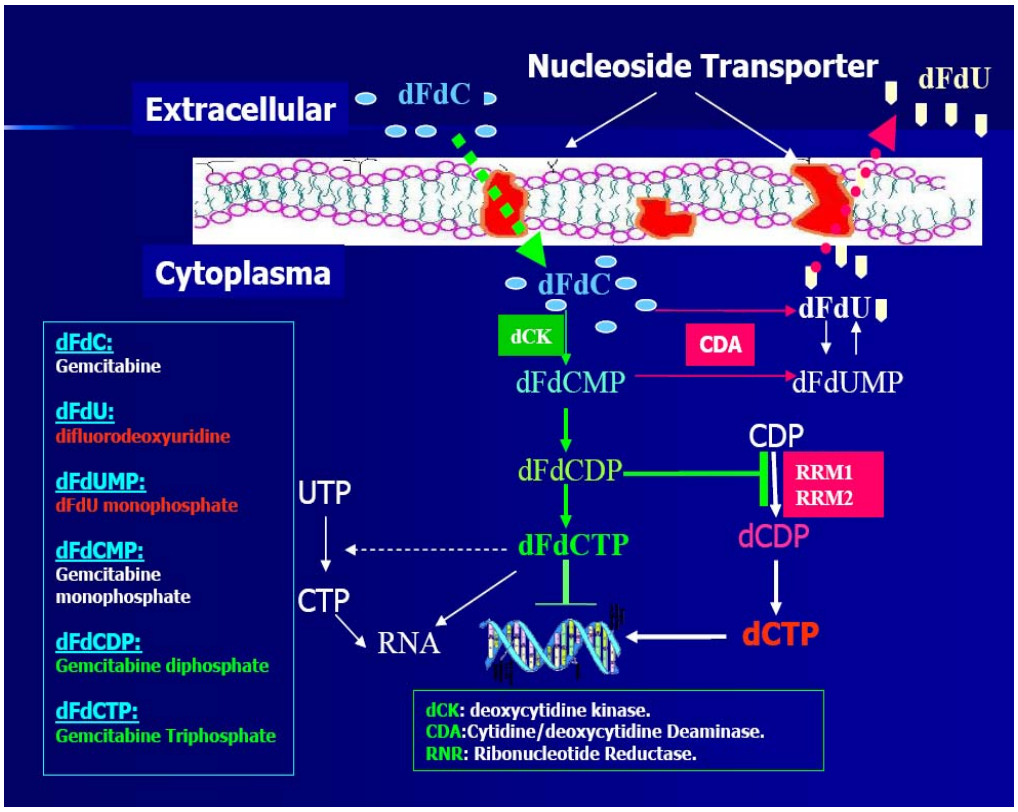


Figure 1.2 Activation Pathways of Gemcitabine

1.5.2 Molecular Pharmacology of Gemcitabine

Cell-cycle kinetic studies have shown that gemcitabine is most active during the S phase. No obvious effect on the G1, G2, or M phases is seen. Due to the competitive inhibition, gemcitabine enters the cell through a saturable carrier-mediated process that is shared by other nucleosides. In addition, this process can even be reversible when normal nucleosides are increased continuously. Gemcitabine can be phosphorylated into its active metabolites once it enters the cell. These active metabolites vary significantly from patient to patient since the activation processes are controlled by a series of enzymes involved in its transportation, activation as well as elimination. In addition, the accumulation of gemcitabine di or triphosphates is also dependent on the infusion rate which is the rationale for proposing prolonged infusion of gemcitabine. [51]

1.6 Pharmacogenetics of Gemcitabine

Gemcitabine is used for several solid tumors including non-small cell lung cancer (NSCLC) but the determinants of toxicity and efficacy are not yet fully understood.

1.6.1 Pathway of Disposition of Gemcitabine Metabolism

The genetic metabolism pathway of gemcitabine to its active form gemcitabine triphosphate and gemcitabine diphosphate is complex (Figure 1.3). Gemcitabine enters the cell via members of the nucleoside transporter family, SLC28 and SLC29. [52, 53] Within the cell, gemcitabine is phosphorylated in a rate-limiting step by dCK to dFdCMP and subsequently by nucleotide kinases to dFdCDP and dFdCTP. Gemcitabine triphosphate is incorporated into DNA by DNA polymerase α and through the process of

masked chain termination inhibits DNA repair and synthesis. Gemcitabine and dFdCMP can be inactivated by CDA and deoxycytidylate deaminase (DCTD) to dFdU and difluorodeoxyuridine monophosphate (dFdUMP), respectively. [12] Additional targets of gemcitabine cytotoxicity are ribonucleotide reductase (RRM1, RRM2) and thymidylate synthase (TYMS) which are inhibited by dFdCDP and dFdUMP respectively. RRM1 converts ribonucleotides to deoxyribonucleotides which are used in DNA synthesis and repair. [48] The inhibition of TYMS results in DNA damage.

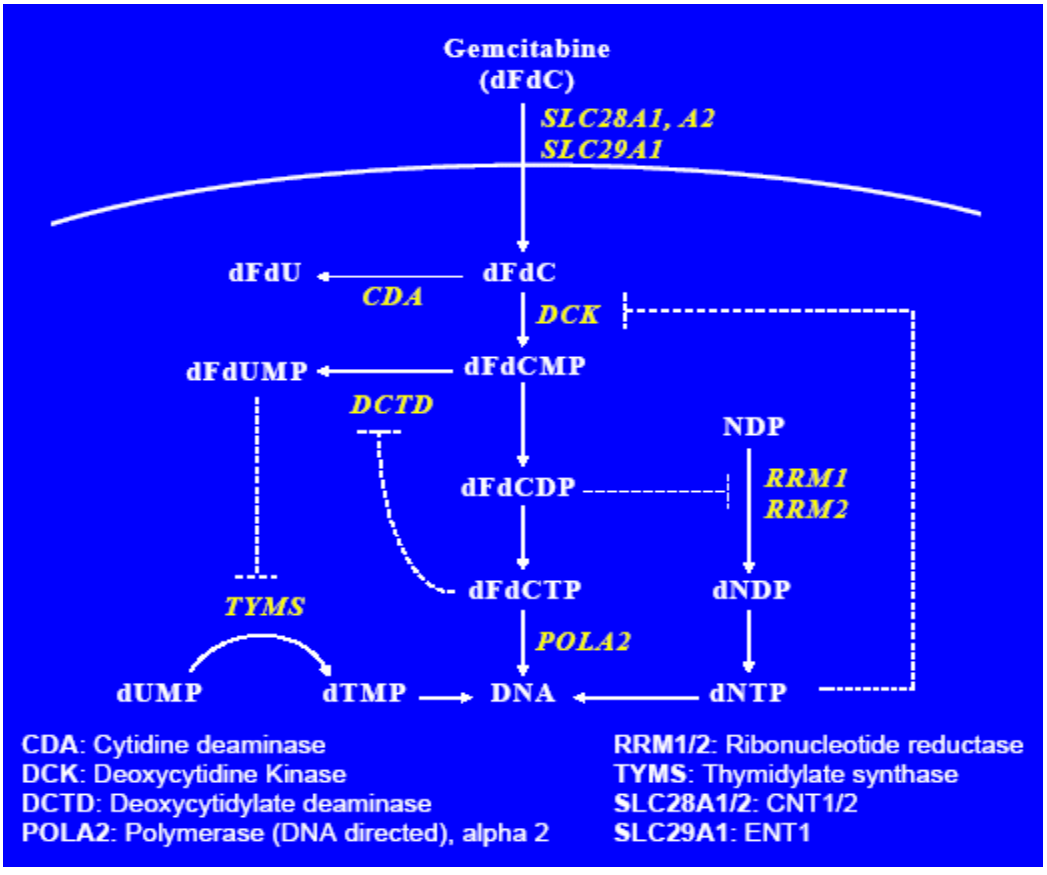


Figure 1.3 Metabolism pathway of gemcitabine to its active metabolites dFdCDP and dFdCTP.

1.6.2 Identification and distribution of SNP

Inherited genetic variation in drug metabolizing enzymes, targets and transporters are associated with inter-patient and inter-ethnic variability in drug effect. Genetic variations may be due to mutations, variation in tandem repeats and single nucleotide polymorphisms (SNPs), which account for over 90% of genetic variation in the human genome.[54, 55] Evaluating the association between gene variants involved in the gemcitabine pathway and clinical outcome is able to elucidate the effect of gene polymorphisms on chemotherapeutic outcome.

1.7 Toxicity of Gemcitabine

The profiles of the general pharmacological effects of gemcitabine were assessed in studies evaluating the cardiovascular and respiratory systems, renal function, the gastrointestinal system, the central nervous system, and the autonomic nervous system using animal models.[56] In general, gemcitabine showed limited organ toxicity but unpredictable severe toxicity such as myelosuppression.

1.7.1 Non-hematology Toxicity

Non-hematologic toxicity comprises fever, chills rigors, hypotension, flu-like symptoms, rash, alopecia, nausea, vomiting, constipation, diarrhea, stomatitis, somnolence, lethargy insomnia, elevated liver enzymes, proteinuria, hematuria, elevated creatinine and dyspnea. Hemolytic uremic syndrome has been reported in several cases. Among these, flu-like symptoms are common but can be relieved by acetaminophen. These results indicated that gemcitabine has a low potential to produce severe adverse pharmacological

effects on organs except for lungs. [57]

1.7.2 Hematology Toxicity

Like that of other antimetabolites, the dose-limiting toxicity of gemcitabine is myelosuppression. Myelosuppression consists of neutropenia, thrombocytopenia and anemia. The frequency of WHO Grade 3-4 adverse effects was summarized by Hui and Reitz. [1] The frequency ranges are 6-51%, 1-14% and 0.2-51% for neutropenia, anemia and thrombocytopenia respectively. These high variations in neutropenia and thrombocytopenia represent a major challenge in management of hematological toxicities during gemcitabine-based chemotherapy. The reasons could be due to different doses, different diseases, and different concurrent therapy as well as different genetic profiles, e.g. mutation of cytidine deaminase. [58]

1.7.3 Models for Gemcitabine-induced Neutropenia

Modelling the relationship between dose and concentration of anticancer drugs with myelosuppression is very important for clinicians to understand interpatient variability and select a better individualized treatment. This is because the use of these drugs is often limited by myelosuppression toxicities. This work can be done by empirical or physiology-based models. The most commonly used pharmacokinetic parameters are peak plasma concentration, the area under the concentration-time curve (AUC), time above certain plasma drug concentration and concentration at steady state (for continuous infusion). [59] For high protein bound compounds, the free drug concentrations would be calculated in evaluating their activities. Empirical neutropenic models have been

established for many drugs such as tipifarnib, irinotecan, etoposide and epirubicin, etc. [60-63] Comparatively, physiology-based models are preferred because ideal physiology-based models are able to separate system parameters, common across drugs, from drug specific parameters. [64-66] However, these modeling procedures are time consuming and well trained modelers are required to build and optimize the pharmacokinetic and pharmacodynamic models.

1.8 Preclinical Research of Gemcitabine

1.8.1 *in vitro* Studies

Nucleoside antimetabolites comprise one of the most effective classes of drugs for the treatment of cancer and viral diseases. Usually, nucleoside analogues are prodrugs and display their activities only after entry into the cell and phosphorylation to nucleotide metabolites. Gemcitabine has been confirmed to exhibit activity on several solid tumors due to its unique multiple mechanisms of action. Gemcitabine is regarded as a new landmark drug of antimetabolites in the past decade.

Preclinical studies revealed gemcitabine had potent and broad spectrum activity against a variety of hematological and solid tumour cell lines like colorectal, renal cell, melanoma and NSCLC cells, etc. The antitumour activity against human myeloid HL-60, T-lymphoid Molt-3, B-lymphoid RPMI-8392 cell lines was 2.6 to 17.3 fold higher than cytarabine after 48 hour incubation. [67-68]

Concurrent addition of deoxycytidine to the cell culture system may cause about a 1000-fold decrease in its biological activity. This implies that the activity of gemcitabine can be competitively inhibited by saturating dCK. [69]

1.8.2 *In vivo* Studies

In human tumor xenografts derived from squamous cell carcinoma of the head and neck, gemcitabine was active.[70] Gemcitabine was tested for its antitumor effect in human tumor xenografts derived from squamous cell carcinoma of the head and neck (SCCHN). At equitoxic doses, gemcitabine was more active in this model than the drugs that are clinically used in SCCHN, i.e., cisplatin, methotrexate, 5-fluorouracil, and cyclophosphamide.

A mode of resistance appeared to be lack of the activating enzyme dCK. Gemcitabine is not affected by the P-glycoprotein. [71] In an *in vivo* induction of gemcitabine resistance mode, an increase in expression of the RRM1 subunit gene was found in resistance phenotype but dCK activity was 1.7-fold decreased. [72] Preclinical studies demonstrated treatment schedule dependency of gemcitabine. The data on the effect of gemcitabine in animal tumour models indicated that the time interval between i.v. push injections was important when intermittent schedules were used and continuous infusions over a 24-hour period might be highly effective for *in vivo* models.[73] It was found that treatment with 120 mg/kg gemcitabine, injected (i.p.) four times at 3-day intervals, was more effective than the schedules of daily (five times 2.5 to 3.5 mg/kg) or weekly (two times 240 mg/kg) injections.

In addition, the lack of cross resistance seen with gemcitabine may contribute to therapeutic synergism when gemcitabine is combined with other agents [74] In general, *in vitro* sensitivity to dFdC was highly related with dFdCTP accumulation and retention, but *in vivo* this relation was less clear. [75] Furthermore, the effect of gemcitabine is also dependent on dose scheduling. Previous mechanistic studies indicate that a continuous-

infusion schedule may be more effective compared to bolus injection. Furthermore, prolonged infusion of gemcitabine can give better antitumor activity and shows promise of being active in clinical trials. [21]

1.9 Clinical Uses of Gemcitabine

Despite the grim prognosis of NSCLC, some progress has been made in defining the role of chemotherapy in this once considered chemo-resistant disease. In the 1970s and 1980s, a number of chemotherapeutic agents were tested in phase II trials, of which several of them fulfilled the criteria of 15% overall response rate, considered to be “active” enough for further evaluation. These include cisplatin, ifosfamide, mitomycin-C, vinblastine, vindesine, and etoposide. [76] Accordingly, a series of combination clinical trials being proposed and tested in Europe and US resulted in superior cisplatin-based combination chemotherapy as a standard treatment of NSCLC. To date, platinum-based combinations are the most widely accepted regimens in the treatment of advanced NSCLC due to a clear improvement on survival in comparison with best supportive care alone.[77] Since the response rates have nearly reached a plateau for the cisplatin combination chemotherapies for NSCLC, novel doublets are continuously being explored by clinician scientists to provide improved treatment efficacy, less toxicities and better quality of life. This has been achieved with the development of third generation of agents which have been active for NSCLC. These agents include taxanes (paclitaxel and docetaxel), gemcitabine, vinorelbine, and irinotecan.[78] The candidate drugs used for combination therapy should possess proven anticancer effect, as well as tolerable toxicities. Among these new anticancer drugs, gemcitabine has been considered a potential agent for

combination chemotherapy due to its unique mechanism of action and relatively "good" toxicity profile.[14]

1.9.1 Single-agent Gemcitabine

Both *in vitro* and *in vivo* studies have demonstrated that gemcitabine is effective on various solid tumours, including NSCLC. [10] Used as a single agent in the treatment of metastatic NSCLC, gemcitabine achieved consistent response rates of 20% and above. Furthermore, the toxicity profile at the doses ranging from 800 to 1250 mg/m^2 was modest, consisting mainly of a short-lasting decrease in leucocytes and thrombocytes, skin reactions (5-8%), peripheral oedema and transient increase in transaminase levels.[79] Nausea and vomiting were mild. Some patients (18.9%) experienced transient flu-like symptoms and mild fever was reported in 37.3% of flu patients. [80] Based on its response rates and modest toxicity which is non-overlapping with other active agents, gemcitabine is now being widely explored in various combination therapies in the treatment of NSCLC.

1.9.2 Gemcitabine plus Platinium Compounds

In vitro studies showed considerable synergy between cisplatin and gemcitabine.[81] Clinically, gemcitabine/cisplatin combination also showed an improved median and one year survival rate compared to single cisplatin, better time-to-disease progression and tumour response rates than its comparator arms in phase III studies. [82, 83] These significant findings reviewed by FDA resulted in the approval of gemcitabine in combination with cisplatin as first-line therapy of patients with advanced NSCLC

patients. The efficacy of the every 4 week regimens was further confirmed by other investigators in the following trials. [84-86] In recent years, a more common schedule is every 3 weeks for gemcitabine 1000 or 1250 mg/m^2 given on day 1 and 8 combined with cisplatin at doses ranging from 75 to 100 mg/m^2. [87-89] These every three weeks schedule studies achieved equivalent response rates that ranged from 31.8%-42% as the every four weeks schedule with a response rates between 25.9% and 54.8% but with less myelotoxicity and better compliance. [90-94]This could be due to the difference in the dose intensity of each schedule of gemcitabine.

Although the gemcitabine and cisplatin combination is currently one of the reference regimens for advanced NSCLC, this regimen has its own limitation due to significant non-hematologic toxicity and difficulty of use in outpatients (the need for hydration and prolonged administration time). In contrast, carboplatin, another platinum analogue, has less non-hematological toxicity associated with cisplatin. The efficacy of gemcitabine and carboplatin combination was confirmed by a recent Phase III trial to compare overall survival in patients with locally advanced or metastatic NSCLC when treated with single-agent gemcitabine versus gemcitabine/carboplatin. [95] In advanced NSCLC, gemcitabine/carboplatin therapy resulted in significant survival benefit compared with single-agent gemcitabine.

In addition, the gemcitabine and carboplatin doublet is effective, with a favorable safety profile, and is well tolerated in the outpatient setting. Even though gemcitabine combined with carboplatin is associated with more hematological toxicity, but the incidence of non-hematological toxicity is significantly lower. Another advantage is that carboplatin can be easily administered in an outpatient setting since no prehydration is needed. [96]

1.9.3 Gemcitabine plus non-platinium agents

Since 1997, platinum-based combination has been recommended by ASCO for treatment of advanced NSCLC. Until 2003, a new guideline has been updated as *"A First-line chemotherapy given to patients with advanced NSCLC should be a two-drug combination regimen. Non–platinum-containing chemotherapy regimens may be used as alternatives to platinum-based regimens in the first line"*. [97] In recent years, a variety of gemcitabine-based combinations with non-platinum agents have been investigated for their efficacy, toxicity as well as quality of life. The efficacies of gemcitabine-based doublets combined with docetaxel, paclitaxel, or vinorelbine compared to any platinum-based doublets or the single-agent schedules have been well documented in several Phase II trials [98-101] and further confirmed by Phase III studies as well. [102-105]

1.10 Nucleoside Transporters

Gemcitabine can be activated to its active metabolites only after entering the cell. Like other nucleoside drugs, gemcitabine is hydrophilic and would not be expected to readily permeate the plasma membrane by passive diffusion. Cellular uptake of gemcitabine requires the presence of specialized plasma membrane nucleoside transporter proteins. [106] Gemcitabine has been shown to be a substrate for five of the nucleoside transporters found in human. These transporters belong to the Solute Carrier families SLC28 (human concentrative nucleoside transporters: hCNT1, hCNT2 and hCNT3) and SLC29 (human equilibrative nucleoside transporters: hENT1 and hENT2). [107] CNTs are thought to play critical roles in nucleoside homeostasis at the organism level by maintaining systemic blood levels of nucleosides through absorption and elimination mechanisms and at the

cellular level by mediating influx of extracellular nucleosides into cells. On the other hand, these transporters play a critical role in drug response, facilitating drug absorption, metabolism, and elimination. Hence, genetic mutation in SLC transporters could be determinants of sensitivity and resistance to gemcitabine and toxicity of gemcitabine.

1.10.1 Effect of Nucleoside Transporters on Activity of Gemcitabine

Gemcitabine requires plasma membrane nucleoside transporter proteins to efficiently enter cells and exert its cytotoxicity. *In vitro* studies have demonstrated that deficiency of human equilibrative nucleoside transporter 1 (hENT1), the most widely abundant and distributed nucleoside transporter in human cells, confers resistance to gemcitabine toxicity. Patients with pancreatic adenocarcinoma with uniformly detectable hENT1 immunostaining have a significantly longer survival after gemcitabine chemotherapy than tumors without detectable hENT1. Immunohistochemistry for hENT1 shows promise as a molecular predictive assay to appropriately select patients for palliative gemcitabine chemotherapy but requires formal validation in prospective, randomized trials. [108]

1.10.2 Effect of Nucleoside Transporters on Excretion of Gemcitabine

Eliminatory organs such as kidney, liver, and intestine defend the human body against potentially harmful effects of xenobiotics by transforming them into less active/inactive metabolites and by excretory transport process. Eventually, most drugs and environmental toxicants are excreted into the urine, either in the unchanged form or as biotransformation products. The mechanisms that contribute to their renal excretion are closely related to the physiological events occurring in the nephrons, i.e., filtration, active

secretion, and re-absorption.[109] The SLC28 family consists of three subtypes of sodium-dependent, concentrative nucleoside transporters, CNT1, CNT2, and CNT3 (SLC28A1, SLC28A2 and SLC28A3, respectively). Each of them has their own substrate specificities. CNT1 is pyrimidine-nucleoside preferring, CNT2 is purine-nucleoside preferring but CNT3 has a more general substrate profile and can transport pyrimidine and purine nuclosides as well. [110] Novel evidence indicates that cytidine is a novel substrate for wild-type CNT2. [111] Both CNT1 and CNT2 are involved in re-absorption of nucleosides.

In epithelia, CNT1 is localized to the apical membrane and works in concert with equilibrative nucleoside transporters localized predominately to the basolateral membranes of these tissues to mediate transepithelial nucleoside flux. Although CNT1 prefers pyrimidine nucleoside, it works on transportation of naturally occurring pyrimidine nucleosides as well as the naturally occurring purine nucleoside, adenosine. [112,113] Many antiviral nucleoside analogues and cytotoxic cytidine analogues including gemcitabine are substrates of CNT1.

Northern blot analysis has confirmed that human CNT2 is present in many tissues such as kidney, liver and heart, etc. [114] CNT2 shows different profile of substrates including antiviral compounds such as didanosine in treatment of HIV and ribavirin in treatment of hepatitis C. Of the nucleoside chemotherapeutic agents, it seems that these compounds are not the substrates of CNT2. However, a recent study demonstrated that gemcitabine is one of the substrates of CNT2. [115] Although each subtype of CNTs shows different substrate specificities, their specificities may become different due to transporter protein conformation changes. For example, the specificity of substrate for CNT1 can be

broadened from pyrimidine to pyrimidine plus purine nucleosides after replacing Ser318 in rat CNT1 with corresponding residue in CNT2 (Gly). This fact implies that the single nucleotide polymorphism which encodes amino acid changes is important in determining the substrate profile for CNTs. This may produce significant clinical implication in interpreting efficacy and toxicity of nucleoside anticancer drugs.

1.11 Chemo-resistance of Gemcitabine

Like other chemotherapeutic agents, treatment effect of gemcitabine is also limited in clinical trials as a single agent or combination regimens due to resistance to gemcitabine. This resistance is believed to be caused by different activity and mutation of several enzymes which control the phosphorylation of gemcitabine and intracellular transportation as well as elimination processes. These include 1) human equilibrative transporter (*hENT1*); [116] 2) multidrug resistance proteins ABCC1 and ABCC5; [117] 3) gemcitabine intracellular phosphorylation by deoxycytidine kinase (dCK) to the active di or triphophate forms; [13] 4) Overexpression of rebonucleotide reductase (RRM1); [118] 5) Decreasing the intracellular normal deoxynucleotide triphosphate pools by inhibition of ribonucleotide reductase. [119] On the other hand, the sensitivity to gemcitabine also depends on the NSCLC pathological characteristics in relation to mutation and deficiency of tumor suppressor genes, silence of proapoptosis genes and overexpression of oncogenes.

1.12 Summary

Lung cancer is the most common form of cancers throughout the world. In addition, advanced NSCLC is usually considered incurable. The development of third generation anti-cancer drugs such as taxanes, irinotecan and gemcitabine brings new hope in the armamentarium of drugs for chemotherapy for NSCLC. For the last ten years, the combination of platinum-based chemotherapy has resulted in significant improvements in treatment of NSCLC patients. Nevertheless, another treatment plateau (response rates of 30-40%, median survival times of 8-10 months and 1-year survival rates of approximately 35% in patients with advanced NSCLC) has been reached even with the use of the third generation agents. [120]Although gemcitabine has been used in solid tumour treatment for more than ten years, there is still room to improve its efficacy by manipulating different infusion rates and dosages with the help of clinical pharmacologically-guided research. These findings will help clinicians to maximize the efficacy of gemcitabine and at the same time, manage the drug toxicity effectively through optimizing pharmacokinetic parameters and tailoring pharmacogenetics findings so as to optimally individualize the treatment for NSCLC patients.

Our study aims are:

1. *in vitro* studies on cytotoxic sensitivities of NPC and NSCLC cell lines to gemcitabine and delineating the determinants for intracellular accumulation and retention of dFdCTP.

2. Pharmacokinetic and pharmacodynamic study in fixed infusion rate of different dose of gemcitabine in combination with carboplatin (Phase I).

3. Pharmacokinetic and pharmacodynamic study of gemcitabine in 2 different infusion rates of gemcitabine with carboplatin in patients with advanced NSCLC (Phase II).

4. Association of pharmacokinetic parameters of gemcitabine in plasma or the intracellular dFdCTP with the various pharmacodynamic endpoints and pharmacogenetic single nucleotide polymorphisms involved in gemcitabine activation pathway.

CHAPTER TWO

Bioanalytical Method Development for Determination of Gemcitabine and Its Metabolites

2.1. Introduction

2.1.1. Quantification of dFdC and dFdU in human plasma using LC-MSMS

Gemcitabine plasma concentrations have been reported to be closely related to rate of accumulation of its intracellular therapeutically active metabolite-gemcitabine triphosphate even though gemcitabine is a prodrug,[14] Plasma quantitation of dFdC is difficult because it has an extremely short half-life due to rapid deamination to dFdU by CDA. [53,121] In addition, simultaneous analysis of dFdC and dFdU is important to define the elimination pathway of parent drug and its metabolites. [16] Furthermore, the ratio of AUC or concentration ratios of dFdU versus dFdC may be useful for evaluating gemcitabine deactivation rate which may be an important marker for drug efficacy. [122]

During our phase I clinical study of gemcitabine, a HPLC-UV method was validated for quantification of gemcitabine and dFdU with the lower limit of quantitation (LLOQ) of 80 ng/ml. [123] However, most of the concentrations from the last sampling points from our Phase II trial study are less than 80 ng/mL. Hence accurate determination of the last sampling points of dFdC during the elimination phase was not possible by using UV detectors because of, the rapid decline of dFdC plasma concentration after the end of infusion and the limitation of UV detection sensitivity. This gap in sensitivity of measurements initiated the development of the current much more sensitive and simpler analytical method using liquid chromatography-tandem mass spectrometry.

In the clinical setting, gemcitabine's unique mechanism of action and its lack of overlapping toxicity with other cytotoxic agents make it as an ideal candidate for combination therapy. [23] Many novel gemcitabine combinations are being tested in

clinical trials resulting in a large number of plasma samples requiring quantitative analysis. Although two mass spectrometric assays were published for determination of dFdC and dFdU in human plasma and urine, [28, 29] the solid phase extraction (SPE) used for sample preparation is laborious and precludes rapid quantifications of dFdC and dFdU in patient plasma samples, especially in clinical laboratories. This is likely to hamper the widespread application of monitoring dFdC and dFdU concentrations in clinical practice due to shortage of trained analytical scientists. In addition, an analogue compound of gemcitabine was used as internal standard which may result in bias pharmacokinetic parameters due to matrix effect. Furthermore, the volume of plasma required for bio-analysis is an important consideration in pharmacokinetic sampling, especially for pediatric clinical trials. In the LC-MS assay for plasma samples, 500 µl was used in quantification of dFdC and dFdU. [28] This sample volume is ten time larger than what we used (50 µl) in current LC-MSMS method. Due to the large difference in polarities of dFdC and dFdU (Figure 2.1), a gradient elution mode must still be utilized to simultaneously quantify both dFdC and dFdU even though tandem MS is a highly selective detector. The key limitation against high-throughput analyses of plasma samples is dependent on the sample preparation.

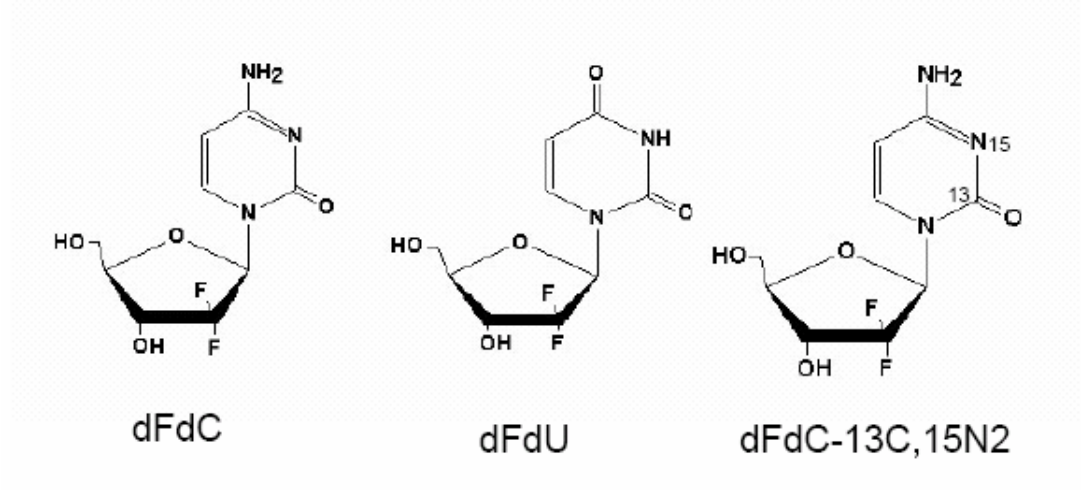


Figure 2.1 The chemical structures of dFdC, dFdU and Internal Standard.

In this study, we developed and validated a highly sensitive and simple liquid chromatography tandem mass spectrometry (LC-MSMS) method for dFdC and dFdU. Sample preparation involved only protein precipitation with acetonitrile, paving the way for high-throughput analysis of clinical samples. In addition, a commercially available isotope gemcitabine was used as internal standard. This ideal internal standard will allow analytical scientists to minimize the possible matrix effects. Therefore, this robust method is most applicable for clinical investigation of the pharmacokinetics of dFdC during elimination phase, to evaluate the AUC ratio of dFdC and dFdU as well as to monitor many samples within a short period. This method has been successfully applied to pharmacokinetics study of phase II clinical trial of gemcitabine in Asian population.

2.1.2. Ion-exchange HPLC determination of dFdCTP in human WBC

As a prodrug, gemcitabine has to go through intracellular conversion to its active metabolites, gemcitabine diphosphate and gemcitabine triphosphate. Current research findings showed that dFdCTP is the main active metabolite and exerts its anticancer effect by incorporation into DNA chain. Several studies have confirmed that *in vitro* and *in vivo* efficacies as well as toxicity of gemcitabine are closely correlated to the intracellular accumulation of dFdCTP. Therefore, a sensitive and accurate analytical method is critical for clinicians to optimize the treatment schedule and correlate the intracellular pharmacokinetics of dFdCTP with pharmacogenetics, pharmacodynamics as well as cytotoxicity. However, bioanalytical method development is a challenge for quantifying intracellular active metabolite, dFdCTP because of 1) the large volume of blood needed for isolating enough white blood cells; 2) tedious procedure on handling, isolating white blood cells and cell counting; 3) sample stability during storage; 4) difficulty in developing sensitive HPLC method.

2.2. Objectives

The aims are to develop and validate a highly sensitive method for plasma dFdC and dFdU quantitation using LC-MSMS and to accurately quantify intracellular metabolite (dFdCTP) using ion-exchange HPLC coupled with UV detector to study pharmacokinetics of gemcitabine.

2.3. Materials and Methods

2.3.1. Reagents and Standards

Gemcitabine hydrochloride (dFdC, LY 188011), Gemcitabine Triphosphate (dFdCTP, LY 264368) and 2', 2'-difluorodeoxyuridine (dFdU, LY 198791) were kindly provided by Eli Lilly. (Indianapolis, IN, USA). The internal standard, Gemcitabine-13C, 5N2 Hydrochloride, was purchased from Toronto Research Chemicals, Inc. (North York, ON, Canada). Tetrahydrouridine (THU) was purchased from Biosciences, Inc. (La Jolla, CA, USA). Ammonium dihydrogen phosphate (AG), phosphoric acid (AG), HPLC grade methanol and acetonitrile were purchased from Merck **(**Darmstadt, Germany). Milli Q water was used for mobile phase preparation.

2.3.2. Sample Collection and Pretreatment

This clinical trial was approved by the institutional review board of National Health Group of Singapore. Blood samples were collected from patients before initiation baseline (0 min) of a 30- to 75-minute gemcitabine infusion, 10 minutes, 30 minutes during infusion, 10 minutes before the end of the infusion, and 30 minutes, 1 hour, 2 hours after the end of the infusion. At each point, 8 ml of blood was drawn into 15-ml

heparined plastic tubes that had been preloaded with 0.1 ml of a 10 mg/ml solution of tetrahydrouridine, a cytidine deaminase inhibitor. Blood samples were centrifuged for 15 minutes at approximately 1,200 ×g at room temperature. The plasma portion of the samples were removed and kept at –20 °C until analysis. The buffy coat was for mononuclear blood cell isolation for quantification of dFdCTP, the intracellular main active metabolite.

2.3.2.1. Plasma Sample Preparation

Fifty microlitre of plasma or calibrator sample, 5 µl of 50 µg/ml aqueous solution of Gemcitabine-13C, 5N2 (internal standard), and 200 µl of acetonitrile were added together in a 1.5 ml Eppendorf tube. The tube was tightly capped and immediately vortex-mixed for 1 minute, and then centrifuged at 10,000×g for six minutes at 4 °C. One hundred microlitre of supernatant was transferred into another Eppendorf tube and dried under nitrogen and reconstituted with 50 µl of 10 mM ammonia acetate buffer solution pH 6.8. After mixing, 40 µl of the mixture was transferred to plastic insert for LC-MSMS analysis.

2.3.2.2. Blood Cell Preparation

2.3.2.2.1. WBC Isolation

The buffy coat (1 ml) was gently collected using Pasteur pipette and transferred into a 15 ml centrifuge tube. Balanced salt solution was added to make the volume of cell suspension to be 6 ml which is equal to the original blood volume used. The cell suspension was mixed using Pasteur pipette. Three milliliter of Ficoll-Paque was added

to another centrifuge tube (2 tubes for each point). Three milliliters of the diluted blood was layered carefully over the Ficoll-Paque. Next, the tubes were capped and centrifuged at 250 ×g at 5 °C for 20 minutes. The upper layer was drawn off carefully while leaving the WBC layer undisturbed. The WBC layer was collected and diluted with saline to 3 ml. Twenty microlitres of cell suspension were diluted with 80 µl of 3% acetic solution and counted with hemacytometer. The number of WBC was calculated as follows:

$$N_{WBC} = 5 \text{ (dilution factor)} \times \text{cell number within each square} \times 10^4 \text{ (cells/ml)}.$$

2.3.2.2.2. Storage of Cell Samples

The remaining isolated WBC except those (20 µl) for counting was centrifuged at 500 ×g at 5 °C for 6 min. The supernatant was decanted off gently and the tube was put upside down on the tissue paper to drain off the water in the tube wall. The tubes were immersed in liquid N_2 and kept in -80 ºC.

2.3.2.2.3. Pre-analytical Preparation of WBC Samples

Eighty microlitres of $HClO_4$ (0.8 M) was added to the tube containing the WBC pellets above, vortex-mixed for 30 seconds and kept on ice for 3 min. Then the tube was centrifuged at 10,000 g at 4 ºC for 2 min. The supernatant was transferred to another centrifuge tube (0.6 ml) and the volume was adjusted to 130 µl. After that, 70 µl of 0.8 M KOH was added and the tube was kept on ice for ten min and centrifuged at 10,000 ×g at 4 ºC for 5 min. The supernatant (150 µl) was transferred into injection insert tube (200 µl) and 100 µl was injected into HPLC system for analysis.

2.3.3. Instrumentation

2.3.3.1. HPLC-MSMS (dFdC and dFdU)

The high-performance liquid chromatographic system was comprised of an Agilent 1100 Binary pump equipped with an Agilent 1100 autosampler injector with 100 µl loop and 1100 column oven set at 20°C (Germany). Chromatographic separations were achieved using a BDS HYPERSIL C_{18} column (2.1×100 mm) (Thermo Hypersil-Keystone, USA) following an Eclipse XDB-C8 guard column (2.1 mm x 50 mm, 5 µm) (Agilent Technologies, USA) with gradient elution of the analytes with an initial mobile phase composition of 2% methanol in 10 mM ammonium acetate buffer pH 6.8 (2:98, v/v). Methanol was increased to 15% in 1 min maintained for 4 min and decreased back to 2% methanol again in 0.5 min. Column was equilibrated at 2% methanol for another 6.5 min before the next injection. The flow rate was set at 0.2 ml /min. Ten microliters of reconstituted supernatant were injected to the HPLC column and the elutant directed to the mass spectrometer turboionspray source without splitting. In order to avoid contaminating the ion source detector, the solvent front eluting in the first 2.5 min was switched to waste container.

LC-MSMS analyses were performed using an API 2000 triple-quadrupole mass spectrometer (Applied Biosystems, MDS SCIEX, Ontario, Canada). The instrument was operated in positive ion mode calibrated by polypropylene glycol. The plasma samples were analyzed by tandem MS using the IonSpray needle at +5500 V and the cluster breaking orifice voltage at 30 V. The ions of dFdC at m/z 264, dFdU at m/z 265.1 and internal standard at m/z 267.0 were passed through the first quadropole (Q1) and the product ions for dFdC (m/z 112.1), dFdU (m/z 113.2) and internal standard (m/z 115.0)

were monitored through the third quadrupole (Q3). The dwell time per channel was 300 ms for data collection. Analyst software (version 1.3) was used to process quantitative data analysis.

2.3.3.2. HPLC-UV (intracellular dFdCTP)

The high-performance liquid chromatographic system consisted of a Hewlett Packard (HP) 1050 quaternary pump equipped with a (HP) 1050 autosampler injector with a 100 µl loop, HP 1100 variable wavelength UV detector and HP ChemStation for data analysis. The analytical column Column is partisphere 5 SAX (Whatman).

The mobile phase was optimized through different buffers, pH values and gradient compositions as well. The optimized analysis conditions were listed as follows:

- HPLC conditions:

  -HPLC column is partisphere 5 SAX (Whatman).

  --Mobile phase consists of solution 1 (0.5M Ammoniun dihydrogen phosphate buffer, pH was adjusted to 3.5 using 85% H_3PO_4) and Solution 2 (0.2 M, H_3PO_4 pH 2.1). The gradient was listed as follows:

Time:	Pump C	Pump D
0’	45%	55%
30’	75%	25%
31’	45%	55%

- Flow rate was 1.5 ml/min and Detection wave length was 275 nm.
- Retention time was 14.5 min for dFdCTP and 17.0 min for ATP.

2.3.4. Standard Solutions and Calibration Curves

2.3.4.1. Gemcitabine and dFdU

Standard stock solutions of dFdC, dFdU and IS prepared in methanol at 1 mg/ml and

were kept at –20 °C. These stock solutions were diluted with water to obtain the concentrations required for preparation of standard working solutions. For dFdC, working solutions of 0.05, 0.1, 0.25, 1, 2.5, 5, 10, 20 µg/ml and for dFdU, working solutions of 0.5, 1, 5, 10, 25, 50, 100 and 200 µg/ml were prepared. A working solution of internal standard was prepared at 5 µg/ml (Table 2.1).

Table 2.1 Calibrator Preparation

calibrators	C1	C2	C3	C4	C5	C6	C7	C8
IS (5 ug/ml)	5 µl	5 µl	5 µl	5 µl	5 µl	5 µl	5 µl	5 µl
dFdC (µg/ml)	0.05	0.1	0.25	1	2.5	5	10	20
Vol (µl)	5 µl	5 µl	5 µl	5 µl	5 µl	5 µl	5 µl	5 µl
dFdU (µg/ml)	0.5	1	5	10	25	50	100	200
Vol (µl)	5 µl	5 µl	5 µl	5 µl	5 µl	5 µl	5 µl	5 µl
Control plasma **(THU, 0.2 g/10ml)**	50 µl	50 µl	50 µl	50 µl	50 µl	50 µl	50 µl	50 µl

Least-squares regression and standard curves weighted according to 1/x (x = concentration) were drawn using linear regression of the peak area ratios of dFdC or dFdU against internal standard obtained from LC-MSMS analysis of standard solution against actual standard concentrations.

The limit of detection (LOD) was defined as the lowest concentration that the analytical assay can reliably differentiate from background levels (S/N > 3). The lower limit of quantification (LLOQ) was defined as the lowest calibrator with the inter-day coefficient of variation < 20%. [124]

2.3.4.2. Gemcitabine Triphosphate (dFdCTP)

Intracellular dFdCTP determination was developed using external method. A series of calibrators were prepared using HONE 1 cell line as a surrogate for control white blood

cells. The HONE 1 cell pellet was treated the same way as white blood cell preparation. 1000 µM of dFdCTP was prepared as stock solution and was stored at -20 °C. This stock solution of dFdCTP were spiked into the alkalined cellular supernatant to prepare a series of working solution with concentration values of 0.4, 1, 1.5, 2, 3, 4, 5, 10 µM. 100 µl of working solutions were injected into HPLC to produce a standard curve. QC samples were prepared as 1.2, 2.5, 7.5 µM. The accuracy and precision were evaluated using four samples at each concentration intra-run and inter-run. Stability of neutralized samples and reproducibility of method will be evaluated to guarantee robustness of analytical intracellular method.

2.3.5. Validation Description

2.3.5.1. Gemcitabine and dFdU in plasma

Quantification was based on the ratios of the peak areas of dFdC and dFdU against that of internal standard. Validation was performed through establishing intra and inter-day accuracy and precision of the method on quality control samples. Five different calibration curves of seven calibrators of dFdC and dFdU were prepared to determine the quality control (QC) samples. To determine intra-day and inter-day precision and accuracy of dFdC and dFdU, the method presented here was validated by analyzing three quality control samples, prepared at the nominal concentrations of 15, 200, 800 ng/ml for dFdC and those of 150, 2000 and 8000 ng/ml for dFdU in blank human plasma. Intra-day variability was tested on five different human plasma QC samples using the same calibration curve. Inter-day variability was tested on five different days using calibration curves obtained daily. The precision of the method at each QC concentration was expressed as a coefficient of variation (%C.V.) by calculating the standard deviation as a

percentage of the mean calculated concentration, while the accuracy of the assay was determined by expressing the percentage of the mean from the true value.

The absolute recoveries at three different plasma concentrations of dFdC (15, 200, 800 ng/ml) and dFdU (150, 2000, 8000 ng/ml) in triplicate and internal standard at 5000 ng/ml were determined by comparing the peak area of dFdC, dFdU or internal standard from samples obtained through complete sample preparation with those obtained from direct injection of equivalent pure substances spiked into post extracted blank plasma. .

2.3.5.2. Matrix effect evaluation

Matrix effect is a common and harmful phenomenon in LC-MS or LC-MSMS procedure. According to FDA bioanalytical method validation guidance for industry, matrix effect should be investigated to achieve good precision and accuracy. The matrix effect [ME(%)] was evaluated according to the following formula: [125]

ME (%) = [Peak Area in control matrix/ Peak Area in neat standard]×100 … (1)

In our current validation procedure, six patient control plasma samples were used for matrix effect evaluation and recovery for three compounds. The standard concentration levels were QC1 and QC3 for dFdC and dFdU and that for internal standard was 500 ng/ml. Three sets of these two concentrations (QC1 and QC3) were prepared. The first set of two QCs (set A) was prepared to evaluate the MS/MS response for neat standards of two analytes (dFdC and dFdU) and internal standard. The second set (set B) was prepared in plasma extracts taken from six different donors. The third set (set C) was prepared in

plasma from the same six sources as in set B, but the plasma samples were spiked before extraction. By comparing absolute area of set B against those of set A, the matrix effect (ion suppression or enhancement) associated with a given lot of plasma can be measured. Similarly, the recovery was determined by comparing the mean peak areas of dFdC, dFdU and internal standard obtained in set C to those in set B at a given concentration.

2.3.5.3. Gemcitabine Triphosphate in the Cell

The stability of dFdCTP was of concern when the white cells were treated with strong acid ($HClO_4$). Different situations were tested for optimizing the sample preparation. We tested how long dFdCTP is stable in strong acid environment ($HClO_4$, 0.4 M). What was a better choice of ratio of hydroxide potassium (KOH) to $HClO_4$ for neutralizing the acidic cellular supernatant after nucleotide extraction? Finally, we further tested how long neutralized dFdCTP would be stable in room temperature while queuing for HPLC analysis.

2.4. Results and Discussion

2.4.1. Gemcitabine and dFdU in Human Plasma

2.4.1.1. Chromatographic Separation

Liquid chromatography-tandem mass spectrometry has advantage for its excellent specificity in biopharmaceutical analysis. In most cases, chromatographic separation is considered by analytical scientists to avoid the possible ion suppression or enhancement during method development. In addition, another aspect has to be kept in mind for dFdC

and dFdU quantification due to very small mass difference between their parent ions with $[M+H]^+$ as 264 amu for dFdC and 265 amu for dFdU as well as their daughter ions with mass transition as 264.0/112.1 for dFdC and 265.1/113.2 for dFdU. Hence, chromatographic conditions should be optimized to achieve good separation, optimized peak shape as well as ion interference free according to following aspects.

Firstly, an optimized gradient mobile phase was needed to elute the dFdC and dFdU with baseline separation. Various pH values of mobile phases, organic modifiers, and elution modes were studied for optimization of baseline separation and run time. Since dFdC is a weak base with pKa values of 3.6. [126] it would exist mainly as a free base when pH value was greater than 5.6. Peak tailing of dFdC in reversed phase HPLC column was minimized by adjusting the pH of mobile phase to pH 6.8 with 10 mM ammonia acetate buffer. Methanol rather than acetonitrile was used because acetonitrile elutes dFdC too rapidly. A gradient elution mode was adopted for baseline chromatographic separation since the hydrophilicity of dFdU was much weaker than those of dFdC. This difference in their hydrophilic properties is mainly attributed to amine group in cytosine ring. (Fig.1). With the optimized chromatographic conditions described, dFdC and dFdU were eluted at about 7.5 and 9.4 min, respectively.(Fig 2) More importantly, a minor isotope peak of dFdC did not interfere with the quantification of dFdU due to the optimized baseline separation. Hence, these optimized chromatographic conditions guaranteed an excellent specificity for this quantitative method.

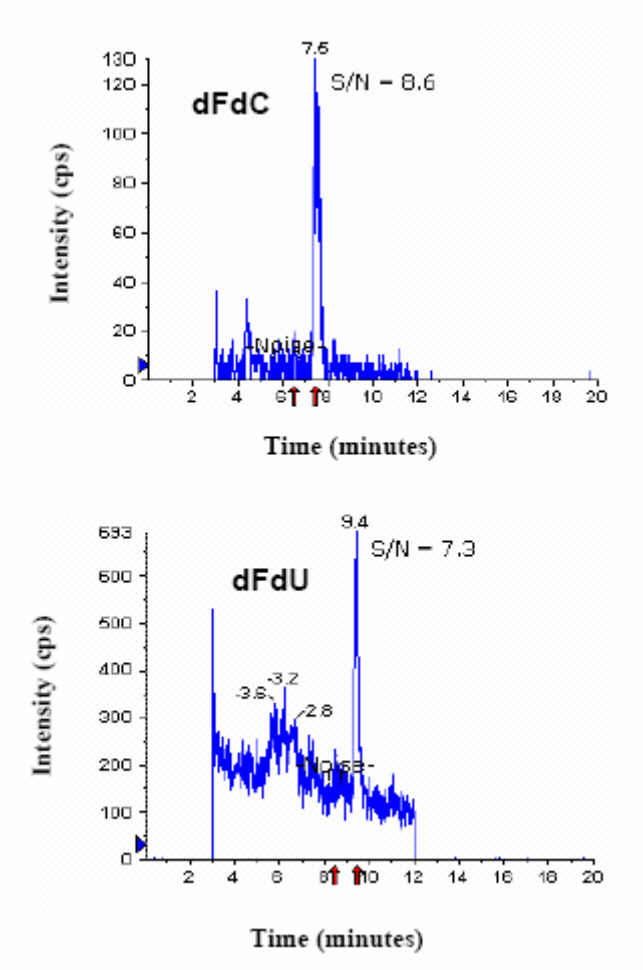


Figure 2.2 The chromatograms of dFdC (upper) and dFdU (lower) at LLOQ in human plasma sample

2.4.1.2. Method Validation of dFdC and dFdU

A simple sample preparation with protein precipitation was used and this was suitable for dealing with a large sample size in a short period. Although nitrogen gas drying was needed following the protein precipitation, the process was fast due to a small plasma sample volume (50 µl) and high percentage (4:1, Acetonitrile/plasma in volume) of acetonitrile used. Moreover, there was good recovery for all three compounds. The mean recovery for these three compounds ranged from 92-98.3% (Table 2). In addition, matrix effect was evaluated through quantification of QC1 and QC2 spiked in six blank human plasmas.

Table 2.2 Intra-day and inter-day precision and accuracy for dFdC and dFdU (n = 5)

Calculated Concentration Precision & Accuracy		Theoretical Concentration (ng/mL)					
		dFdC	dFdU	dFdC	dFdU	dFdC	dFdU
		15	150	200	2000	800	8000
Intra-day (n = 5)	Conc.(ng/mL) (SD)	14.1 (0.4)	150.6 (9.3)	188.0 (15.8)	1996.0 (64.3)	787.2 (35.4)	7588.0 (510.5)
	Precision (%CV)	2.8	6.2	8.4	3.2	4.5	6.7
	Accuracy (%)	93.7	100.4	94.0	99.8	98.4	94.9
Inter-day (n = 5)	Conc.(ng/ml) (SD)	14.3 (1.0)	149.2 (17.4)	200.2 (24.4)	2048.8 (217.6)	817.0 (49.1)	8504.0 (717.1)
	Precision (%CV)	6.9	11.7	12.2	10.6	6.0	8.4
	Accuracy (%)	95.2	99.5	100.1	102.4	102.1	106.3

The matrix effect [ME(%)] was calculated according to the formula mentioned experimental section. No serious matrix effect was observed under our optimized chromatographic conditions with ESI (electrospray ionization) interface using six patient control plasma samples (Table 2.3). Good linearity was achieved for concentration ranges

of 5-2,000 ng/ml for dFdC and 50-20,000 ng/ml for dFdU based on the current LC-MS/MS conditions. The correlation coefficients r^2 for dFdC and dFdU were 0.9969 (95% confidence interval, 0.9957-0.9981) and 0.9980 (95% confidence interval, 0.9969-0.9990), respectively. The lower limit of quantitation was 5 ng/ml and 50 ng/ml for dFdC and dFdU, respectively. This LC-MS/MS method is much sensitive than our previous HPLC-UV assay by using as little as 50 μL of human plasma. Their signal-to-noise ratios are 8.6 and 7.3, respectively. The assay sensitivity was more than adequate for all clinical samples with the last sampling time of 120 min after the end of dFdC infusion.

Table 2.3 Matrix effect and recovery tested in patient control plasma at two concentration levels (n = 6).

dFdC	Mean Peak Area						ME(%)		RE (%)	
nominal conc. (ng/ml)	dFdC set A	dFdC set B	dFdC Set C	IS set A	IS set B	IS Set C	dFdC	IS	dFdC	IS
15	11902	11731	10915	410235	423167	389833	98.6	103.2	93.0	92.1
800	524333	464667	443667	392667	345333	339500	88.6	87.9	95.5	98.3
dFdU	**Mean Peak Area**						**ME(%)**		**RE (%)**	
nominal conc. (ng/ml)	dFdU set A	dFdU set B	dFdU Set C	IS set A	IS set B	IS set C	dFdU	IS	dFdU	IS
150	9317	8008	7745	406169	412667	389833	86.0	101.6	96.7	94.5
8000	580000	497000	457333	392667	345333	339000	85.7	87.9	92.0	98.2

Set A: Neat standards of two analytes (dFdC and dFdU) and internal standard were dissolved in mobile phase; Set B: dFdC, dFdU and internal standard were dissolved in plasma extracts taken from six different donors; Set C: dFdC, dFdU and internal standard were spiked in plasma from the same six sources as in set B, then the spiked plasma QC samples will go through extraction.

The accuracy and precision of this method were evaluated from the QC samples. The precision and accuracy of dFdC and dFdU for QCs were listed in Table 2. The intra and inter-day precisions for dFdC and dFdU were ≤9 and ≤13 and their accuracy ranged from 94 to 102 for quality control samples.

The sample stability was judged by decrease of sample concentrations after one year storage of plasma samples at -20 ºC. Both dFdC and dFdU showed good stability (decrease in concentrations is less than 10%) over the period of one year.

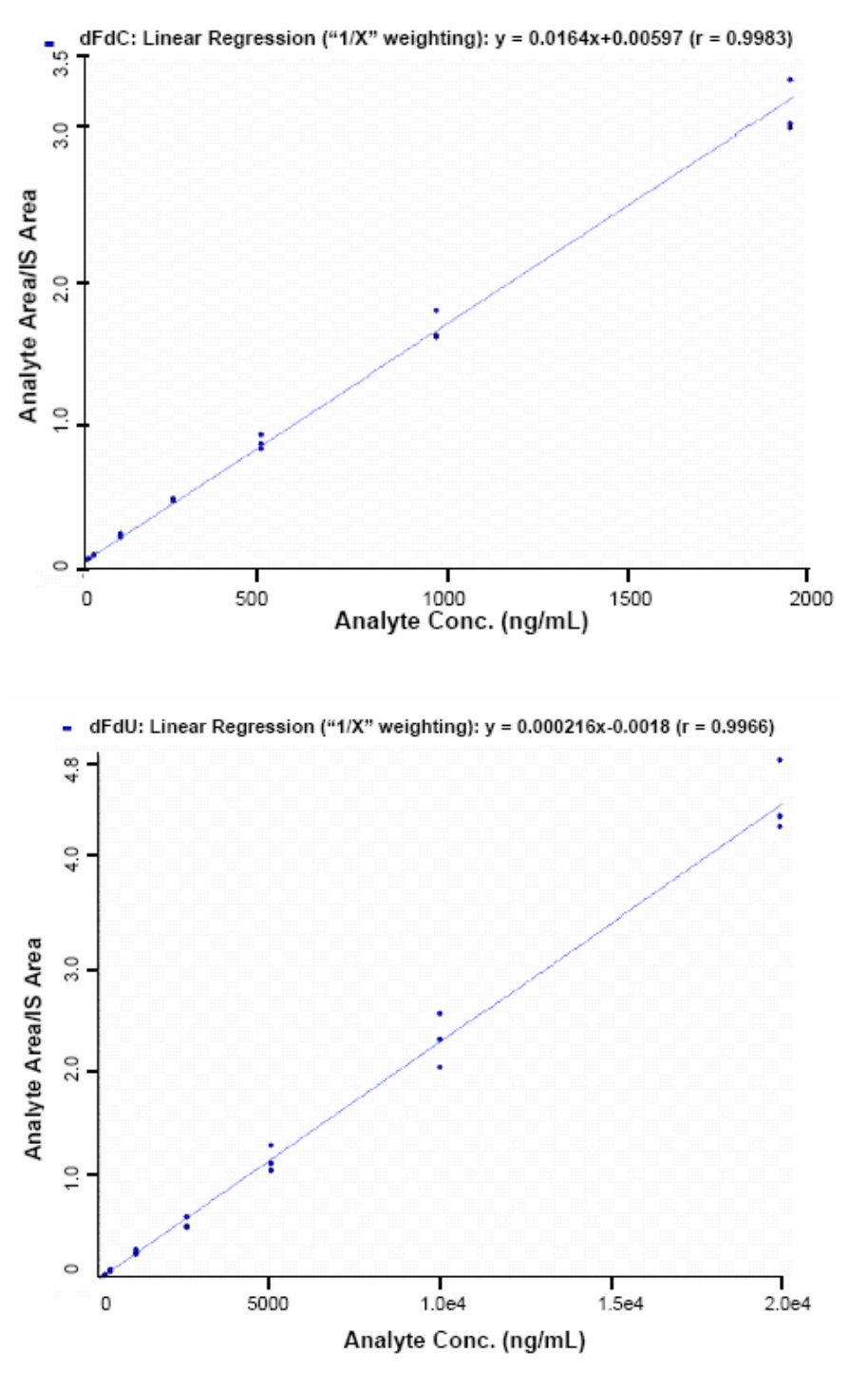


Figure 2.3 Standard Curve for dFdC (upper, n = 3) and dFdU (lower, n = 3)

This LC-MSMS method is much more sensitive than our previous ion-pair HPLC method[16] where most concentrations of the last sampling point (2 h) are below its LLOQ of gemcitabine (80 ng/mL). The method has been successfully used in multi-centre clinical trial of dFdC at a dosage of infusional dFdC given at a constant rate of 10 mg/m^2/min over 75 min or at 1000 mg/m^2 in 30-min, when combined with a fixed dose of carboplatin. The sensitivity of this method is 16 fold higher than our previous HPLC-UV method. [123] More importantly, a micro-volume of plasma (50 µL) is needed so that it is suitable for pediatric clinical trials since the safety of dFdC treatment in children has not been established. [30]

2.4.2 Gemcitabine Triphosphate

2.4.2.1 Chromatographic Separation

Figure 2.4 shows a clear baseline chromatographic separation of dFdCTP from other cellular endogenous substances such as ATP which was eluted at 17.5 min.

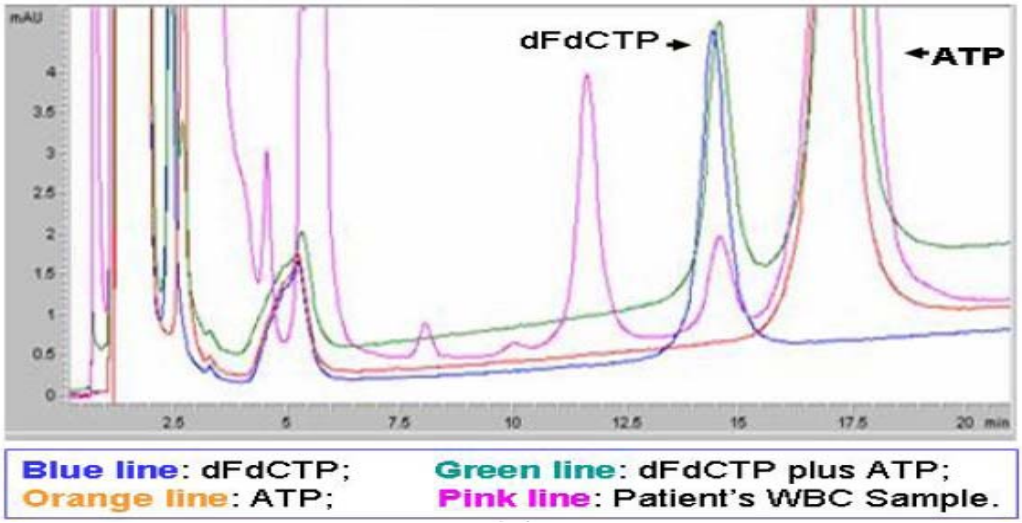


Figure 2.4 HPLC chromatogram of dFdCTP in Human Sample Analysis

2.4.2.2 Method validation for determination of gemcitabine triphosphate

Figure 2.5 showed a good linearity for quantitation of dFdCTP in saline. Due to the difficulty in getting human WBC, one of NPC cell line, HONE1, was used for method validation. According to validation table 2.4, the intra-run and inter-run were well validated with accuracy and precision in the range of 90 to 105%.

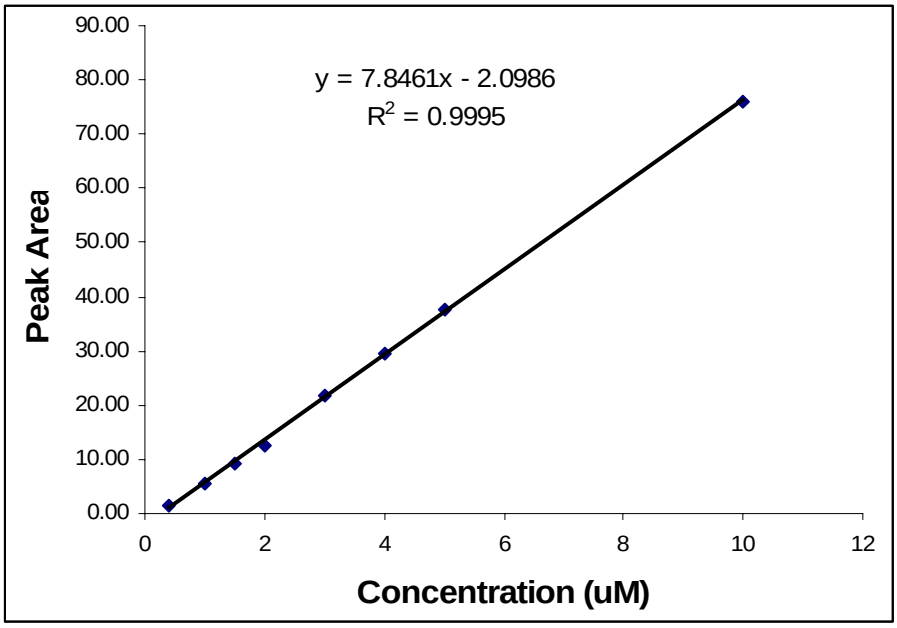


Figure 2.5 Mean Standard Curve of dFdCTP (HONE 1 cells as matrix)

Table 2.4 dFdCTP method development and validation using HONE1

Nominal Concentration (ng/ml)	Found Concentration Mean ± SD (ng/ml)	Precision (%)	Accuracy (%)
	Intra-run		
1.2	1.26	104.8	4.06
2.5	2.27	90.9	2.82
7.5	7.25	96.7	2.71
	Inter-run		
1.2	1.22	6.62	98.7
2.5	2.45	9.85	99.2
7.5	7.49	8.68	99.3

2.4.2.3. Optimization of dFdCTP extraction from human WBC

Initially, 10 μM dFdCTP in PBS was used for stability testing. When treated with $HClO_4$ (0.4 M) alone, dFdCTP would degrade quickly after 15 min whether on ice or in room temperature. In contrast, the alkalined dFdCTP was very stable (Figure 2.6).

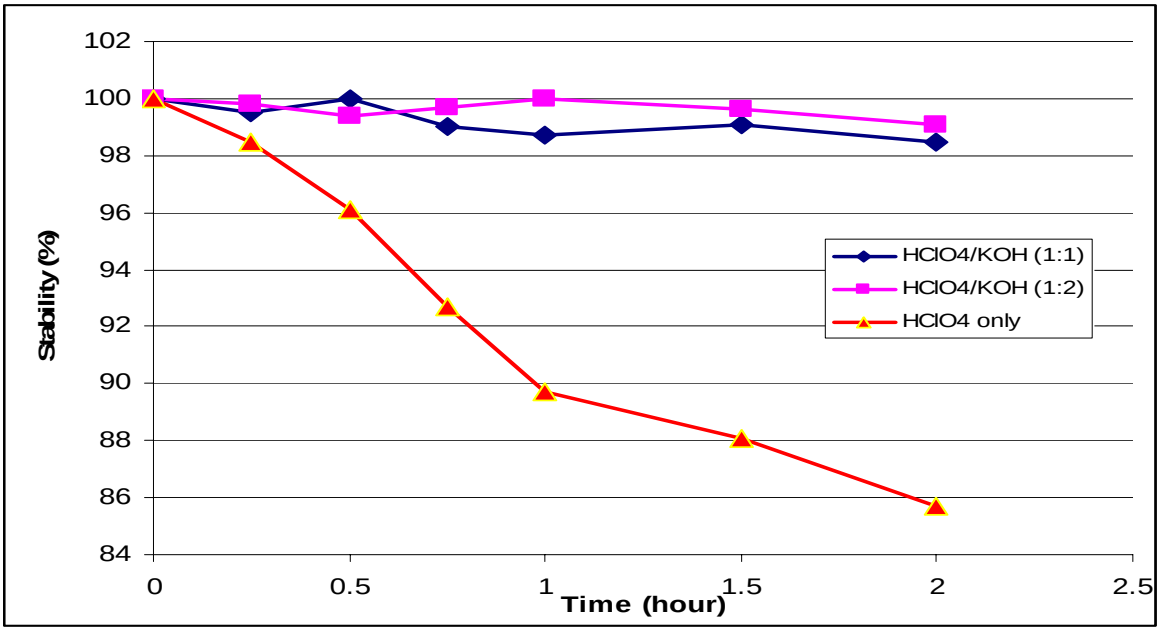


Figure 2.6 Stability of dFdCTP in solution of HClO4 (0.4M) alone and various concentrations of KOH

In order to evaluate the stability of dFdCTP for a longer time, two different concentrations of KOH (0.4M, 0.8 M) were tested. When $HClO_4$ solution was neutralized with an equivalent concentration of KOH (0.4 M), dFdCTP became more stable compared to that in $HClO_4$ solution. Although dFdCTP degraded slowly at first 5 hours, the percentage of degraded dFdCTP would degrade 10% after 11.5 hours and up to 36% after 24 hours (Figure 2.7). By increasing the concentration of KOH from 0.4 to 0.8 M, the stability of dFdCTP improved tremendously with decreased degrade rate of dFdCTP to 8.5 % from 36.2 % after 24 hours. Using this $HClO_4$ (0.4 M)/ KOH (0.8 M), the prepared samples were stable for at least 12 hours (Figure 2.7) suitable for overnight storage of samples in room temperature for injection.

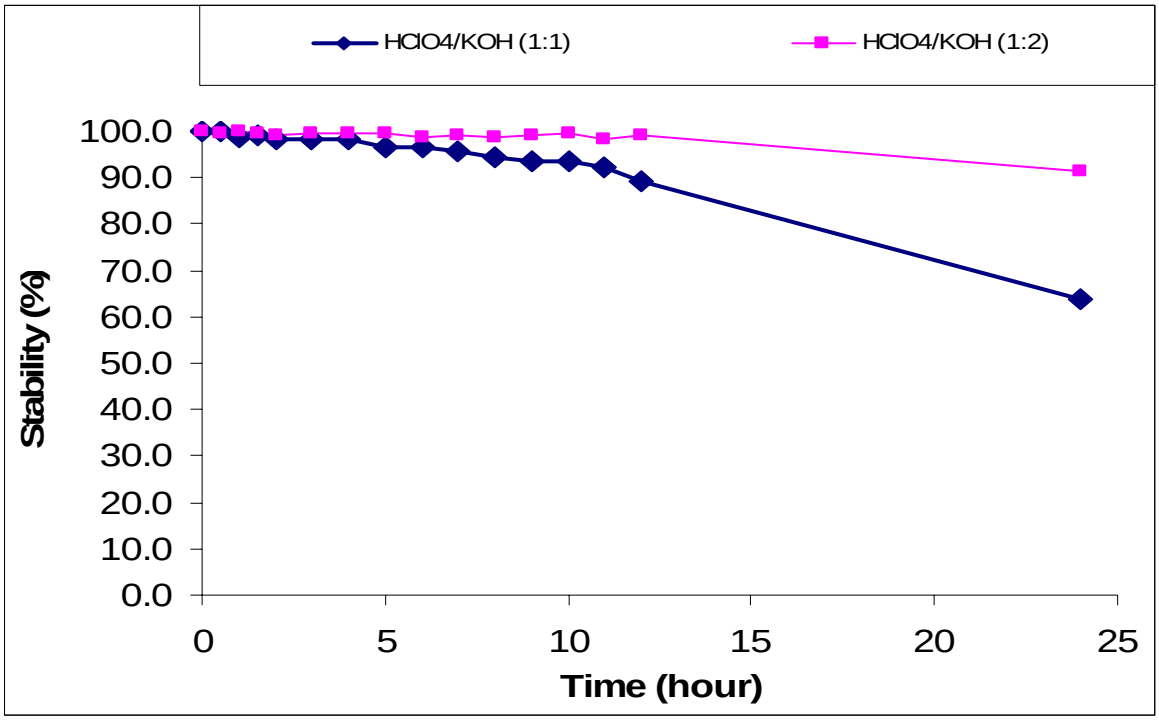


Figure 2.7 Stability of dFdCTP in mixture solution of HClO4 (0.4M)/ KOH (0.4M) and HClO4 (0.4M)/ KOH (0.8M)

2.5. Conclusions

A highly sensitive LC-MS/MS method was validated and applied to a Phase II clinical plasma determination of gemcitabine and its deaminated metabolite. The sensitivity of this method is 16 fold greater than our previous HPLC-UV method. More importantly, only a micro-volume of plasma is needed so that it is suitable for pediatric clinical trials. In addition, a simple sample preparation procedure with acetonitrile precipitation is advantageous for high-throughput analysis for a multi-centre clinical trial context. Its simplicity and sensitivity will greatly improve the clinical applicability.

Several studies have indicated that *in vitro* and *in vivo* efficacy of gemcitabine may be closely correlated to the intracellular accumulation of dFdCTP. Therefore, a sensitive and accurate analytical method is critical for oncologists and scientists to optimize the treatment scheduling. This intracellular dFdCTP quantitation is a platform for oncologists to evaluate the pharmacokinetics of gemcitabine more efficiently and provide a useful tool to correlate the pharmacokinetics to pharmacodynamics or pharmacogenetics in Asian population.

Chapter Three

In vitro Study of Gemcitabine as A Single Agent or in Combination Therapy

3.1. Introduction

Gemcitabine is a prodrug. It is transported through the cell membrane via nucleoside transporters because it is highly hydrophilic. [106] Gemcitabine is converted to its active phosphorylated metabolites, dFdCDP and dFdCTP or to inactive metabolites like dFdUMP and dFdU within the cell. The cytotoxicity of gemcitabine is highly dependent on the accumulation of dFdCTP since this metabolite is the main component (more than 95%) in the active metabolite pool. [127] The mechanisms of action of dFdCTP include its incorporation into DNA chain to stop DNA synthesis in S phase of cell proliferation cycle and competition with deoxycytidine triphosphate (dCTP) to inhibit DNA polymerase. [14] Thus, the efficacy of gemcitabine is mainly determined by accumulation and retention of intracellular dFdCTP.

Another minor active metabolite, dFdCDP, is a potent inhibitor on ribonucleotide reductase. It exerts its anticancer effect through decreasing dCTP pool by inhibiting the activity of ribonucleotide reductase. This will interfere with DNA synthesis indirectly.

Many factors are involved in the active conversion of gemcitabine to dFdCTP. Deoxycytidine kinase catalyses the rate-limiting reaction of gemcitabine activation which involves phosphorylation to gemcitabine monophosphate (dFdCMP). According to Michaelis-Menten Equation ($V = Vmax[S]/([S]+Km)$, [S] being substrate concentration), dCK will be saturated when substrate (gemcitabine) concentration, [S], is much greater than Km. In this case, $V \approx Vmax$. This means that dFdCTP concentration will be expected to reach a plateau after a period of exposure with a certain concentration of gemcitabine in cell culture. The minimum concentration which saturates dCK is defined as the cut-off value for gemcitabine. Accordingly, this concentration is considered

optimal to achieve maximum treatment effect.

In this section, we designed an *in vitro* experiment to define this optimal concentration of gemcitabine in culture medium.

An additional objective of this *in vitro* experiment was to study the synergistic activity of histone deacetylase inhibitor (PXD101) with gemcitabine cytotoxicity on NPC and NSCLC cell lines. The determinants of sensitivity and resistance to gemcitabine are not fully elucidated. Many factors involving intracellular dFdCTP and dFdCDP accumulation and metabolism can affect the efficacy of gemcitabine. Although several papers have proposed possible mechanisms of gemcitabine intrinsic and acquired resistance, the progress on reversing this resistance is very limited, especially for clinical strategy.

PXD101 is one of novel histone deacetylase inhibitors (HDACi) which acts through disturbing tumour growth via regulating histone acetylation and restoring silenced tumor suppressor genes. However, the HDACi-regulated genes necessary and/or sufficient for their biological effects remain undefined. At the moment, PXD101 is undergoing phase II clinical trials in a variety of disease indications including hepatocellular carcinoma. Recently, several papers reported that PXD101 can potentiate several current chemotherapeutic agents such as 5-FU, paclitaxel and carboplatin *in vitro* and *in vivo*. [128, 129] Two histone-deacetylase inhibitors, trichostatin A and SAHA, were also reported to enhance gemcitabine-induced cell death in pancreatic cancer. [130, 131] This prompted us to test if a novel HDAC inhibitor, PXD101, could produce a synergistic effect with gemcitabine on other tumours e.g. NPC and NSCLC.

3.2. Objectives

- To evaluate the incubation time of gemcitabine on the effect of intracellular dFdCTP accumulation;
- To determine the minimum concentrations of gemcitabine in culture medium that optimize intracellular dFdCTP accumulation;
- To screen sensitivity of different cell lines to gemcitabine or gemcitabine combined with PXD101;
- To explore the mechanism of the synergistic effect of gemcitabine and PXD101 on different cell lines.

3.3. Materials and Methods

3.3.1. Drug and chemicals

Gemcitabine hydrochloride (dFdC, LY 264368), Gemcitabine Triphosphate (dFdCTP, LY 264368) were kindly provided by Eli Lilly & Co. (Indianapolis, IN, USA). The internal standard, Gemcitabine-13C, 5N2 Hydrochloride, was purchased from Toronto Research Chemical (Canada), Tetrahydrouridine (THU) was purchased from Biosciences, inc. La Jolla, CA 92039-2087. MTS + PES reagent (CellTiter 96® Aqueous One Solution Cell Proliferation Assay, Promega, USA). PXD101 (N-hydroxy-3-[phenylsulphamoylphenyl] acryl amide) was kindly provided by TopoTarget (Oxford, UK). RPMI-1640 media (GIBCO, Invitrogen corporation, USA); Fetal Bovine Serum (Sigma, St Louis, MO, USA; 100 U/ml penicillin (GIBCO, Invitrogen corporation, USA); 100 μg/ml streptomycin, USA); Phosphate-buffered saline (PBS) (NUMI, National University of Singapore). Propidium iodide (PI) and Triton X-100 were purchased from Sigma-Aldrich

(USA). HPLC grade methanol and 70% ethanol were purchased from Merck Darmstadt (Germany). All other chemicals were of analytical grade and commercially available, unless otherwise specified.

3.3.2. Cell lines and cell culture

Nasopharyngeal carcinoma cell lines include CNE1, HK1, HONE1 and Lung Cancer cell lines include H1299 and H292 were provided Dr Hsieh from Singapore Johns Hopkins International Medical Centre.

Cells were routinely grown in culture in RPMI 1640 medium containing of 10% fetal bovine serum and 100 units /ml penicillin and 100 µg/ml streptomycin. The cells were kept growing at 37 °C in an incubator in a humidified atmosphere containing 95% air and 5% CO2.

3.3.3. Growth inhibition study

1. Seeding the cells in triplicate in 96-well plate. The plating cell number was set as 2000-2500 cells/well except for HK1(5000 cells/well);
2. Cell suspension was prepared at cells density of 25,000 cells/ml and 50,000 cells/ml for HK1 with 100 µl of cell suspension for each well of 96-well plate;
3. After incubating 24 hrs, 100-µl drug solution or blank medium was added into each well of 96-well plate;
4. 48 or 72 hours later, 20 µl of MTS/PES was added into each well for incubation for 3 hours;

5. Measurement of OD value on wavelength 490 nm with reference wavelength set at 650nm;
6. At least five drug concentrations were used to determine the IC50 values.

3.3.4. dFdCTP and dFdC quantitation

Analytical determinations of dFdCTP and dFdC were the same as those described in Chapter II. The only difference was that the samples are from *in vitro* experiment. Sample collection and preparation were described as follows.

3.3.4.1. dFdC sampling and preparation

Eppendorf tubes (1.5 ml) were prepared for culture medium sample collection by adding 50 µl of THU (cytidine deaminase inhibitor) solution and stored in a covered box at -20 °C. The sampling times were as follows:

Table 3.1 Culture media sampling time point for determination of dFdC

Unit	**Minutes**				**hours**									
Time point	0	10	20	30	1	1.5	2	3	4	6	8	24	30	48

These samples were used for determination of dFdC concentration left in the medium at different incubation time. At above mentioned time points, 50 µl of medium from the culture flask (75 ml containing 15-ml medium) was transferred into a THU containing Eppendorf tube. After centrifuging at 10,000 g for ten minutes, the 90-ul supernatant was transferred into HPLC insert and 20 ul supernatant was injected into LC-MSMS system

for quantifying dFdC and using established LC-MSMS method. A series of calibrators in blank media for dFdC were prepared to establish standard curves. 5 µl of Gemcitabine-13C, 5N2 (5 ug/ml) was added into each tube as internal standard. Since the sample collected in Eppendorf tube was diluted, the final concentrations should be equal to 2-fold calculated concentrations.

3.3.4.2. Cell harvesting and preparation

The cell pellets were collected at 1, 4, 8, 24 and 48 hrs for determination of dFdCTP accumulation after incubation with various concentrations of gemcitabine in the culture medium. The cells were trypsinized and washed with PBS. After centrifugation at 200 g for 5 min, the supernatant was fully discarded. Then, 100 µl PBS was added into the 15 ml centrifuge tube and the cell pellets were homogenized and transferred into another Eppendorf tube. This tube was frozen immediately in liquid nitrogen and then stored in freezer at - 80°C for dFdCTP quantitation.

The sample for HPLC analysis was prepared according to the following protocol.

i). Add 80 µl of HClO4 (1M) into cell pellet containing tubes;

ii). Vortex for 30 seconds;

iii). Centrifuged at 10,000 g for 2 min;

iv). Transfer the supernatant into another Eppendorf tube containing 30 ul of KOH (3 M);

v). Vortex 30 seconds;

vi). Kept on ice for 20 minutes;

vii). Centrifuged at 10,000 g for 5 min;

viii). 100 µl of supernatant was injected into HPLC system for analysis.

3.3.5. Titration of gemcitabine concentration for maxium accumulation of dFdCTP

A nasopharyngeal carcinoma cell line, HONE1, was used as the *in vitro* model to evaluate the effects of gemcitabine's exposure concentrations in culture medium and incubation time on the accumulation of dFdCTP inside the cells. In this study, the concentrations of gemcitabine used were increased from 2 μM to 100 μM. The incubation time was set at 1, 4, 8, 24 and 48 hr. At each time point, the cells were trypsinized and collected for determination of intracellular dFdCTP and dFdC concentrations as described previous section 3.3.4.

3.3.6. Combination Study

In order to evaluate the drug interaction, two methods were used to measure the the effect of PXD101 on cytotoxicity of gemcitabine; the Modulate Effect for enhancement or the Combination Index for antagonistic, synergistic or additive effect .

For enhancement effect of PXD101 on gemcitabine's anticancer activity, the Modulate Effect (ME%) was defined as:

Modulate Effect (%)= (IC50 of dFdC/IC50 of dFdC combined with PXD101)×100

The Combination Index (CI) was defined as:

Combination Index (CI) = (Am)50/(As)50 + (Bm)50 + (Bs)50

Where (Am)50 is the concentration of drug A necessary to achieve 50% inhibitory effect in the combination; (As)50 is the concentration of the same drug that will produce the identical level of effect by itself; (Bm)50 is the concentration of drug B that will produce a 50% inhibitory effect in the combination, and (Bs)50 is the concentration of drug B that will produce the same level of effect by itself.

CI > 1 indicates antagonism;

CI < 1 indicates synergy; and

CI = 1 indicates an additive Effect [132]

3.3.7. DNA content measurement

Fix cells with ethanol

1. Prepare the fixative by filling 12×75 mm-centrifuge tubes with 4.5 ml of 70% ethanol. Keep tubes on ice.

2. Collect cells and suspend 10^6 to 10^7 cells in 5 ml PBS in a centrifuge tube.

3. Centrifuge cells 6 min at 200 g.

4. Thoroughly re-suspend cells in 0.5 ml PBS using a Pasteur pipette. (Note: It is important to achieve a well-dispersed suspension).

5. Transfer the cell suspension into the tubes containing 70% ethanol. Keep cells in fixative ≥ 2 hrs. (Note: Cells suspended in 70% ethanol can be stored at 0 °C to -20 °C for several months).

Stain cells with propidium iodide (PI)

6. Centrifuge the ethanol-suspended cells 5 min at 200 g. Decant ethanol thoroughly.

7. Suspend the cell pellet in 5 ml PBS, wait 60 seconds and centrifuge 5 min at 200 g.

8. Suspend cell pellet in 1 ml PI/Triton X-100 staining solution with RNase A. Keep either 15 min at 37 °C or 30 min at room temperature.

Perform Flow Cytometric Analysis

9. Set up and adjust the flow cytometer for excitation with blue light and detection of PI emission at red wavelengths.

10. Measure cell fluorescence in the flow cytometer. Use the pulse width-pulse area signal to discriminate between G2 cells and cell doublets and gate out the latter.

11. Analyze the data using DNA content frequency histogram deconvolution software.

3.4. Results and discussion

3.4.1. Gemcitabine's chemical stability in culture medium without cells

Gemcitabine (20 µM) was incubated in culture medium in incubator at 37 °C for 5 days. 100-µl media containing gemcitabine were sampled at the start point, 24 h and 120 h after incubation. The drug analysis was processed with HPLC-UV. The result showed that gemcitabine was stable at least for 5 days in the cell culture incubation condition (Figure 3.1.)

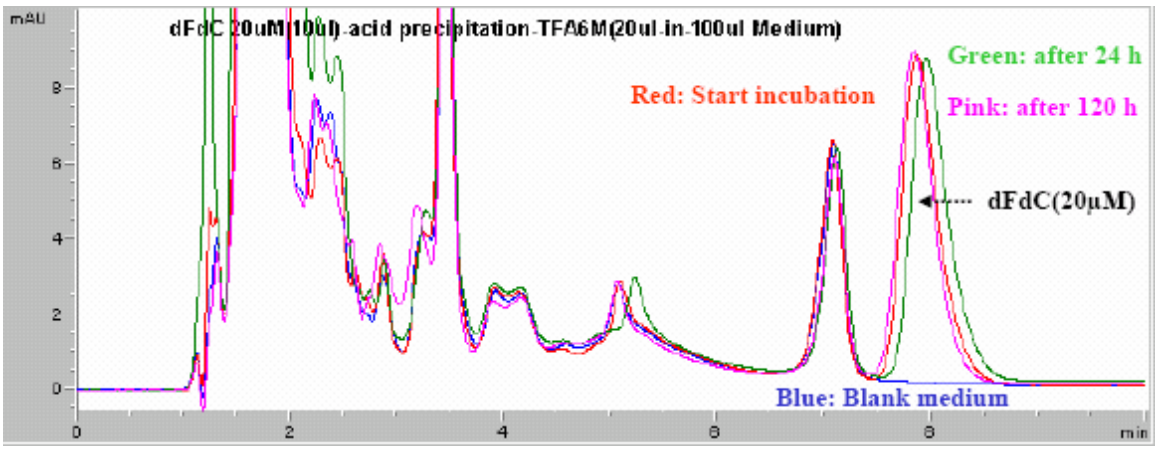


Figure 3.1 Determination of gemcitabine stability in incubation culture medium

3.4.2. Gemcitabine's sensitivity on NPC cell lines

Gemcitabine's sensitivity on NPC cell lines were shown as follows (Table 3.2). Gemcitabine was active against all three NPC cell lines. HK-1 was the most sensitive, followed by HONE1 and CNE-1. The Cmax of gemcitabine in the clinical setting could be as high as 64.6 μM when dosed at 1000 mg/m^2 with 30 min infusion but the Cmax for fixed dose rate infusion of gemcitabine were reduced significantly to the range of 18-35 μM (Table 1.1). Thus, we could conclude that NPC cell lines were sensitive to gemcitabine *in vitro* since their IC_{50} values are much less than the generally acceptable target 10-20 μM plasma concentration for gemcitabine. [15, 133]

Table 3.2 IC_{50} of NPC cell lines to gemcitabine after 72 h incubation

NPC Cell lines	HK1	HONE1	CNE1
(n = 3) IC50 ± SD (μM)	1.30 ± 0.23	2.10 ± 0.38	2.62 ± 0.41

3.4.3. Impact of incubation time on IC_{50} of gemcitabine for HK1

Incubation time was expected to be an important factor on the drug activity since gemcitabine is a prodrug and time would be required for it to be transported and converted into its active metabolites inside the cells. In order to understand the effect of time course on gemcitabine's activity, HK1 was used as the cell model to evaluate the role of incubation time on IC_{50} of gemcitabine. HK1 cells were incubated for various incubation times ranging from 4h to 72h. The IC_{50} values were labeled in Figure 3.2. The

results showed that IC_{50} values of gemcitabine changed dramatically in terms of incubation time. If the incubation time was equal to or less than 24 hrs, the IC_{50} could not be attained up to 100 µM gemcitabine. When the incubation time was increased to 48 hrs, the IC_{50} value was dramatically decreased to 2.6 µM. If the incubation time was further increased to 72 hrs, the IC_{50} was decreased to 1.3 µM which was only half of that with 48 h incubation. This phenomenon might be because that gemcitabine itself is a produrg which needed to be converted into its active metabolites, gemcitabine diphosphate and triphosphate, to play the effects against cancer cells through inhibiting ribonucleotide reductase and stopping DNA elongation. Besides, gemcitabine produced a pro-apoptosis effect on cancer cells through interfering S-phase transition.

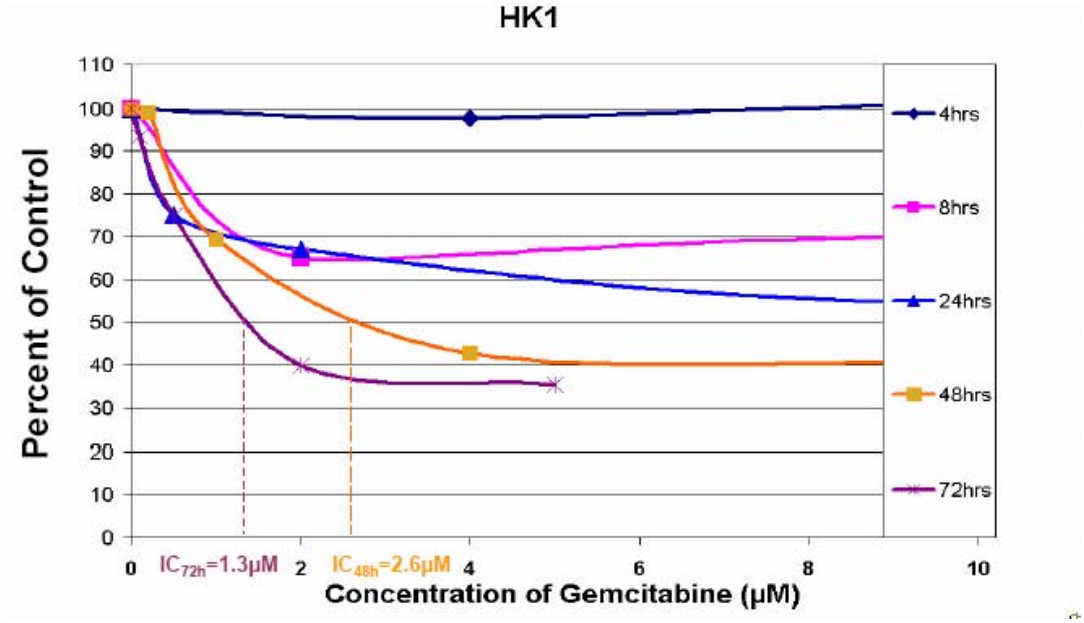


Figure 3.2 Effect of incubation time on the inhibition of HK1 by gemcitabine

3.4.4. Effect of incubation time and concentration of dFdC on intracellular accumulation rate of dFdCTP using HONE1 cell model

We have learned from the IC_{50} values of gemcitabine with HK1 cells experiment that incubation time is a key factor on cell proliferation. However, we have no idea on the kinetics of dFdCTP accumulation during the incubation period. Since gemcitabine's activation involved multiple steps, and exposure time would be expected to play an important role on its activity through modulating the intracellular accumulation and elimination of dFdCTP. This effect was evaluated through determining kinetic change of dFdCTP concentrations using various concentrations of gemcitabine. HONE1 cell line was used because it would grow faster than HK1 cells. The cells were incubated with the indicated concentrations of dFdC, the accumulation concentrations of dFdCTP were measured after variable incubation times. There was an increase in intracellular concentration of dFdCTP with increasing concentrations of dFdC in culture medium. Our finding was similar to the results from other investigators. [14] But the accumulation of dFdCTP reached a plateau after 8 h incubation when the initial incubation concentration of dFdC was 10 μM or above. After 8 h incubation, the intracellular concentrations of dFdCTP were shown to peak in the indicated time points (Figure 3.3). This implied that the activation process of dFdC to dFdCTP might be saturable at this point, assuming that Vmax was reached. The concentration of substrate (dFdC) had no influence on formation rate of dFdCTP.

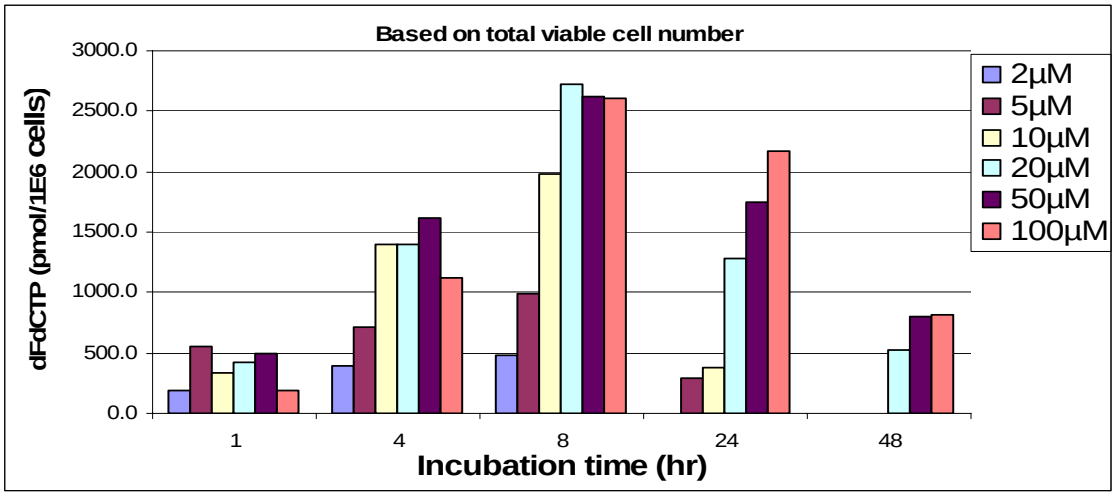


Figure 3.3 Effect of incubation time on the accumulatin of dFdCTP in HONE1 with various concentrations of gemcitabine.

When the incubation time was extended to 24 h, dFdCTP could not be detected in the cell pellets exposed to 2 μM of dFdC. There were two postulated reasons for this phenomenon. The first one was that the elimination rates of dFdCTP showed different kinetic characteristics, linear elimination was proposed for low concentrations of dFdCTP. And elimination seemed to become saturated at high concentrations of dFdCTP. [127] We could expect that the elimination rate of dFdCTP was fast when the initial concentration of dFdC in culture medium was less than 50 μM. For example, when dFdC was at 2 μM, all the dFdCTP inside the cells would be completely eliminated and finally converted to its non-active metabolite (dFdU) after a certain time (24 h) (Figure 3.3). However, we could observe that dFdCTP elimination pattern was changed when dFdC concentration was ≥ 50 μM. Over exposure dFdC could induce an inhibition of deaminase, resulting in a decrease of dFdCTP catabolism. The elimination could be switched to concentration-dependent kinetics from linear elimination. If the incubation time was further increased up to 48 h, the dFdCTP was only detected in the cells with equal or greater than 20 μM of dFdC and dFdCTP showed a similar concentration for

both 50 and 100 µM dFdC. On the other hand, the intracellular dFdCTP concentrations were dependent on the constant exposure of dFdC in the culture medium. With increase of dFdC concentrations, an exposure window for dFdC at a minimum 2 µM would be increased significantly (Figure 3.4). This could provide enough dFdC for intracellular phosphoralation.

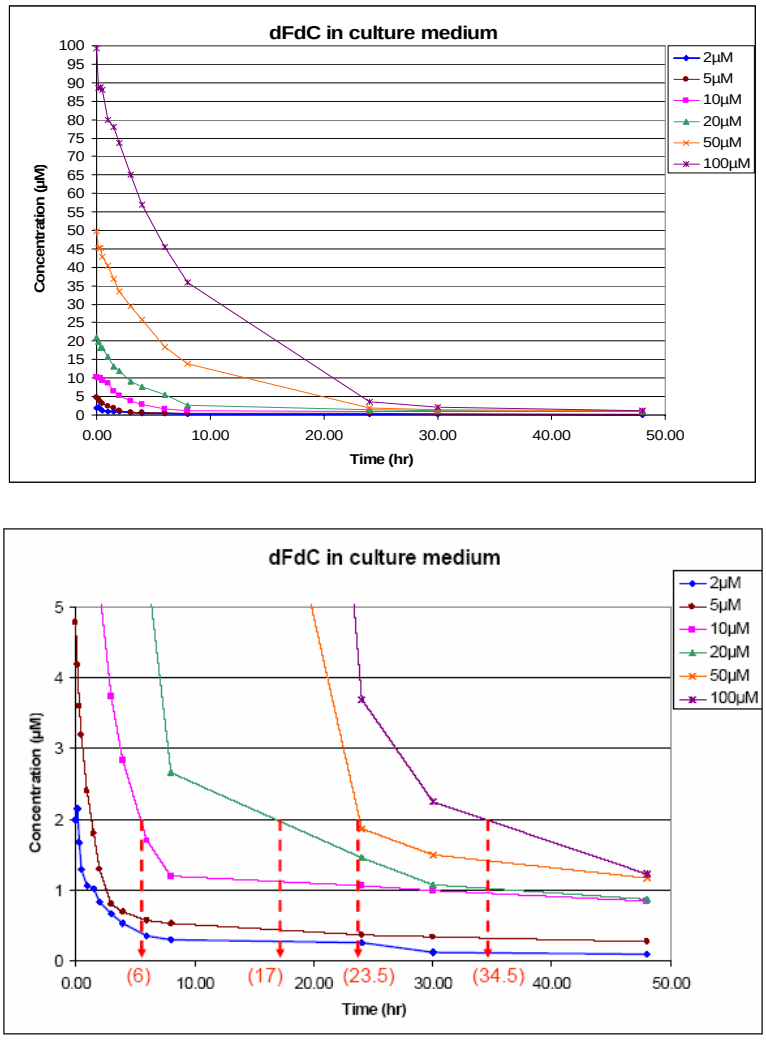


Figure 3.4 Kinetics of dFdC in culture medium for variable incubation concentrations of gemcitabine (upper: full concentration scale; lower: enlarged for concentrations below 5 µM).

3.4.5. Effect of dFdC concentration on cell viability with an increasing exposure time

In order to understand the association of dFdCTP accumulation with cell viability, the cultured cells were trypsinized and counted at indicated time points. The cells were incubated in 5% of trypan blue. Then the total cell number and viable cell number were counted under microscope. The viability was calculated by the ratio of viable cell number versus total cell number. The cell viabilities for cells incubated in various concentrations of dFdC were shown in Figure 3.5. When incubation time was ≤ 8 h, cell viability would be maintained at a relatively high level. No difference existed among different concentrations of dFdC. With the increase of incubation time, the cell viability decreased quickly, especially for those cells incubated in 10 μM or higher concentration of dFdC. However, the cell viability values were nearly the same when the concentrations of dFdC were in the range of 10 to 100 μM. This trend was in accordance with the results of dFdCTP accumulation. It was found that the cell's survival was mainly dependent on the exposure durations of dFdC rather than its incubation concentrations as long as the concentrations of dFdC were above 2 μM during incubation. In addition, a high initial concentration of dFdC (≥ 10 μM) resulted in a significant change on cell viability after 48 h incubation.

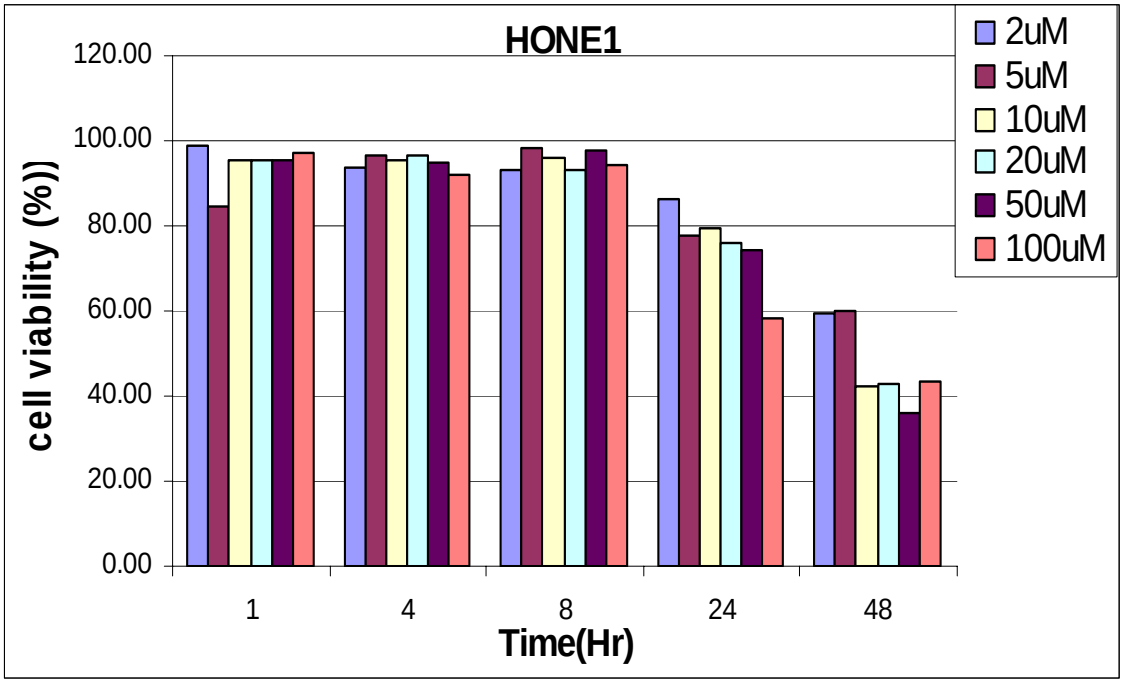


Figure 3.5 The influence of incubation time and gemcitabine concentration on the viability changes of HONE1

3.4.6. Combination of gemcitabine with PXD101

As a novel HDAC inhibitor, PXD101 showed a good anticancer activity with a tolerable toxicity. It has been used as a single agent in several tumors in the clinical setting. At the same time, various combinations with other current anticancer agents are being explored and some of these combinations have been confirmed as effective combination with synergistic effects.

In this study, we investigated the efficacy of gemcitabine and PXD101 combination using different cell models including CNE1, H292 and H1299. CNE1 is one of NPC cell line and H292 and H1299 are NSCLC cell lines. Since gemcitabine was also used clinically for NPC and NSCLC, therefore the results would potentially be relevant for planning future clinical trials.

3.4.6.1. CNE1 cell model

Gemcitabine was shown to be active to CNE1 with IC_{50} of 2.61 μM after 72 h incubation. This concentration is within the clinical therapeutic range. If CNE1 cells were exposed to 2 μM of PXD101 together with different concentrations of gemcitabine simultaneously, the IC_{50} of gemcitabine was dramatically decreased to 0.48 μM (Figure 3.6). The enhancement effect was obvious with a modulate effect of 5.3 (Modulate Effect = IC_{50} of dFdC / IC_{50} of dFdC+PXD101 = 5.3).

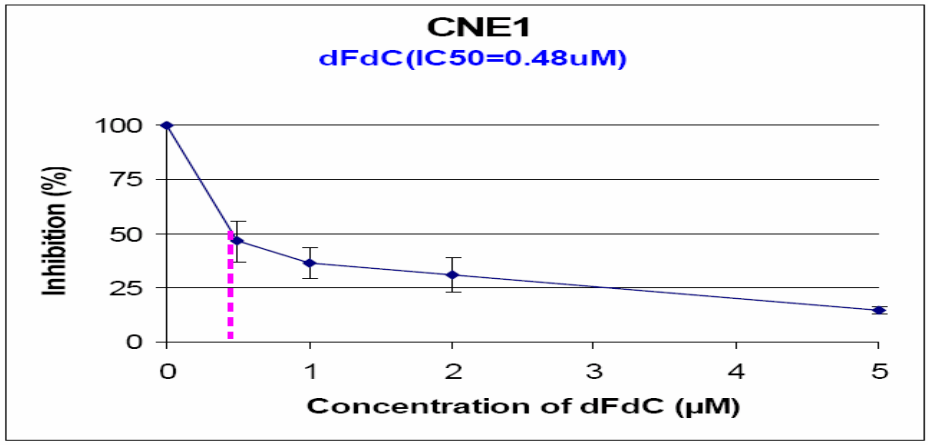


Figure 3.6 IC_{50} of gemcitabine to CNE1 with PXD101 (2 μM) after 72 h

3.4.6.2. H292 cell model

H292 is one of non-small cell lung cancer cell lines and has been frequently used as a lung cancer model. In order to evaluate the synergistic effect of gemcitabine and PXD101 on lung cancer, we measured the IC_{50} values of gemcitabine in single or combination with PXD101 (2.5 μM). After 72 h incubation, the IC_{50} values were 3.5 μM and 0.43 μM for gemcitabine alone and gemcitabine and PXD101 combination, respectively. Thus, the modulate effect can be calculated as follows:

Modulate Effect = IC_{50} of dFdC / IC_{50} of dFdC+PXD101 = 8.1

According to clinical pharmacokinetic parameters of PXD101 in human plasma, the peak concentration could reach up to 200 μM. The concentrations of PXD101 in patients' plasma were kept at a concentration higher than 2.5 μM for the first 2 hours when the dosage administered was 1200 mg/m^2 by 30 min i.v. infusion according to our phase I trial. In addition, PXD101 at this dosage (1200 mg/m^2) was also well tolerable by cancer patients in our current Phase I study on hepatocarcinoma. Thus, 2.5 μM of PXD101 was expected to have very mild toxicity but efficacious when used as single agent. We used this concentration of PXD101 (2.5 μM) to test the synergistic effect .The results showed that there was a strong synergistic effect between gemcitabine and PXD101 (Figure 3.7).

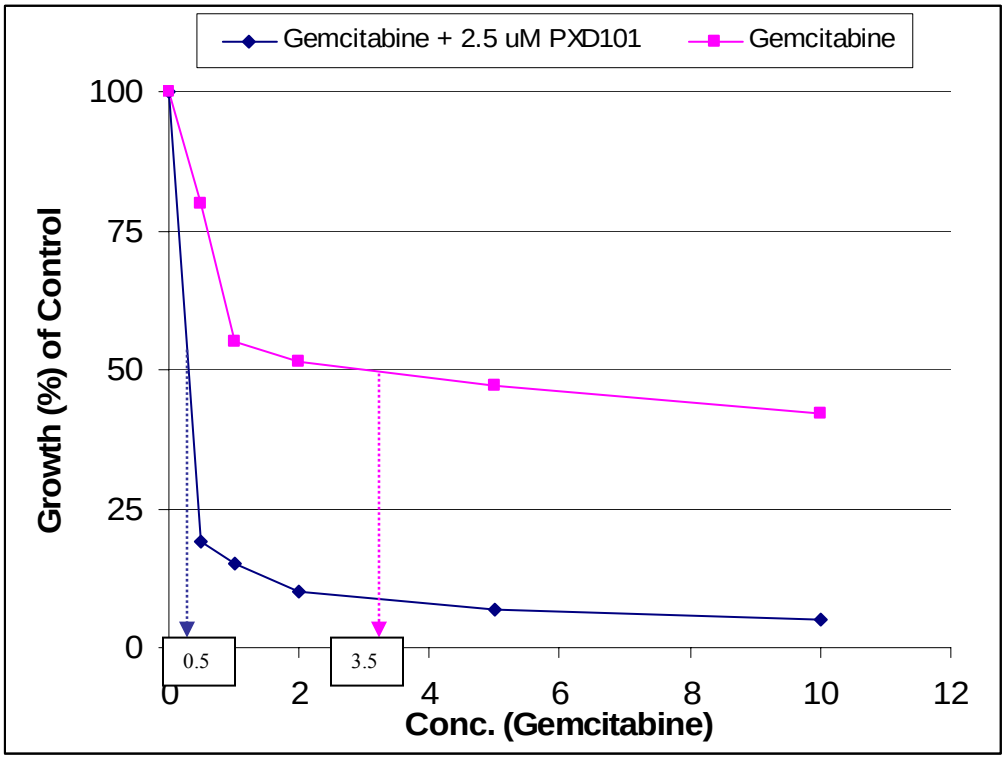


Figure 3.7 IC_{50} of gemcitabine alone to H292 and IC_{50} of gemcitabine combined with PXD101 (2.5 µM)

This synergistic effect was further confirmed with cell morphological characters and cell density (Figure 3.8). The cells in A are H292 without any treatment after 72 h incubation. The cells in B are H292 treated with 5 µM of gemcitabine alone after 72 h incubation. The cell proliferation was inhibited by gemcitabine at 5 µM but there was no obvious

change on cell morphology. The cell in C is H292 treated with 2.5 μM of PXD101 after 72 h incubation. The cell proliferation was also inhibited like that in B. However, a significant difference was shown in D using the combination. The cell density was much less compared to those treated with either single agent. This indicated that the cell growth was not only inhibited but the cells were killed by this combination of gemcitabine and PXD101.

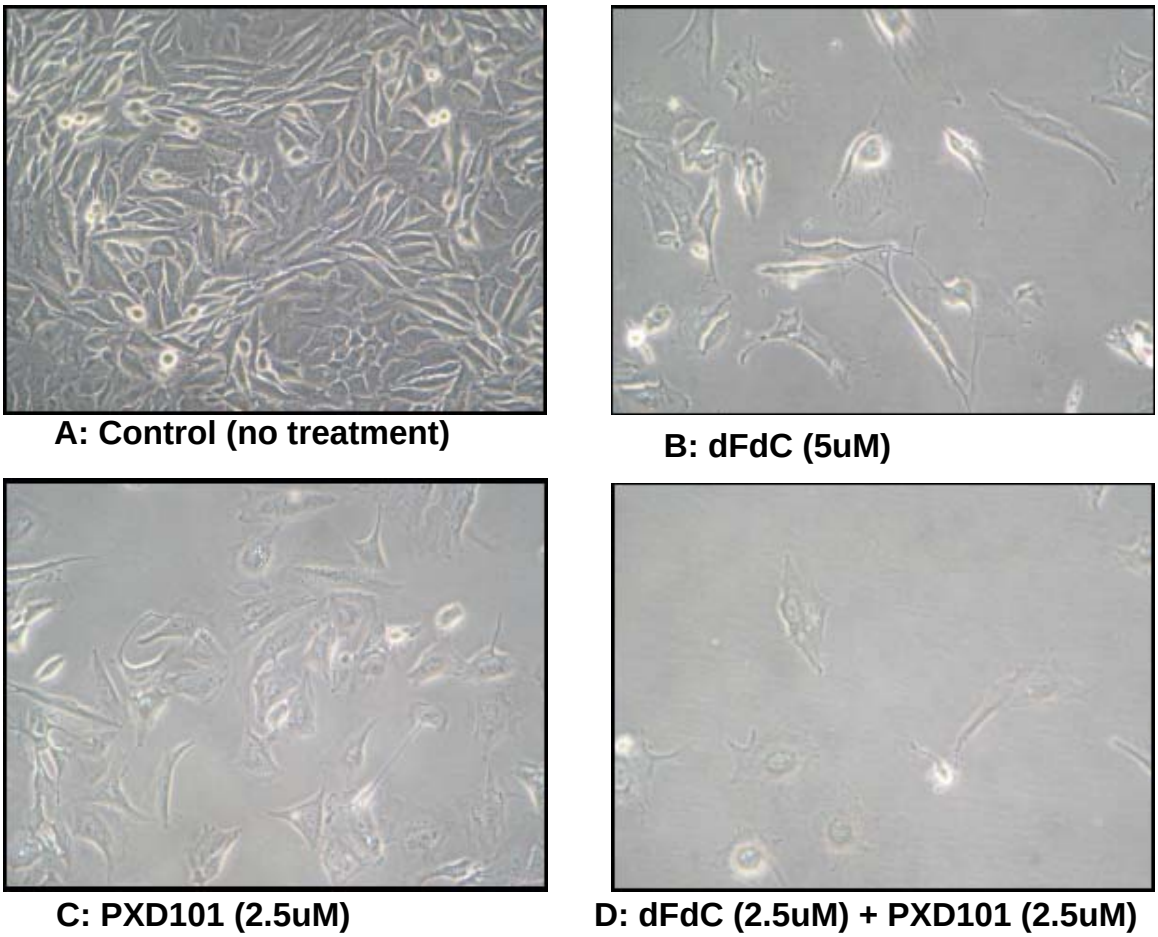


Figure 3.8 Microscope observation on H292 treated with indicated concentrations of gemcitabine, PXD101, or both after 72 h incubation

As discussed in previous parts, the synergism was detected between gemcitabine and PXD101. In order to understand the mechanism, a DNA content experiment was designed to observe cell cycle change as well as percentage of the apoptosis cell population. The results were shown in Figure 3.9. M1, M2, M3 and M4 represent sub G1, G1, S and G2/M, respectively. For the control cell (without any treatment), there was no cell in sub G1. When the cell was treated with gemcitabine (5 μM) alone, the G1 was significantly decreased and the S phase increased. This indicated that the DNA synthesis in S phase was blocked when the cell was treated with gemcitabine. On the other hand, high percentage of sub-G1 resulted in cell apoptosis. When PXD101 alone was used, the obvious change was that a relative high of G2/M was observed. This implied that the cell was mainly on G2/M arrest. Lastly, the combination of gemcitabine and PXD101 resulted in significant apoptosis and S phase arrest.

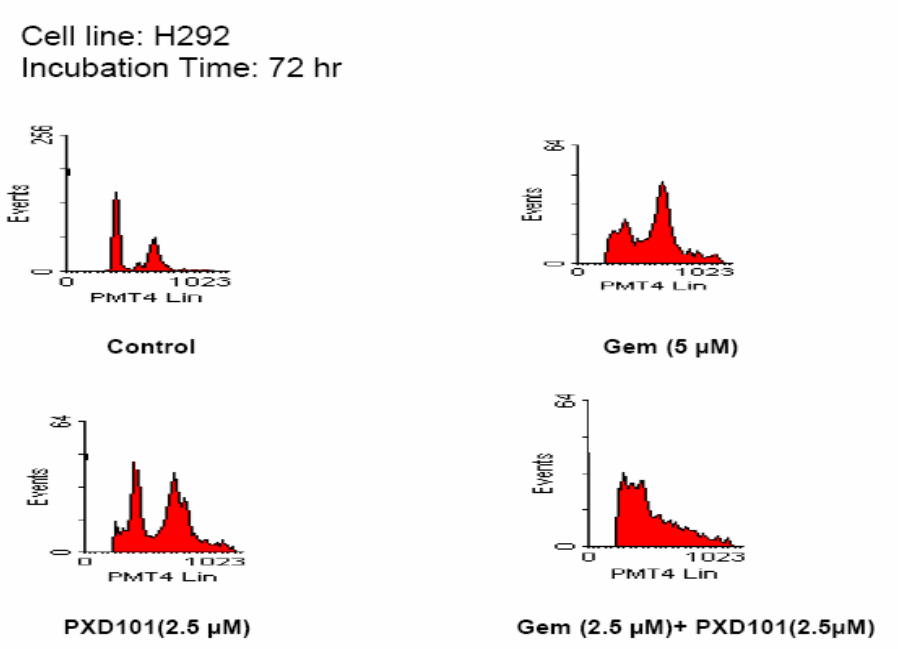


Figure 3.9 Flowcytometry of H292 treated with indicated drugs

3.4.6.3. H1299 cell model (Drug resistant NSCLC)

*p*53 being one of tumor-suppressor genes, responsible for directing repair of damaged DNA or committing a cell to apoptosis, is mutated or otherwise altered in more than 50% of lung cancers, including 40% to 70% of non-small cell lung cancers. A majority of clinical studies suggested that lung cancers with *p*53 alterations carry a worse prognosis and lack of p53 is an important factor attributed to drug resistance. H1299 is a NSCLC cell line without p53 expression. Any novel chemotherapy would have high clinical implication if its potency could be demonstrated in p53 mutant or null cell lines like H1299. Herein, we tested the combination of gemcitabine and PXD101 on the cytotoxicity on H1299. In order to measure the combination index (CI), the IC_{50} values of gemcitabine and PXD101 were measured separately. The IC_{50} of PXD101 is 3.2 μM for H1299 (Figure 3.10).

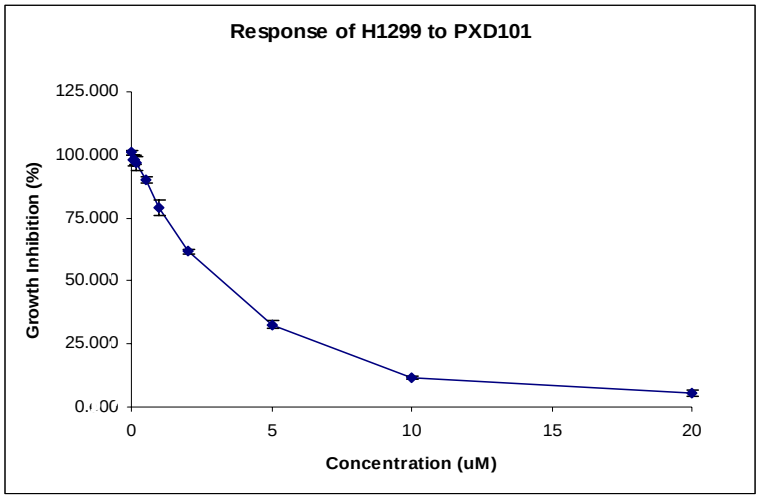


Figure 3.10 IC_{50} of PXD101 on H1299 after 72 h incubation

The IC_{50} value of gemcitabine on H1299 was 88.99 μM (Figure 3.11). In combination study, PXD101 was fixed as half of its IC_{50} (1.6 μM). A serial of gemcitabine concentration was applied to test the IC_{50} of gemcitabine combined with 1.6 μM of PXD101. In this case, the IC_{50} value was dramatically decreased 6898 fold from 89 μM to 0.013 μM. This experiment demonstrated the combination of gemcitabine and PXD101 showed a very strong synergistic effect on this drug resistant cell.

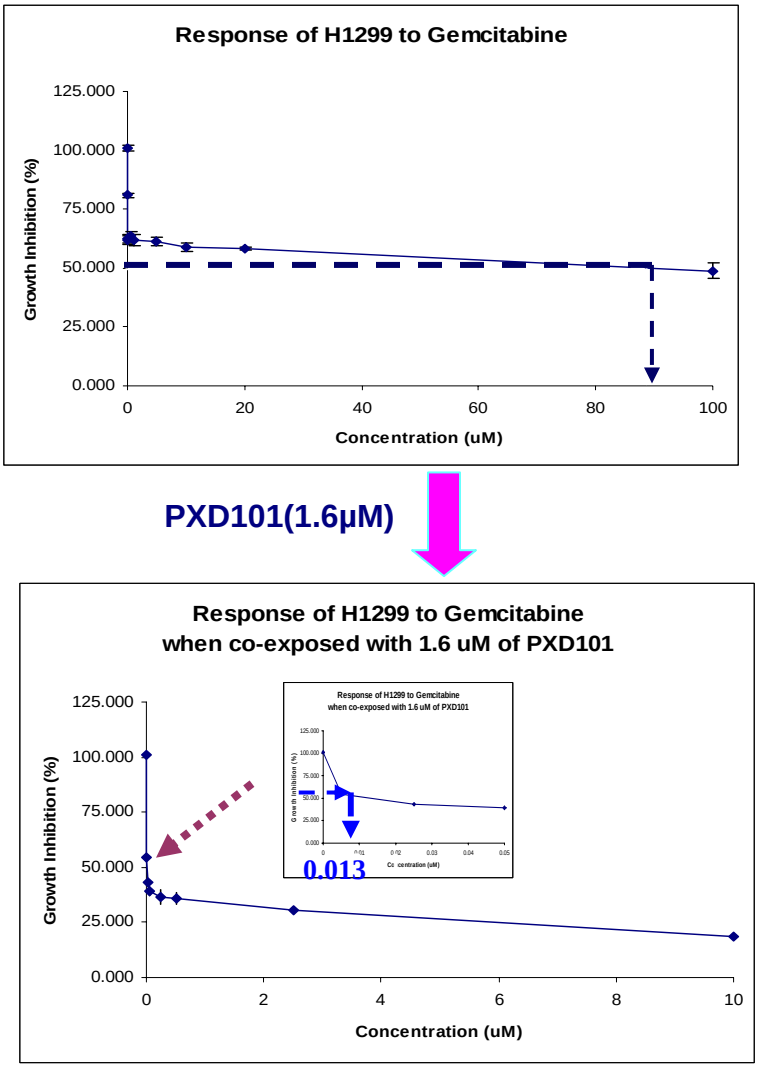


Figure 3.11 IC_{50} of gemcitabine combined with 1.6 μM of PXD101 on H1299 after 72 h incubation

The modulate effect and CI were calculated and listed as follows (Table 3.3.). Obviously, this enhancement effect was much more significant than p53 wild-type cell (H292). CI was equal to 0.5001. This also suggested a very strong synergistic effect of gemcitabine and PXD101 on H1299.

Table 3.3 IC_{50} of gemcitabine, PXD101 or in combination on H1299 after 72 h

Drug	IC50 (μM)	Modulate Effect (%)	Combination Index
PXD101	3.2	NA	NA
Gemcitabine	88.99	NA	NA
Gmcitabine+ PXD101	0.0139	6898	0.5001

This strong synergistic effect was also confirmed through microscope observation (Figure 3.12). The cell in A is H1299 without any treatment after 72 h incubation. The cell in B is H1299 treated with 5 μM of gemcitabine alone after 72 h incubation. The cell density was less compared to control but a quite number of cancer cells still survived after being exposed to 5 μM of gemcitabine for 72 h. The cell in C is H1299 treated with 2.5 μM of PXD101 after 72 h incubation and the cell density was in the same magnitude as in B. The cell morphology looked undamaged after being incubated in 2.5 μM of PXD101 for 72 h. However, the situation was changed significantly in drug combination D. The cell morphology was changed and the cell density was much less compared to those treated with either single agent. The results indicated that this combination of gemcitabine and

PXD101 was effective for tumor cells regardless to the status of p53. In terms of overcoming drug resistance, this finding has an important clinical implication to identifying the combination of gemcitabine and PXD101 as a promising combination for further evaluation NSCLC.

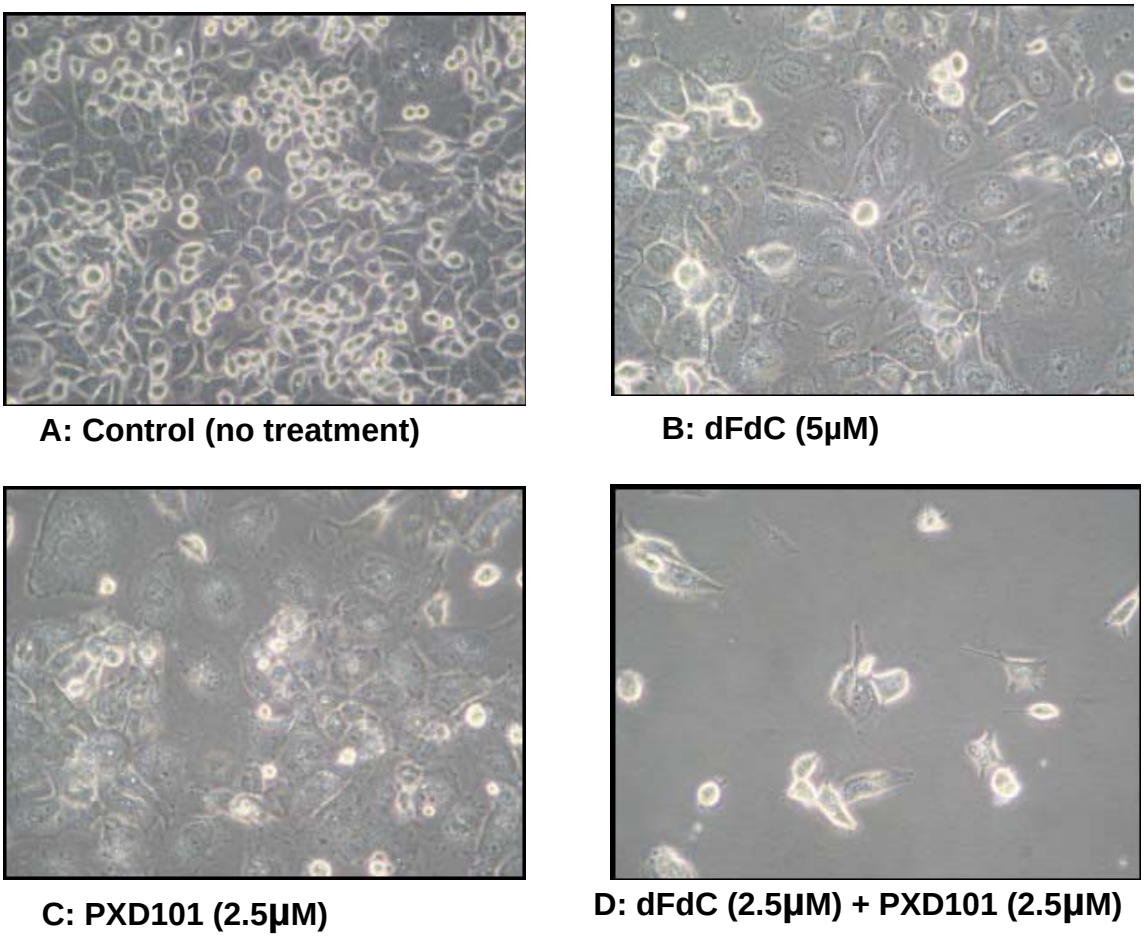


Figure 3.12 Microscope observations on H1299 treated with indicated concentrations of gemcitabine, PXD101, or both after 72 h incubation

The cell cycle changes of H1299 were shown as follows (Figure 3.13). For the control cell (without any treatment), there was no cell in sub G1 phase. When the cell was treated with gemcitabine (5 μM) alone, some portion of G1 was shifted to S phase and G2/M was obviously decreased. This indicated that the cells were arrest in S Phase and DNA synthesis was blocked when the cell was treated with gemcitabine. At the same time, a significant increase in sub-G1 resulted in cell apoptosis. When PXD101 alone was used, the cells were arrested at G2/M phase. But the the percentage in sub-G1 was relatively lower compared to gemcitabine. However, the combination of gemcitabine and PXD101 resulted in a significant increase on both cell apoptosis and S phase arrest.

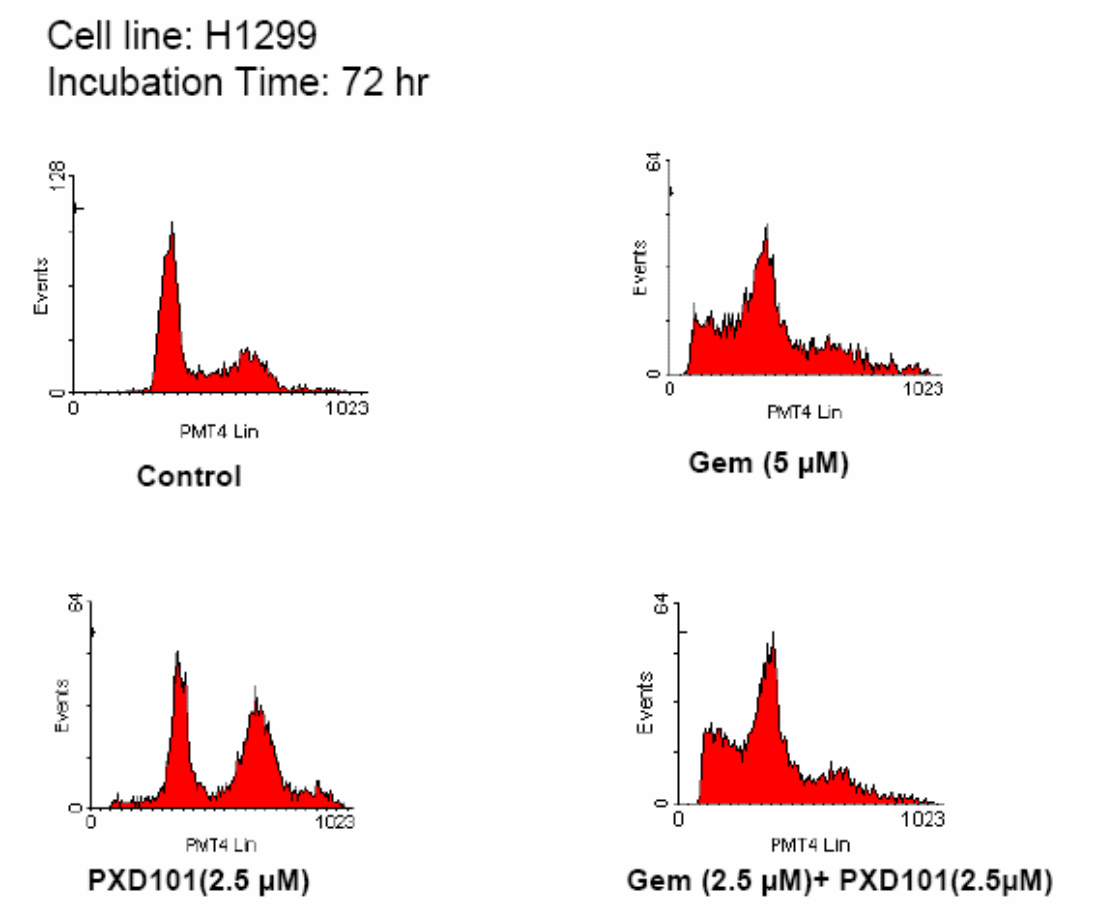


Figure 3.13 Flowcytometry of H1299 treated with indicated drugs

Furthermore, the percentage of apoptosis cells was statistically significant higher in combination of gemcitabine and PXD101 compared to gemcitabine alone (Figure 3.14).

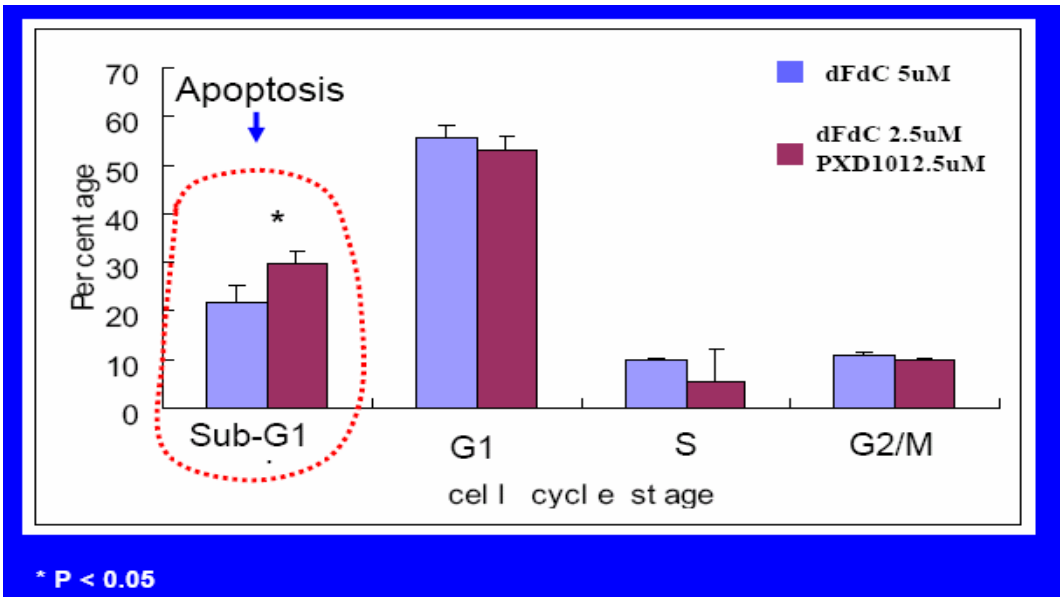


Figure 3.14 Cell cycle changes of H1299 treated with gemcitabine alone or in combination of gemcitabine and PXD101

3.5. Conclusions

Our *in vitro* experimental results suggested that the duration of incubation was the determinant for intracellular dFdCTP accumulation when the concentration of dFdC of incubation medium was ≥ 2 μM. A plateau concentration of intracellular dFdCTP would be achieved after 8 h incubation when initial incubation concentration of dFdC was ≥ 10 μM. In accordance with this observation, the cell viability was reduced by the same magnitude with 48 h incubation when the exposure concentration of dFdC was in the concentration range of 10-100 μM. The viability was due to the combined effect of dFdCTP accumulation level and retention duration (incubation time). With regards to combining PXD101 with gemcitabine, the potent synergistic effect was verified using different cell models (NPC and NSCLC) especially with the p53-null resistant lung cancer cell line (H1299). This combination would be used *in vivo* to test its effect on solid tumors. This novel combination might have high potential to become a very promising chemotherapy for cancer treatment.

CHAPTER FOUR

Pharmacokinetics & Pharmacodynamics of Fixed Dose Rate Infusion of Gemcitabine in Combination with Carboplatin in NSCLC

4.1. Introduction

Developments in the clinical administration of gemcitabine are focusing on efforts to extend the duration of exposure to the drug to counteract its rapid metabolism in the circulation. [134] The infusion of gemcitabine at 10 mg/m^2 per min has demonstrated increased tumor efficacy in a randomized phase II study of advanced pancreatic cancer.[135] A higher median intracellular gemcitabine triphosphate concentration is achieved in the fixed dose rate schedule. However, infusion of gemcitabine at a fixed dose rate generally results in higher toxicity like increased myelosuppression and hepatic dysfunction than a standard 30-min infusion.[86] In addition, more phase II studies of infusion gemcitabine at 10 mg/m^2 per min have suggested tolerability and a favorable response rate in combination with platinum compounds in patients with advanced NSCLC have been reported.[136, 137]

The pharmacologic advantage of administering gemcitabine at a fixed dose rate of 10 mg/m^2/min and the proven efficacy of combination with carboplatin in NSCLC provided the basis for our study.

We conducted a phase I-II study of fixed dose rate (10 mg/m^2 per min) gemcitabine in combination with fixed AUC-dose carboplatin in patients with advanced-stage NSCLC. The study was to establish the maximal tolerated dose (MTD) of gemcitabine, to evaluate the toxicity and to determine the pharmacokinetics of plasma gemcitabine in this regimen.

4.2. Objectives

In this chapter, a phase I clinical trial was conducted to titrate the dosing of infusion gemcitabine administered at 10 mg/m^2 per min and evaluate the response rates and toxicities of this fixed rate infusion of gemcitabine in combination with carboplatin in advanced NSCLC. In addition, pharmacokinetic parameters of gemcitabine at different doses would be calculated and analyzed. And finally, the toxicities would be associated with doses and pharmacokinetic parameters.

4.3. Methodology

4.3.1. Patient selection

The eligibility criteria for study entry included histologically or cytologically confirmed stage IIIB or stage IV NSCLC. Patients were required to have measurable or evaluable disease and to have received one or no prior chemotherapy for advanced disease. Previous neoadjuvant or adjuvant chemotherapy, or chemotherapy given concurrently with radiotherapy for non-metastatic disease was allowed if the last dose had been administered 6 months or more before study entry. Patients who had received prior platinum and/or gemcitabine were excluded. Patients with symptomatic central nervous system metastases requiring steroid were excluded. Prior radiotherapy was allowed as long as the indicator lesion(s) was not within the previous radiation field and the last dose of radiotherapy had been completed at least 3 weeks before study entry. Patients were required to have a Karnofsky performance status of ≥70%, WBC count ≥3500/μL, neutrophils ≥2000/μL, platelet count ≥100,000/μL, hemoglobin ≥9 g/dL, serum creatinine

<133μmol/L or creatinine clearance >30 ml/min, serum bilirubin not more than 1.5 times the upper limit of normal (ULN), serum transaminase levels not more than twice ULN (not more than five times ULN if liver metastases were present), life expectancy >3 months, and age ≥18 years. The study was approved by the institutional review board and all patients were required to provide written informed consent.

4.3.2. Treatment plan

Treatment consisted of carboplatin on day 1 followed by fixed dose rate of infusion gemcitabine on days 1 and 8 every 21-day cycle. 5-Hydroxytryptamine-3 receptor antagonists were routinely used as antiemetics. Prophylactic growth factors were not used routinely. Carboplatin was given at a dose to target an area under the curve (AUC) of 5 mg/ml×min over 1 h. [138, 139] The dosing of carboplatin was calculated according to Calvert formula, with AUC of 5 mg/ml×min used as the end point [*Dose (mg) = Target AUC × (GFR+25)*]. The GFR was calculated according to the Cockroft-Gault formula [*GFR (ml/min) = (140-age) × Body weight (in kg)/0.81 × serum creatinine (μmol/l)*]. For female, the correction factor is 0.85 (×calculated GFR). Gemcitabine was infused at a constant rate of 10 mg/m^2 per min. The starting dose level of gemcitabine was 600 mg/m^2 with subsequent 150 mg/m^2 increments to 750 and 900 mg/m^2. The duration of infusion is increased according to the following schema (Figure 4.1).

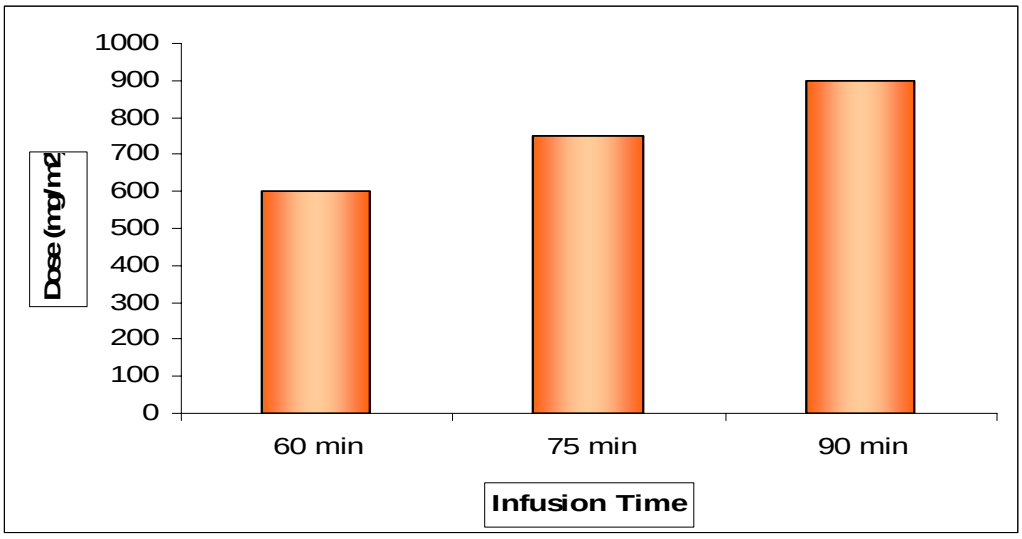


Figure 4.1 Treatment doses of gemcitabine

The duration of infusion was identical for days 1 and 8 in any individual patient. Doses were assigned at registration and no dose escalation was allowed in an individual patient. Treatment-related toxicity was evaluated after each cycle. The dose limiting toxicity (DLT) was defined based on toxicities experienced during the first cycle of chemotherapy only. Cohorts of at least three patients were treated at each dose level. Dose escalation proceeded if no patients developed DLT after the first cycle. If one of three patients experienced DLT, a further three patients were treated at that level. Dose escalation was stopped if one-third of patients at a given dose level developed DLT. The last patient at each dose level was evaluated for first cycle toxicity before a new patient was entered into the next dose level. DLT was defined as grade 4 neutropenia for 7 days or more,

grade 4 thrombocytopenia, grade 3 neutropenia with fever or grade 3 thrombocytopenia with active bleeding, failure to recover from toxicity to receive a second cycle of chemotherapy despite a delay of more than 1 week from the scheduled day and non-hematologic toxicity of grade 3 or more (except for reversible elevation of transaminases, nausea, vomiting and alopecia). MTD was defined as the dose level immediately below the level that resulted in at least one-third of patients experiencing DLT. If DLT was experienced, responding patients were allowed to continue treatment with dose reduction. Dose modifications were based on weekly blood counts and assessment of toxicity. Toxicities were graded according to the National Cancer Institute Common Toxicity Criteria, version 2. (http://ctep.cancer.gov/forms/CTCv20_4-30-992.pdf). On day 22 of each cycle, for neutropenia of grade 1 or more and/or platelets <100,000/μL, treatment was delayed for 1 week. On day 8 of each cycle, for neutropenia of grade 3 or more and thrombocytopenia of grade 2 or more, the gemcitabine dose was reduced by 25% and maintained for the next cycle, and for grade 4 neutropenia and/or grade 3/4 thrombocytopenia, gemcitabine was omitted and then decreased by 25% for the next cycle after marrow recovery and carboplatin was also reduced by 10% for the next cycle. The nadir count of the previous cycle also influenced dose adjustment for the next cycle. Gemcitabine was reduced by 25% and carboplatin by 10% for a grade 4 neutropenia with fever, or grade 4 neutropenia for more than 7 days or thrombocytopenia of grade 3 or more with bleeding or platelets <25,000/μL. Patients requiring a third dose reduction were taken off study. Patients who experienced a non-hematologic toxicity of grade 3 or more, except for nausea, vomiting, fatigue and reversible elevation of transaminases, were taken off study.

4.3.3. Patient evaluation

Before initiation of chemotherapy, all patients underwent a history and physical examination and determination of performance status. A complete blood count with differential, serum biochemistry, urinalysis, and ECG were obtained at baseline for each patient. Chest radiography, thoracic and abdominal computed tomography (CT) scans were performed as required for assessment of measurable or evaluable disease. CT scan of the brain and bone scan was performed if clinically indicated. Patients were assessed weekly throughout treatment by complete blood count, serum biochemistry and recording of toxicities. Tumor response evaluation was performed after every two cycles according to the RECIST criteria.[140] Patients with at least stable disease or better continued with treatment to a maximum of six cycles. If DLT was experienced, responding patients continued treatment with a reduced dose. Patients with progressive disease were withdrawn from the study.

4.4. Results

4.4.1. Patient Characteristics

A total of 15 patients were enrolled into the study. Their median age was 57 years (range 35 to 81 years) and 11 were male. Of the 15 patients, 3 and 12 had stage IIIB and IV disease, respectively, and 13 had a Karnofsky performance status (KPS) of at least 90% and 2 a KPS of 70–80%. (Table 4.1) Adenocarcinoma was the predominant histologic subtype (n=10). All patients were chemonaive. All patients were assessed for toxicity. A total of 51 cycles were administered. The median number of cycles per patient administered was three (range one to six). The relative dose intensities of gemcitabine at levels 1, 2 and 2A were 78%, 82% and 85%, respectively.

Table 4.1 Karnofsky performance scale and explanation

Scale	Summary
100: Normal, no complaints; no evidence of disease	Able to carry on normal activity; no special care needed
90: Able to carry on normal activity; minor signs or symptoms of disease	
80: Normal activity with effort; some signs or symptoms of disease	
70: Cares for self; unable to carry on normal activity or to do active work	Unable to walk; able to live at home and care for most personal needs; a varying amount of assistance is needed
60: Reqires occasional assistance but is able to care for most of his needs	
50: Requires considerable assistance and frequent care	
40: Disabled; requires special care and assistance	Unable to care for self; requires equivalent of institutional or hospital care; disease may be progressing rapidly
30: Severely disabled; hospitalization is indicated although death not imminent	
20: Very sick; hospitalization necessary; active supportive treatment is necessary	
10: Moribund; fatal processes progressing rapidly	
0: Dead	

4.4.2. Toxicity

At dose level 1, with infusion of gemcitabine over 60 min, no DLT were observed in three patients. The MTD was exceeded at dose level 2 (90 min). The DLT observed was grade 3 liver failure in one patient and grade 3 thrombocytopenia with hematemesis in the second patient. A study amendment was made to include an intermediate dose level (level 2A) of gemcitabine 750 mg/m^2 over 75 min. At this level, one out of six patients experienced DLT, which was grade 3 neutropenia with failure to recover in time to receive the second cycle of chemotherapy. The frequency of cycle 1 grade 3/4 hematologic toxicity was low (Table 4.2). However, when all cycles of chemotherapy at a given level were analyzed, a different hematologic profile was observed. At level 1 (60 min), 50% of cycles were complicated by a nadir neutrophil count of grade 3 or 4. At levels 2 and 2A, 20% and 43% of cycles, respectively, were complicated by neutropenia grade 3 or 4. There were no episodes of febrile neutropenia. Cumulative thrombocytopenia, in contrast, was not as frequent (Table 4.3). Non-hematologic side effects including fatigue, nausea, vomiting, constipation and fever were mild and not dose-dependent (Table 4.4). One patient developed grade 3 vomiting. Aspartate transaminase was mildly elevated in six patients but was not clinically significant and was reversible. Transient fever and rash were uncommon. One patient developed a non-hematologic DLT, manifested by grade 3 clinical liver failure (asterixis) at level 2. This patient had a previous history of heavy alcohol intake ceasing 5 months prior to study entry. At the time of chemotherapy, he had a grade 2 hypoalbuminemia, but liver function was otherwise normal. On day 15 of cycle 1 of chemotherapy, he developed confusion and asterixis. He had a grade 3 hypoalbuminemia, grade 1 transaminases and

grade 2 hyperbilirubinemia. The patient recovered with supportive care but did not receive further therapy and was subsequently withdrawn from the study.

Table 4.2 Cycle 1 hematologic toxicities by dose level (n = 15)

Dose Level	No. of patients	Neutropenia grade			Thrombocytopenia grade		
		1/2	3	4	1/2	3	4
1 (60 min)	3	1	0	0	1	0	0
2 (90 min)	6	1	0	1[a]	1	1[a]	0
2A(75 min)	6	3	2	0	1	0	0

[a]Same patient

Table 4.3 Cumulative grade 3/4 hematologic toxicities by dose level
Numbers in parentheses are the percentage of courses having the indicated toxicity (n = 15)

Dose Level	No. of patients	Cycle	Neutropenia grade		Thrombocytopenia grade	
			3	4	3	4
1 (60 min)	3	8	1 (12)	3 (38)	3 (38)	0 (0)
2 (90 min)	6	20	3 (15)	1 (5)	2 (10)[a]	0 (0)
2A(75 min)	6	23	10 (43)	0 (0)	0 (0)	0 (0)

Table 4.4 Non-hematologic toxicity (n = 15)

Side Effect	NCI CTC grade	
	1/2	3/4
Nausea	6	1
Vomiting	5	0
Constipation	4	0
Anorexia	1	0
Mucositis	1	0
Hyperbilirubinemia	2	0
Elevated alanine transaminase	4	0
Elevated aspartate transaminase	6	0
Clinical liver failure	0	1[a]
Peripheral neuropathy	3	0
Weight loss	3	0
Rash	2	0
Fatigue	10	0
Fever	2	0
Alopecia	4	0

[a]DLT

4.4.3. Response

Of the 15 patients, 10 were evaluable for response. A partial response was documented in two patients (20%) and stable disease was seen in five patients. One partial response was seen at level 1 and one at level 2A.

4.4.4. Pharmacokinetic data

The mean pharmacokinetic parameters for gemcitabine based on serial plasma concentration-time data from six patients at dose level 2A and one patient at level 2 are shown in Table 4.5. Plots of time against mean gemcitabine and dFdU concentrations at 75 min and 90 min infusions of gemcitabine are shown in Figure 4.2. Plasma concentrations of gemcitabine were above 10 μM between 20 and 90 min in all patients.

Table 4.5 Pharmacokinetic parameters of plasma dFdC.
Values are means ± SD (only one full data set available at 900 mg/m^2)

Dose (mg/m^2)	$AUC_{0\text{-}inf}$ (μg/mL·min)	Cl (L/min)	Cmax (ng/mL)	T1/2 (min)	Vss (L)
750 (n = 6)	315.4 ± 98.8	4.11 ± 1.20	4.79 ± 1.26	15.0 ± 3.5	76.0 ± 38.3
900 (n = 1)	730.9	2.24	9.16	12.4	34.3

Note: AUC: area under the concentration-time curve; Cl: clearance; Cmax: maxium concentration; T1/2: half-life; Vss: volume steady state.
Blood sampling scheme was described on page 34, Chapter II.

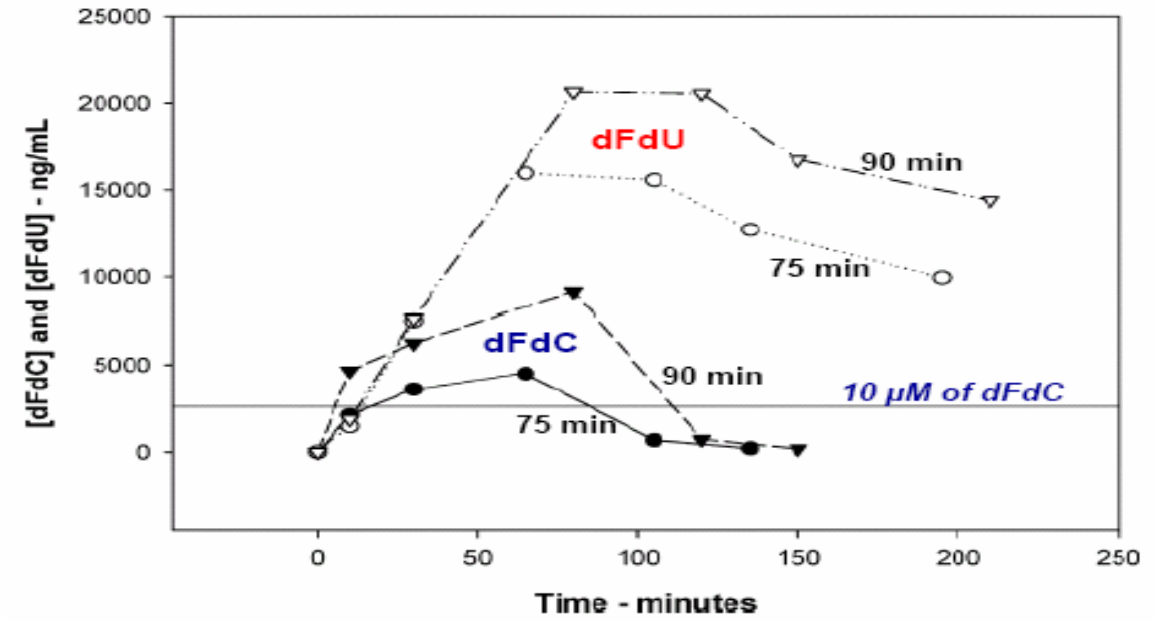


Figure 4.2 Plots of time vs mean gemcitabine and dFdU concentrations with 75-min and 90-min infusions of gemcitabine (10 mg/m^2/min). The horizontal line represents the gemcitabine plasma concentration of 10 µmol/L (2630 ng/ml). The plots for the 75-min infusion represent the mean concentration values (n = 6) at each time point. The data for 90-min is from one patient (●gemcitabine, 75-min infusion; ○dFdU, 75-min infusion; ▼gemcitabine, 90-min infusion; Δ dFdU, 90-min infusion).

4.5. Discussion

In this study, we determined that the MTD for fixed dose rate infusion of gemcitabine at 10 mg/m^2 per min was 900 mg/m^2 when given in combination with carboplatin at a targeted AUC of 5 mg/mL×min. The dose-limiting toxicities encountered included thrombocytopenia, neutropenia and liver dysfunction.

The 750 mg/m^2 dose of gemcitabine was found to be tolerable based on the occurrence of DLT in one out of six patients, in whom there was failure to recover from neutropenia for retreatment in the first cycle. As observed in previous study, [86] there was increased and cumulative hematopoietic toxicity with prolonged infusion. Hepatotoxicity had also been reported to be more frequent with longer infusions of gemcitabine [141] and was reflected by the frequency of elevated transaminases in our study patients, but this resulted in DLT in only one patient.

For any chemotherapy combination to be feasible, recommended dosing should allow maintenance of relative dose intensity with repeated dosing without dose delay due to cumulative toxicity. In our study, there was an increase in hematopoietic toxicity with repeated dosing, but the relative dose intensity was still an acceptable 85% and three out of six patients at the recommended phase II dose completed six cycles of treatment. Although the recommended phase II dose of gemcitabine in our study was 750 mg/m^2, it appeared that by infusing gemcitabine at 10 mg/m^2 per min, hematological toxicities especially neutropenia, were similar to regimens using 30-min administration of gemcitabine at 1000 mg/m^2 higher doses.

The pharmacokinetics showed that gemcitabine plasma concentrations relevant for optimizing intracellular phosphorylated gemcitabine were achieved in most patients after

10 min of infusion. Clearance of gemcitabine was rapid, with a mean terminal half-life of 15 min. The patient with DLT from neutropenia at 75 min infusion did not have the highest AUC of plasma gemcitabine. This is expected of antimetabolites like gemcitabine, where intracellular active metabolites reflect better clinically observed pharmacodynamics. With constant rate infusion, it might be possible to increase the duration of exposure to pharmacologically relevant concentrations of active metabolites, resulting in better cytotoxicity.

In our Phase I study, there were high frequencies of grade 3/4 neutropenia. Based on data analysis, the frequency of grade 3/4 neutropenia did not increase proportionally with dosage of gemcitabine (Table 4.6). On the contrary, the frequency of grade ¾ neutropenia showed an inverse correlation (Fig. 4.3). The possible reasons would be explored from phenotypic and genotypic factors in our phase II study.

Table 4.6 Reverse effect of dosage of gemcitabine and frequencies of severe neutropenia (grade 3/4). (Numbers in parentheses are the percentage of course having the indicated toxicity).

Dose Level (10 mg/m2/min)	Dose (mg)	No. of Patients	Cumulative Cycle	Neutropenia (Grade ¾)
1 (60 min)	600	3	8	5 (50)
2A(75 min)	750	6	23	10 (43)
2 (90 min)	900	6	20	4 (20)

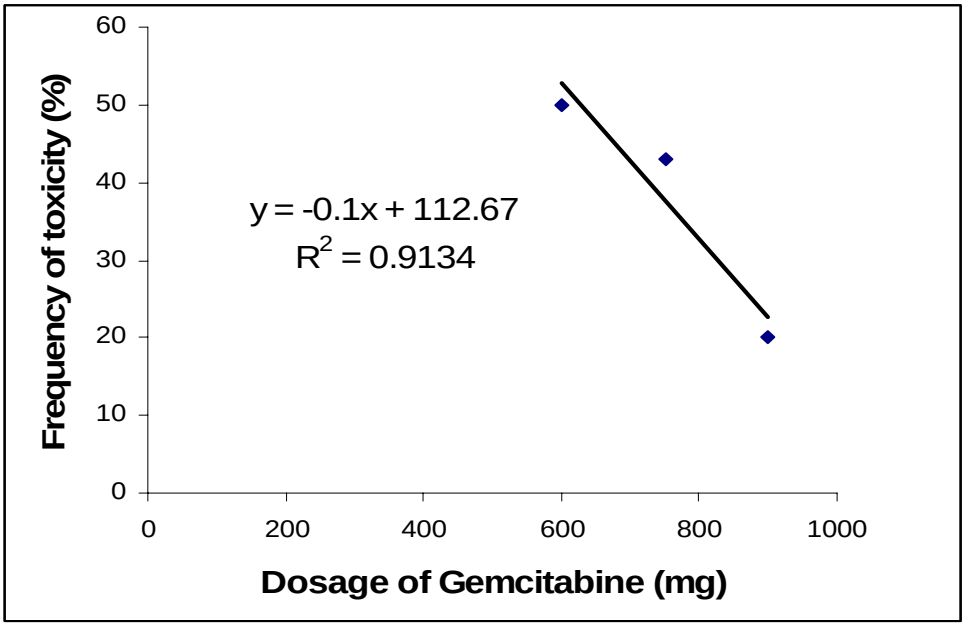


Figure 4.3 Correlation of toxicity rate (%) and dosage of gemcitabine

4.6. Conclusions

A relative new regimen of gemcitabine administered as a 75-min infusion at a constant rate of 10 mg/m^2 per min in combination with carboplatin was found to be tolerable and active in Asian NSCLC patients in our Phase I trial. Pharmacokinetic studies demonstrated that the target plasma gemcitabine concentration above 10 μM was achieved from 20-90 min during the 75 min infusion of gemcitabine at the stated constant rate regimen.

CHAPTER FIVE

Pharmacokinetics & Pharmacodynamics of Gemcitabine at Two Infusion Rates in Combination with Carboplatin in NSCLC

5.1. Introduction

Preclinical studies showed that intracellular dFdCTP accumulation is dependent on the total exposure time and rate of administration of gemcitabine in human cell lines and xenografts [142, 143]Hence, the efficacy and toxicity of gemcitabine are influenced by the dose and dosing schedule [144] The clinical benefits from fixed dose rate infusion of gemcitabine at 10 mg/m^2/min have also been reported in a randomized phase II study for patients with pancreatic adenocarcinoma, where higher intracellular dFdCTP accumulation in peripheral blood mononuclear cells was achieved compared to the standard 30-min infusion .[16] Since there were evidences to support prolonged infusion schedule of gemcitabine resulting in favourable activity of gemcitabine and carboplatin combination in advanced NSCLC, [145] we conducted a randomized phase II study of gemcitabine in combination with carboplatin in patients with advanced NSCLC utilizing two different infusion rates of gemcitabine. The dose for fixed dose rate infusion of gemcitabine was based on our previous phase I study.[15]

5.2. Objectives of the study

Our study aims were to analyze plasma and cellular pharmacokinetics of gemcitabine and its metabolites; to evaluate the response rate, overall survival and toxicity of carboplatin and gemcitabine given at a fixed rate infusion (arm A) and standard 30-minute infusion (arm B) and to correlate the pharmacokinetic parameters of gemcitabine and its metabolites with toxicity of gemcitabine and tumor shrinkage.

5.3. Methodology

5.3. 1. Patient selection

The Eligibility criteria included histologically or cytologically confirmed NSCLC with measurable disease, stage IIIB unsuitable for radical radiotherapy or stage IV disease. Patients were required to have a Karnofsky performance status (KPS) of ≥ 70%, age ≥ 18 years, life expectancy > 3 months, hemoglobin ≥ 9g/dL, white blood cell count ≥ 3500/μL, neutrophils ≥ 2000/μL, platelet count ≥ 100 000/μL, serum creatinine < 133 μmol/L or creatinine clearance > 30 mL/min (based on the Cockcroft formula), serum bilirubin ≤1.5 times the upper limit of normal (ULN), and serum transaminase levels ≤ two times ULN or ≤ five times ULN if hepatic metastases were present. The study was approved by the institutional review board of each participating centre and all patients gave written informed consent. Previous chemotherapy for advanced disease was not allowed. Prior neoadjuvant or adjuvant chemotherapy or chemotherapy given with radiotherapy for non-metastatic disease was allowed if the last dose was administered ≥6 months before study entry. Patients were excluded if they had received prior gemcitabine therapy or had symptomatic central nervous system metastases requiring steroids. Prior radiotherapy was allowed provided the indicator site(s) had not been irradiated and the last dose of radiation therapy had been completed ≥3 weeks before study entry.

5.3.2. Treatment Plan

Patients were randomly assigned to the following two treatment arms: gemcitabine 750 mg/m^2 over 75 min at a constant infusion rate (arm A) or gemcitabine 1000 mg/ m^2 over 30 min (arm B) on days 1 and 8 every 3 weeks. An infusion pump was used to ensure

exact infusion time. In both arms, carboplatin targeting AUC of 5 mg/ml×min was given over 1 h on day 1 prior to the gemcitabine infusion. Stratified randomization was performed using the minimization method based on study site, KPS (90–100% versus 70–80%), and disease stage (IIIB versus IV). Dose modifications were based on weekly blood counts and toxicity. On day 22 of each cycle, for grade ≥ 1 neutropaenia and/or platelets < 100 000/ μL, treatment was delayed for 1 week. On day 8 of each cycle, for grade 3 neutropaenia and grade 2 thrombocytopaenia, the dose of gemcitabine was reduced by 25% and maintained for the next cycle, and for grade 4 neutropaenia and/or grade 3/4 thrombocytopaenia, gemcitabine was omitted and decreased by 25% for the next cycle following marrow recovery and carboplatin was also reduced by 10% the next cycle. Gemcitabine was also reduced by 25% and carboplatin by 10% for a grade 4 neutropenic fever or grade 4 neutropaenia for > 7 days or thrombocytopaenia grade ≥ 3 with bleeding or platelets <25000/μL. Patients requiring a third dose reduction, or experienced a nonhaematologic toxicity of > 3 (except for nausea, fatigue, or reversible elevation of transaminases) were taken off study.

5.3.3. Patient Evaluation

Prior to chemotherapy, patients underwent a history and physical examination, chest X-ray, chest and abdominal computed tomography (CT) scans, complete blood count (CBC), serum biochemistry, urinalysis, and ECG. Additional radiological imaging was performed if clinically indicated. A physical examination, recording of toxicities, serum biochemistry was performed prior to each cycle of therapy. Weekly CBC was obtained during each cycle.

5.3.4. Early Phase Tumor Response

Tumor response was evaluated after every two cycles. Patients with stable disease or better continued with treatment to a maximum of six cycles. Confirmed responses required repeat CT scans at least 4 weeks later. Early detection of tumor shrinkage could provide a valuable marker for oncologists to decide whether to switch patient's treatment at the early phase of chemotherapy.

We used tumor shrinkage data from first two cycles mainly because on the one hand, there was usually a delay time for tumor response and there would be few cases to observe significant dimension changes of tumors in the first cycle of chemotherapy. Hence, tumor response would not be evaluated after the first cycle. On the other hand, it would be too late to switch chemotherapy treatment for many patients if oncologists got the information on the tumor shrinkage after routine six cycles of chemotherapy. In order to fill this gap, we associated the plasma concentration ratios of metabolite/gemcitabine with early phase tumor shrinkage data to identify potential disease progression markers for gemcitabine-based chemotherapy. Tumor response was assessed according to standard RECIST criteria (Table 5.1)

Table 5.1 RECIST response criteria for evaluation of target lesions

* Complete Response (CR):	Disappearance of all target lesions
* Partial Response (PR):	At least a 30% decrease in the sum of the LD of target lesions, taking as reference the baseline sum LD
* Progressive Disease (PD):	At least a 20% increase in the sum of the LD of target lesions, taking as reference the smallest sum LD recorded since the treatment started or the appearance of one or more new lesions
* Stable Disease (SD):	Neither sufficient shrinkage to qualify for PR nor sufficient increase to qualify for PD, taking as reference the smallest sum LD since the treatment started

If the value is less than 30 %, the case was taken as non-responders including PD and SD. The others (equal or bigger than 30 %) were regarded as responders including CR and PR according to RECIST criteria. [140]

5.3.5. Pharmacokinetic Analysis

This study plan was to determine the relationships between pharmacodynamic measures (hematologic toxicity, tumour shrinkage), against the plasma pharmacokinetics of gemcitabine, and cellular pharmacokinetics of gemcitabine using peripheral mononuclear cells. The pharmacokinetic information obtained will enable correlative study with the clinical endpoints like toxicity and efficacy. We studied gemcitabine and dFdU concentrations in the plasma, as well as the concentrations of dFdCTP in the peripheral mononuclear cells. All pharmacokinetic sampling was done in the first cycle of treatment.

5.3.5.1. Plasma dFdC and dFdU concentrations

Ten millilitres of blood were drawn at 0 hours (baseline), 10 minutes, 30 minutes, 10 minutes before the end of the infusion, and 30 minutes, 1 hour, 2 hours after the end of the infusion. The blood was drawn into 10 ml tubes (green topped) containing heparin and 5 μmol tetrahydrouridine. The tubes were then centrifuged at 3300 rpm for 15 minutes and the supernatant plasma was transferred to cryo tubes for immediate storage at –80 ^{o}C. Samples were labelled with the patient's study number, dosage and sampling times and protocol number (Table 5.2). A pharmacokinetic form would accompany the plasma samples for pharmacokinetic analysis.

Table 5.2 PK form of infusional gemcitabine

Phase I-II Trial of Infusion Gemcitabine in Combination with Carboplatin in Chemo-naïve Advanced Non-Small Cell Lung Cancer Protocol Ref: CTRG L08/00

Principal Investigator: Dr Goh Boon Cher

Oncologist-In-Charge: _____

Patient No: _____

Patient's Weight:_____kg Height: _____cm BSA: _____m^2

Total drugs dosage

Date:_____Cycle _____Day_____

Carboplatin:_____Time started: _____Time ended:_____

Gemcitabine:_____Time started: _____Time ended:_____

Samples for Plasma dFdC and dFdU PK

No. Time blood sample taken				Parameters		
	Sampling Time	Protocol Time	Actual Time	Time	Pulse	B/P
1	0 min					
2	10 min					
3	30 min					
4	-10 min					
5	Post 30 min					
6	Post 1 hour					
7	Post 2 hours					

Remarks: _____

_____

_____

_____

Gemcitabine and dFdU concentraions in plasma were measured using LC-MSMS method described in chapter two, section 2.3.3.1. Briefly, to a 1.5 ml Eppendorf tube was added 50 µl of plasma samples or calibrators, 5 µl of 50 µg/ml aqueous solution of Gemcitabine-13C, 5N2 (internal standard), and 200 µl of acetonitrile. The tube was tightly capped and immediately vortex-mixed for 1 minute, and then centrifuged at 10,000×g for six minutes at 4 °C. One hundred µl of supernatant was transferred into another Eppendorf tube and dried under nitrogen and reconstituted with 50 µl of 10 mM ammonia acetate buffer solution pH 6.8. After mixing, 40 µl of the mixture was transferred to plastic insert for LC-MSMS analysis.

5.3.5.2. Intracellular dFdCTP levels

Samples of blood from first cycle were collected to assay intracellular dFdCTP. Five samples per patient, obtained 10 min, 30 min, 10 min before completion of infusion, and 30 min, 1 h, and 2 h after completion of the infusion, were assayed for dFdCTP.

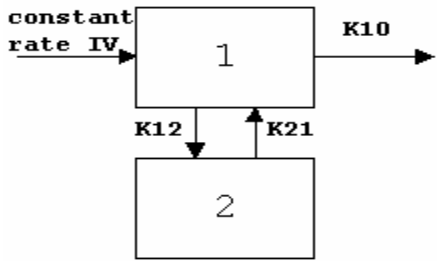
Plasma was first separated from the blood by centrifugation at 3300 rpm for 20 minutes. Mononuclear cells were then isolated by Ficoll-Hypaque density gradient centrifugation and deoxyribonucleoside triphosphates were extracted with 0.4 N $HClO_4$, and the acid-insoluble material was removed by centrifugation. The supernatant was then carefully neutralised with potassium hydroxide and kept on ice for 20 min for $KClO_4$ precipitation. The precipitate was then removed by centrifugation. An ion-exchange high performance liquid chromatography method was then used to separate and quantitate the dFdCTP as described in chapter two, section 2.3.3.2.

5.3.5.3. Pharmacokinetic calculation

Noncompartmental analysis was performed using WinNonlin 5.2. (Pharsight Corporation) to calculate the pharmacokinetic parameters, clearance (CL), half-life of the terminal disposition phase (t½), and volume of distribution at steady state (Vss) for gemcitabine and dFdCTP. Area under the concentration-time curve (AUC) was estimated using the log-linear trapezoidal option from time 0 to infinity. Derivation of the rate constant for the terminal phase, k, was done with the final three sampling time points and extrapolation of the last measured concentration to infinity. Clearance (CL), half-life (t½) and steady state volume of distribution (Vss) were computed.

In addition, compartmental analysis was also used to fit and simulate the plasma gemcitabine concentrations through changing the infusion time. According to time-concentration curve visualization and AIC criteria, two compartment modelling (model 10) was adopted to calculate modeling pharmacokinetic parameters of dFdC concentrations and good fitting was achieved using a 1/Y weighting model. After that, simulation was processed according to the calculated pharmacokinetic parameters for different infusion time to evaluate the effect of infusion time on blood concentrations of dFdC.

2 compartment IV-Infusion, macro-constants, no lag time, 1st order elimination



$$C(T)=A*(EXP(-ALPHA*T)-EXP(-ALPHA*TSTAR))+B(EXP(-BETA*T)-EXP(-BETA*TSTAR))$$

5.3.6. Statistics

Pharmacokinetic data between the two treatment arms and between male and female within same treatment schedules were compared using the Student's t-test. The t-test was analyzed as two tailed distribution and two-sample unequal variance. The sample size was calculated based on the statistical selection theory. Assuming a 90% probability of correctly choosing the best treatment, and anticipating a baseline response rate of 40%, to detect a 15% superiority of the best treatment, a sample size of 37 patients per treatment arm was needed.

Efficacy parameters were evaluated according to intent-to-treat (ITT) analysis. Survival and TTP (time to progression, were measured as the time from randomization until death, disease progression or last contact) were calculated using the Kaplan-Meier technique. Survival was calculated from the date of randomization to the date of death or last follow up. TTP was defined as the time from randomization until disease progression, or last contact. The difference between the two treatment groups was tested by log-rank test.

In the phenotypic study, the difference in plasma concentration ratio of dFdU:gemcitabine between responders and non-responders was tested by Mann Whitney test. The frequency difference of the ratio larger than 500 distributed in responders and non-responders was tested by Chi Square test.

A p value of <0.05 was considered to be statistically significant.

5.3.7. Hematological toxicity modeling

Toxicities were evaluated every cycle using the National Cancer Institute Common Toxicity Criteria, version 2.0.

Dependent variables included absolute Neutrophil Count Nadir (ANCnadir) for 1st cycle (ANCnadir_c1) and over all cycles (ANCnadir_all) and Platelet Count Nadir (PLTnadir) for 1st cycle (PLTnadir_c1) and over all cycles (PLTnadir_all). The natural logarithm transformation of the nadir neutrophil and nadir platelet values was used as a dependent variable for exploring pharmacodynamic relationships. The reason for this lies in the possible existence of polynomial relationships (powers of the predictor variables) between the predictor variables and the dependent variable [59] Separate Linear regression analysis was used to relate ANCnadir_c1, lnANCnadir_c1, ANCnadir_all, lnANCnadir_all, PLTnadir_c1, lnPLTnadir_c1, PLTnadir_all, and lnPLTnadir_all to the independent variables. Independent pharmacokinetic variables thought to affect hematological toxicity were considered for this correlation analysis, including dFdC exposure assessed by the first dose, AUC or body surface area normalized AUC, CL and duration of exposure of dFdC above 5 or 10 μM as well as intracellular dFdCTP exposure assessed by AUC and body surface area normalized AUC.

Since our patients were assigned into two cohorts, 30 min infusion group and 75 min infusion group, a within group toxicity association analysis was used to exclude the confounding factor caused by different infusion schedules. All the linear regressions were performed using SPSS Version 13.0 through stepwise approach.

Chi-square test was used to compare severe hematologic toxicities in arm A and arm B.

5.4. Results

5.4.1. Patient characteristics

Between July 2001 to February 2004, 76 patients were accrued from Singapore and Australia (Table 5.3). The study was approved by the institutional review board of each participating centre and all patients gave written informed consent. One patient withdrew consent after randomization and did not receive treatment.

Table 5.3 Patient characteristics

Treatment	Arm A (n = 38) Fixed dose rate	Arm B (n = 37) Standard infusion
Age (years)		
Median	55	62
Range	39-77	32-76
Sex		
Male	24	29
Female	14	9
Disease Stage		
IIIB	7	7
IV	31	31
KPS status (%)		
90-100	31	31
70-80	7	7
Histology		
Adenocarcinoma	19	26
Squamous cell	5	4
Large cell	4	4
Others	9	4

5.4.2. Treatment

A total of 322 cycles of chemotherapy was administered (150 in arm A and 172 in arm B) with a median number of four cycles (range 0 to 6). In arm A, gemcitabine was omitted in 3.7% and reduced in 21.3% of courses. In arm B, 2% and 15.7% of gemcitabine doses were omitted or reduced respectively. In both treatment arms, the most common reasons for dose omission and reduction were neutropaenia and thrombocytopaenia. Carboplatin was reduced in 18.7% of doses in arm A and 12.8% in arm B. The relative dose intensity (RDI, the dose intensity that was delivered compared with the intended dose intensity) of gemcitabine was 83% and 84% in arms A and B respectively. [146] RDI has been taken as an important prognostic factor for survival in diffuse large cell lymphoma treated with multidrug regimens. [147]

5.4.3. Toxicity

Seventy-five patients were assessed for toxicity (Arm A, n = 38, Arm B, n = 37). Grade 3/4 anaemia and neutropaenia was similar in both treatment arms (Table 5.4) whilst grade 3/4 thrombocytopaenia was more frequent in arm A (69% versus 50%), this, however, was not statistically significant with Pearson Chi-square test (p = 0.10). Two episodes of neutropaenic fever were reported, one in each treatment arm. Significant non-hematological toxicities were infrequent and tolerable in both treatment arms (Table 5.5). There were no treatment related deaths.

Table 5.4 Hematological toxicities (% of patients)

	Arm A	Arm B	p-value
Toxicity	**Grade 3/4**	**Grade 3/4**	
Anaemia	**31**	**33**	**1**
Neutropaenia	**68**	**75**	**0.61**
Thrombocytopaenia	**69**	**52**	**0.10**

Table 5.5 Non-hematological toxicities for grade 3 or 4 (% of patients)

Toxicity	Arm A		Arm B	
	Grade 3	Grade 4	Grade 3	Grade 4
Anorexia	**3**	**0**	**3**	**0**
Nausea	**5**	**0**	**0**	**3**
Vomiting	**5**	**0**	**0**	**0**
Diarrhoea	**0**	**3**	**0**	**0**
Constipation	**3**	**0**	**3**	**0**
Fatigue	**8**	**3**	**5**	**0**
Rash	**5**	**0**	**13**	**0**
ALT	**0**	**0**	**3**	**0**
Musculoskeletal	**0**	**0**	**0**	**3**

5.4.4. Efficacy

5.4.4.1. Response Rate and Survival

Five patients (three in arm A, two in arm B) did not undergo tumor assessment because of early disease progression (three patients), lost to follow-up (one patient) and withdrawal of consent (one patient). All patients were included in the response assessment as per ITT (Intent-to-Treat) analysis. No patient had a complete response. Thirteen patients (34%, 95% CI 26–59%) in the fixed dose rate arm and sixteen patients (42%, 95% CI 20–51%) in the 30-minute arm had partial responses. The median follow up was 233 days. The median TTP (Time to Progression) was 160 days (95% CI 96–210 days) in arm A and 157 days (95% CI 116–214 days) in arm B (Figure 5.1). No significant difference was seen between the two treatment groups (p = 0.73, log-rank test HR 1.08, 95% CI 0.68–1.73) The median survival and one year survival rate was 212 days (95% CI 176–263 days) and 31.6% respectively for patients in arm A and 287 days (95% CI 191–394 days) and 35.6% respectively for patients in arm B.

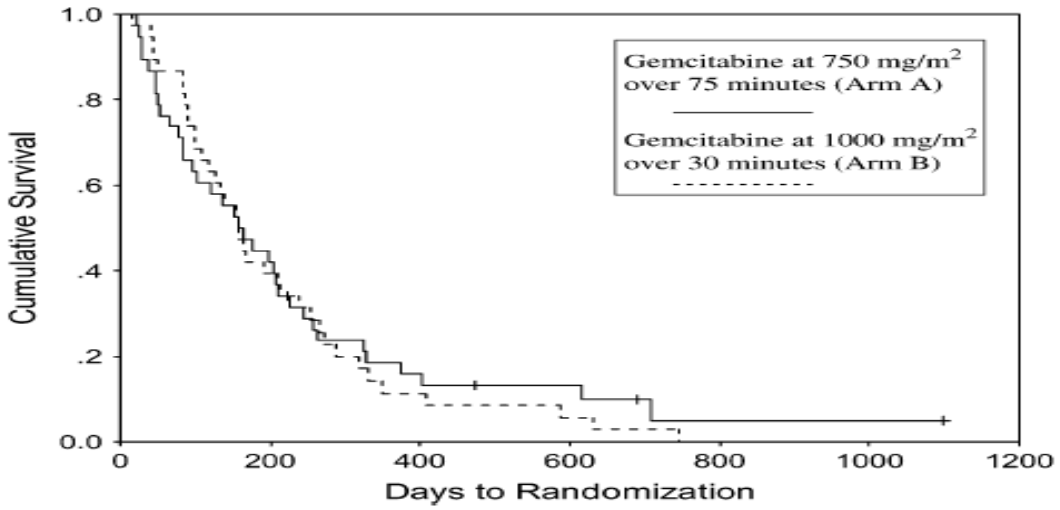


Figure 5.1 Progression free survival

5.4.4.2. Early Phase Tumor Response

The tumor was measured at day 1 before gemcitabine chemotherapy and first two cycles later. The tumor shrinkage percentage was calculated according to following formula 1:

$$Percentage\ Shrinkage\ (\%) = (Diameter_{day1} - Diameter_{day42})/Diameter_{day1} \times 100 \quad \textbf{... (1)}$$

In consideration of slight check time difference on tumor size measurement due to logistic or physical reasons, we set an acceptable range for this tumor measurement time after first two cycles as day 42 ± 7. There were 58 subjects were from Singapore. Among them, five patients (three in arm A, two in arm B) did not undergo tumor assessment because of early disease progression (three patients), lost to follow-up (one patient) and withdrawal of consent (one patient). Among 53 patients left, four patients were withdrawn after 1st cycle of gemcitabine in combination with carboplatin from the study due to disease progression. Thirteen subjects were found to have shrinkage percentage ≥30 and these were considered as responders while there were 36 non-responders with shrinkage percentage <30.

5.4.5. Pharmacokinetic data

5.4.5.1. Non-compartmental Anaysis

Plasma gemcitabine were analyzed in 58 patients using non-compartmental analysis (29 in each arm, all from Singapore) and intracellular dFdCTP was determined in 33 patients (arm A 15 patients, arm B 18 patients). Peak plasma gemcitabine concentrations occurred earlier in arm B than in arm A (Table 5.6A). In arm A, Cmax of gemcitabine was 20.8 ± 17.2 μM at 51 min compared to 41.2 ± 13.9 μM at 29 min in arm B (Table 5.6B). Mean gemcitabine AUC was 1345.9 ± 1112.6 μM×min and 1432.4 ± 528.9 μM×min in arm A

and B, respectively. Terminal elimination was similar, with a mean clearance of 164.0 ± 64.0 l/h/m^2 in arm A and 181.6 ± 74.5 l/h/m^2 in arm B (Table 5.6A). The volume of distribution of gemcitabine was 65.0 ± 37.2 L in arm A and 74.5 ± 41.2 L in arm B, indicating that irrespective of the infusion rate, gemcitabine was widely distributed in the tissues.

Table 5.6 Pharmacokinetic parameters of (A) plasma gemcitabine and (B) intracellular gemcitabine triphosphate. Values are expressed as mean ± SD, Cmax maximum concentration, Tmax time to maximum concentration, AUC area under the concentration time curve, Vss volume of distribution at steady state, CL clearance.

A: Plasma gemcitabine pharmacokinetic parameters		
	Arm A (n = 29)	**Arm B (n = 29)**
Dose gemcitabine (mg)	**1193 ± 145.3**	**1600 ± 197.5**
Cmax (µM)	**20.8 ± 17.2**	**41.2 ± 13.9**
Tmax (min)	**51.3 ± 17.2**	**28.8 ± 8.6**
$AUC_{0\text{-}inf}$ (µM*min)	**1,345.9 ± 1112.6**	**1,432.4 ± 528.9**
AUC extra (%)	**0.3 ± 0.3**	**0.3 ± 0.3**
Vss (L)	**65.0 ± 37.2**	**74.5 ± 41.2**
CL (L/h)	**261.8 ± 106.7**	**293.0 ± 128.1**
CL (L/h/m2)	**164.0 ± 64.0**	**181.6 ± 74.5**
t1/2 (min)	**18.2 ± 4.2**	**17.1 ± 3.1**

B: Intracellular gemcitabine triphosphate pharmacokinetic parameters		
	Arm A (n = 29)	**Arm B (n = 29)**
Cmax (µM)	**173.9 ± 77.3**	**224.8 ± 73.8**
Tmax (min)	**1.6 ± 0.4**	**0.9 ± 0.2**
Apparent $AUC_{0\text{-}inf}$ (µM*min)	**35079 ± 18216**	**32249 ± 11267**
AUC extra (%)	**32 ± 7**	**28 ± 5**
Apparent CL (L/h)	**8.6 ± 3.4**	**11.0 ± 4.1**
Apparent CL (L/h/m2)	**5.2 ± 2.0**	**7.0 ± 2.6**

The pharmacokinetic profiles of dFdC and the inactive dFdU were shown in Figure 5.2 and that of intracellular dFdCTP accumulation was shown in Figure 5.3. Higher intracellular dFdCTP accumulation was observed in Arm B but was unlikely to be at saturation point according to our previous *in vitro* results (Chapter three), showing that the incubation time is the determinant of intracellular dFdCTP accumulation and retention. The saturation of intracellular dFdCTP was only achieved after 8 h incubation when applied concentration of dFdC was ≥ 10 μM. In contrast, our clinical infusion time was one seventh of the exposure time of in our *in vitro* experiment.

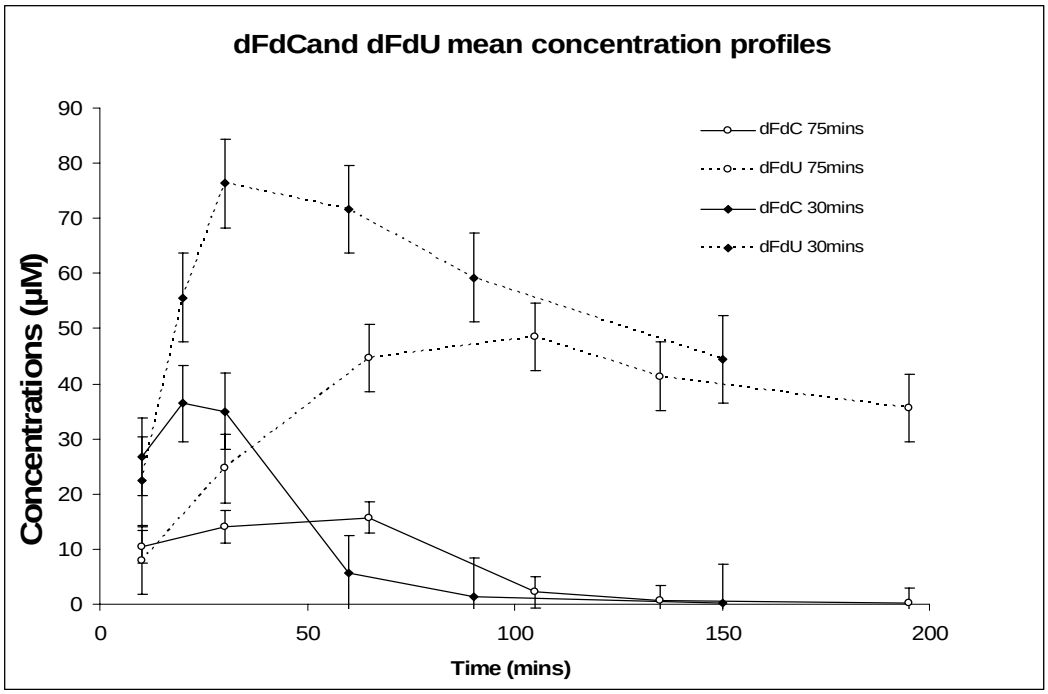


Figure 5.2 The pharmacokinetic profile of gemcitabine and dFdU in plasma

Thus, the increase of infusion time from 30 min to 75 min might enhance the intracellular accumulation of dFdCTP, but its contributory effect could be compromised by the dose reduction (1000 mg/m^2 in 30-min arm vs 750 mg/m^2 in 75-min arm). Intracellular dFdCTP AUC was similar at 35 079 ±18 216 μM×min and 32 249 ± 11 267 μM×min in arms A and B (p = 0.08, t-test), respectively (Table 5.6B). Their pharmacokinetic profiles were shown in Figure 5.3.

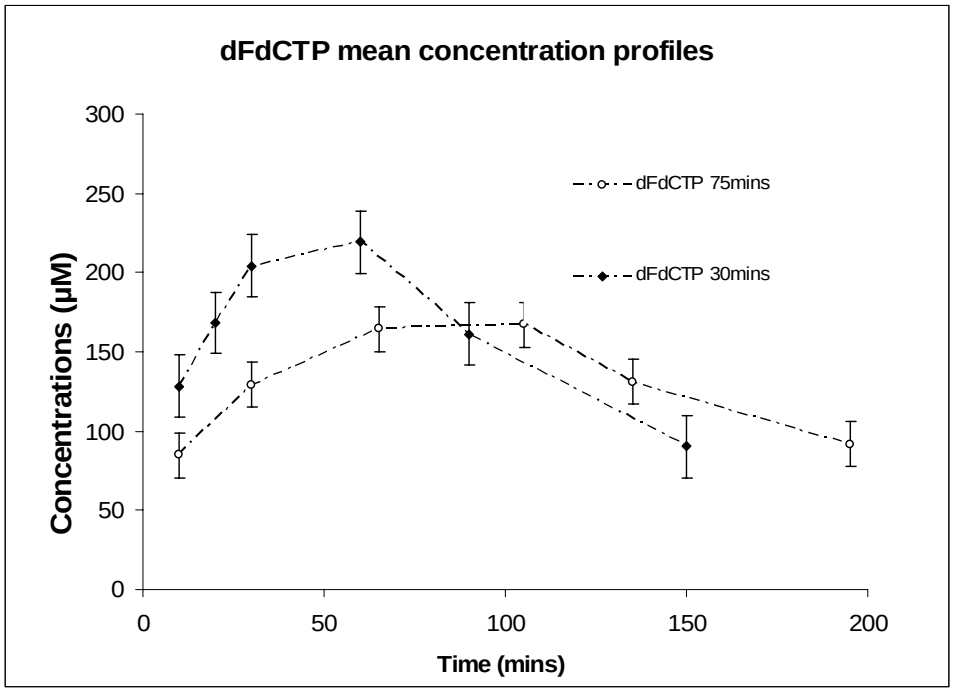


Figure 5.3 The pharmacokinetic profile of dFdCTP in PBMC

Figure 5.7 showed the plasma concentration ratios of dFdU/gemcitabine with time. Since gemcitabine was metabolized very fast in plasma, its plasma concentration would decrease quickly after stopping infusion. Thus, the range of the plasma concentration ratios of dFdU/gemcitabine increased quickly with increase of sampling time after infusion.

Table 5.7 Plasma concentration ratio of dFdU/gemcitabine in NSCLC patients

Sampling Time	Subjects	Range	Minimum	Maximum	Mean
10 min	**48**	**2.07**	**.06**	**2.13**	**.7475**
30 min	**51**	**13.71**	**.95**	**14.66**	**2.5090**
(-)10 min	**52**	**4.29**	**.79**	**5.08**	**2.2357**
(+) 30 min	**52**	**123.58**	**4.25**	**127.83**	**27.7444**
(+) 60 min	**50**	**403.78**	**7.76**	**411.54**	**94.0594**
(+) 120 min	**49**	**1149.88**	**106.12**	**1256.00**	**484.1816**

5.4.5.2. Compartmental Analysis on Gemcitabine Plasma Concentration

Most previous studies report plasma pharmacokinetic parameters of gemcitabine using non-compartmental analysis (NCA). This is due to the limitation of gemcitabine determination sensitivity, especially in elimination phase where the concentrations are very low. The disadvantage of NCA is that the data obtained are not useful for simulations of different infusion rates before initializing new clinical trials. Recent development in LC-MSMS applications has resulted in enhanced sensitivity. We utilized compartmental modeling to fit our data. In our model selection exercises, one and two compartment models with or without weighting were compared. Three compartment models could not be tested due to the limitation of sampling points. According to the

results of concentration-time profile visualization and minimized Akaike Information Criterion (AIC), a two-compartment model (model 10) with 1/Y weighting provided the better fitting model and was used for the plasma concentrations of gemcitabine. The pharmacokinetic parameters for two treatment schedules were listed in Table 5.8. The dose of gemcitabine applied in arm A is 30 % less than that in arm B. The differences in AUC were in the same magnitude as their doses. The clearances and Vss in both arms were similar. Maximum concentrations in arm B were significant higher than those in arm A. AIC criteria from 1/Y weighting model was much lower than those without weighting in most cases and other weightings.

Table 5.8 Difference of Pharmacokinetic parameters of plasma gemcitabine between arm A (75-min infusion) and arm B (30-min infusion).

PK Parameters	Arm A (n = 24)	Arm B (n = 24)
Dose (mg)	1203.50 ± 160.48	1612.50 ± 203.50
AUC (min×μM)$_{0\text{-inf}}$	1236.14 ± 517.98	1594.02 ± 528.81
Cmax (μM)*	16.15 ± 6.69	42.56 ± 13.79
Cl (L/min)	4.32 ± 1.73	4.22 ± 1.40
Vss (L)	65.38 ± 33.97	57.90 ± 24.30
$T_{1/2\beta}$ (min)	20.10 ± 14.71	15.94 ± 9.56
AIC Criteria_no weighting	-10.54 ± 15.09	0.79 ± 15.40
AIC Criteria_weighting_(1/Y)	-22.15 ± 13.01	-18.07 ± 10.93

* p value < 0.05

5.4.6. Hematological models

Although seventy-six patients were accrued in this study, fifty-eight patients with plasma pharmacokinetic data of gemcitabine and dFdU were available for analysis. Among them, thirty-three patients with dFdCTP intracellular concentrations were available for hematologic toxicity association analysis with dFdCTP exposure. Initially, we analyzed the pooled data from both arms. No relationship was found between neutrophil nadir /platelet nadir and gemcitabine pharmacokinetic parameters or dose. Hence, an individual correlation analysis was carried in individual arm. Among the parameters tested, only AUC of dFdCTP in arm B was identified to have a modest linear relationship with natural log neutrophil nadir (r = -0.51) (Table 5.9) and was also found to be associated with in natural log platelet nadir (r= -0.692) (Table 5.10, Figure 5.4). However, arm A did not show such relationship.

Table 5.9 Arm B Univariate linear regression of PK parameters tested with the lnANCnadir_all

PK parameters	r	intercept	β	*p* value	F value
Cl_{dFdC}	-0.095	6.528	-0.030	0.722	0.131
Cl_{dFdC_BSA}	-0.232	6.793	-0.129	0.371	0.850
AUC_{dFdC}	0.265	5.802	0.000	0.304	1.132
AUC_{dFdC_BSA}	0.095	6.171	0.000	0.712	0.142
AUC_{dFdCTP}	-0.365	7.110	-0.000023	0.151	2.295
AUC_{dFdCTP_BSA}	-0.515	7.110	-0.000048	**0.034**	5.415

Table 5.10 Arm B Univariate linear regression of covariates tested with the lnPLTnadir_all

PK parameters	r	intercept	β	*p* value	F value
Cl_{dFdC}	-0.263	4.226	-0.124	0.309	1.110
Cl_{dFdC_BSA}	-0.263	4.226	-0.124	0.309	1.110
AUC_{dFdC}	0.212	3.439	0.000	0.412	0.711
AUC_{dFdC_BSA}	0.155	3.548	0.000	0.549	0.375
AUC_{dFdCTP}	-0.698	5.015	-0.000038	**0.002**	14.256
AUC_{dFdCTP_BSA}	-0.692	4.933	-0.000055	**0.002**	13.782

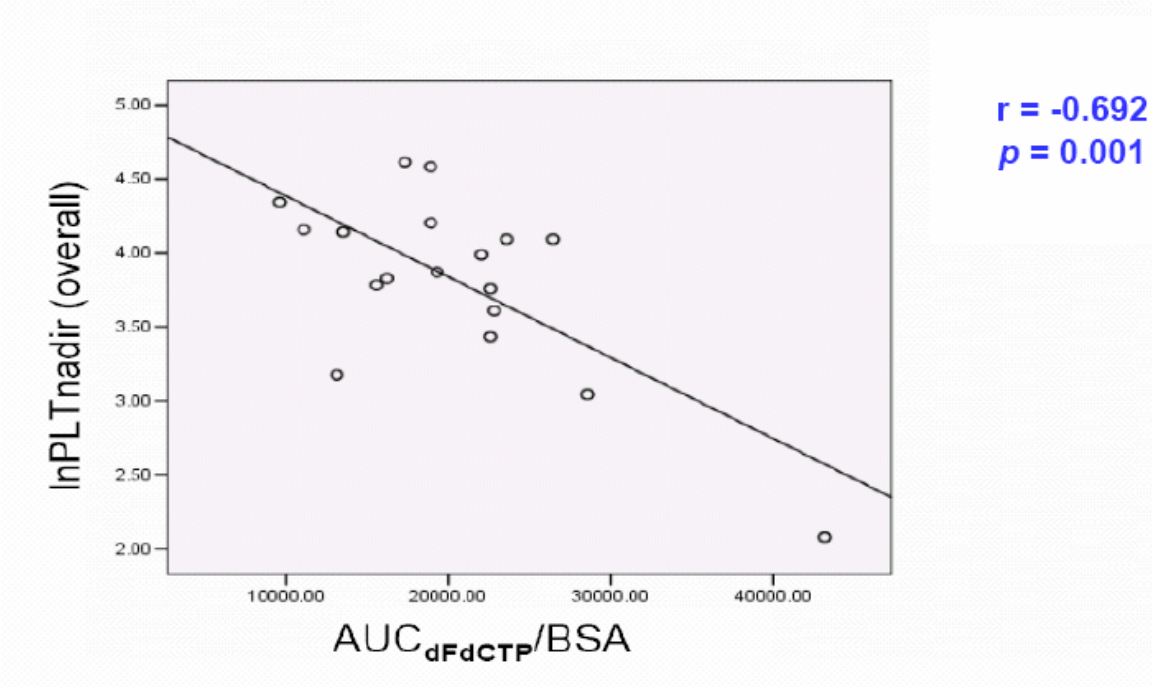


Figure 5.4 Association of thrombocytopenia with dFdCTP exposure for arm B

5.4.7. Correlation of dFdU/gemcitabine ratios with demography & tumor shrinkage

To investigate whether there is any correlation between metabolite/parent ratios and demography as well as response, the dFdU/gemcitabine ratios were computed for each of sampling times (Table 5.7). Pearson correlation and t test showed no correlation and effect of demography on the concentation ratios of dFdU/gemcitabine (Table 5.11).

Table 5.11 Effect of demographic factors on plasma concentration ratio of dFdU versus gemcitabine

Variables	10 min	30 min	(-)10 min	(+) 30 min	(+) 60 min	(+) 120 min
Age*	r = 0.230 p = 0.116	r = -0.119 p = 0.406	r = -0.124 p = 0.381	r = -0.029 p = 0.841	r = 0.002 p = 0.991	r = -0.028 p = 0.846
BSA*	r = 0.110 p = 0.457	r = 0.280 p = 0.46	r = -0.055 p = 0.697	r = 0.018 p = 0.898	r = -0.001 p = 0.993	r = 0.092 p = 0.521
Sex	p = 0.817	p = 0.060	p = 0.521	p = 0.956	p = 0.143	p = 0.292
M (Mean±SD)	0.76 ± 0.42	2.78 ± 2.54	2.18 ± 1.10	27.63 ± 26.50	81.73 ± 54.79	430.04 ± 269.10
F (Mean±SD)	0.73 ± 0.36	1.92 ± 0.374	2.36 ± 0.82	27.98 ± 17.94	120.25 ± 93.70	548.83 ± 400.11

*Pearson Correlation Coefficient

When the metabolite/parent ratios at the different sampling times were statistically tested (Mann-Whitney U test) with early phase tumor response (section 5.4.4.2), it was found that the ratios at 120 min were significantly different (Table 5.12 and Fig. 5.5). The ratios of the responders were much smaller than those of the non-responders.

Table 5.12 Relationship between responders and plasma concentration ratio of dFdU versus gemcitabine

Sampling Time	10 min	30 min	(-) 10 min	(+) 30 min	(+) 60 min	(+) 120 min
Mann-Whitney U	193.500	201.000	181.500	233.000	197.000	125.000
Wilcoxon W	298.500	306.000	286.500	338.000	302.000	216.000
Z	-1.831	-1.102	-1.636	-.549	-1.062	-2.555
Asymp. Sig. (2-tailed)	.067	.270	.102	.583	.288	.011

a Grouping Variable: responder

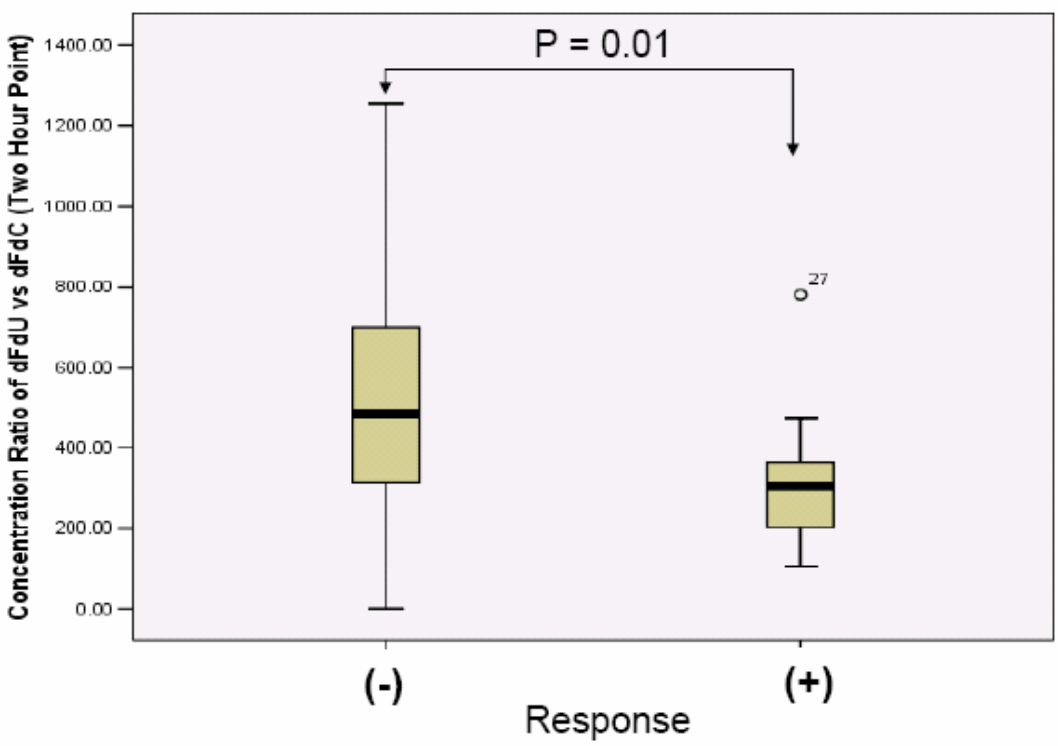


Figure 5.5 Box plot of response vs concentration ratios of dFdU and gemcitabine at 120 min after infusion

In order to predict the responders or non-responders, a frequency histogram was drawn according to concentration ratios of dFdU/gemcitabine at 120 min (Fig. 5.6).

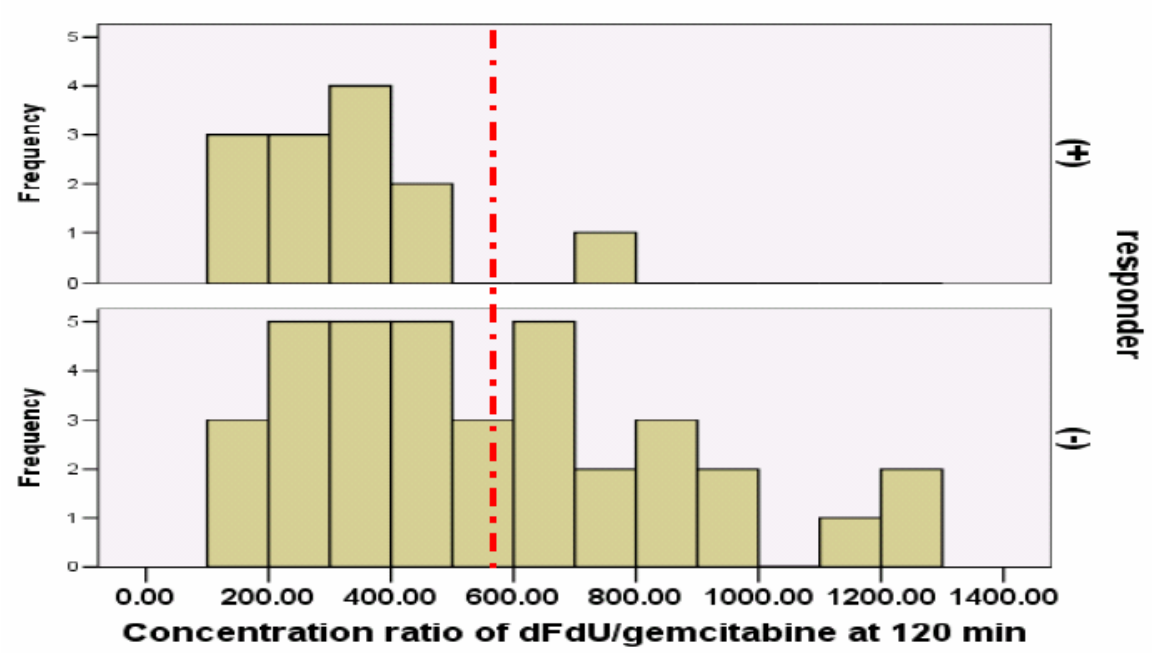


Figure 5.6 Frequency histogram for the concentration ratios of dFdU/gemcitabine at 120 min (red line represents midpoint of concentration ratios of dFdU/gemcitabine).

The ratios of dFdU/gemcitabine were widely distributed in the range of 106 to 1256. The middle value with 575 was labeled as a red line in Figure 5.6. This line possessed a different meaning for each group. For non-responders, this line nearly separated this group equally. It could not be used as a marker to identify non-responders from the study cohort. However, there was a different situation for responders. Among 13 responders, only one subject (7.69%) had the ratio of dFdU/gemcitabine bigger than 575. This result

indicated that in general responders to gemcitabine treatment were most likely to have low dFdU/gemcitabine ratios. This result also suggested that gemcitabine's deamination rate could be a determinant factor in chemotherapeutic activity where a larger ratio represents faster deamination rate.

Through Figure 5.6, the red line could distinguish non-responders from the all subjects. When the ratio was larger than 575, there would be 94% (16 out of 17) probability to predict this subject as a non-responder. In addition, a ratio of 500 would be a better cutoff value to predict non-responders. In this case, the probability to predict non-responders was increased nearly to 95% (18 out of 19).

Hence, the cutoff ratio was set as 500, subjects with ratios < 500, would be slow deaminators, while those with ratios ≥ 500, would be fast deaminators. In the responders, only one in thirteen (7.69%) had a value >500. On the contrary, there was a much higher percentage (50%, 18 over 36) with >500 in non-responders. This difference was statistically significant Chi Square test ($p = 0.004$). This finding provided an important marker in evaluating the efficacy of gemcitabine at an early phase (2^{nd} cycle) of gemcitabine chemotherapy. For example, if the ratio is ≥ 500, it might imply that the patient might not respond to gemcitabine and alternative anti-cancer drugs could be tried.

5.5. Discussion

This randomized phase II study compared the pharmacokinetics of plasma gemcitabine and intracellular dFdCTP in a large set of patients receiving gemcitabine either as a fixed dose rate or as a standard infusion rate in combination with carboplatin. In addition, pharmacodynamics and toxicities were also compared between the two arms. Early phase response was evaluated and a potential progression marker was explored to predict non-responders to gemcitabine combination therapy with carboplatin in NSCLS patients.

5.5.1. Phase II pharmacokinetic study of gemcitabine dosing 10 mg/m^2/min for 75 min or 1000 mg/m^2 for 30 min

One of main objects of this study was to evaluate the pharmacokinetics of plasma gemcitabine and intracellular dFdCTP between the two treatment arms. According to Table 5.6, although both $AUC_{0\text{-}inf}$ and CL in arm A were 89% of those in arm B, these differences were not statistically significant ($p = 0.38$ for $AUC_{0\text{-}inf}$ and $p = 0.32$ for CL). However, the mean exposure time above 10 μM (Figure 5.2) was 80 min and 50 min for arm A and arm B, respectively. This suggested that fixed rate infusion might be more efficient than standard 30 min infusion.

On the other hand, the $AUC_{0\text{-}inf}$ of intracellular dFdCTP was similar in both treatment arms despite the dose of gemcitabine being 25% higher in arm B. Consistent with previously reported studies, [18, 46, 148] the pharmacokinetic data demonstrated that the 30-min infusion arm was a pharmacologically less efficient method of administering gemcitabine compared to a fixed dose rate of 10 mg/m^2/min. In arm B, a higher mean plasma gemcitabine Cmax of 41.2 uM was reached, a value that was well outside the

concentration range known to optimize the rate of gemcitabine phosphorylation.

5.5.2. Phase II pharmacodynamics and toxicities of gemcitabine dosing 10 mg/m^2/min for 75 min or 1000 mg/m^2 for 30 min

The response rates in both study arms were consistent with the established efficacy of this regimen in NSCLC. Similar outcome measures of response rates, time to disease progression, survival and toxicities were found using carboplatin with gemcitabine at 750 mg/m^2 in a 75-min infusion or at 1000 mg/m^2 in a 30-min infusion in the treatment of advanced NSCLC. Response rates were reported to range from 29% to 42% in studies using carboplatin and 30-min infusion gemcitabine [97, 121, 149] and 34% to 47% in studies of gemcitabine administered at 10 mg/m^2/min with carboplatin or cisplatin. [17, 150] Whilst significant myelosuppression was seen in both treatment arms, the frequency of neutropaenic fever and bleeding from thrombocytopaenia was low. The relative-dose intensity (RDI, a scientific term refers to the amount of a particular chemotherapy given over a specific time in relation to what was originally ordered) of gemcitabine in arm A of 83% was similar to that in arm B (84%) and compared favorably with the 75% for gemcitabine reported in a phase II study of carboplatin and gemcitabine 1200 mg over 120 min in NSCLC. [151] Therefore, a fixed dose rate infusion of gemcitabine with carboplatin was feasible without major cumulative toxicities. Previous studies using a prolonged gemcitabine infusion schedule reported elevation in hepatic transaminases; [12] this, however, was not seen in our study and could be related to duration of the infusion.

As phosphorylation of gemcitabine was more efficient in the fixed dose rate arm, a lower dose of gemcitabine could be administered, with the resultant benefit of reduced

chemotherapy costs. This advantage, however, might be offset by increased charges associated with a longer infusion time and nursing costs as well as greater inconvenience. Regarding to hematological toxicity modeling, logarithm transformation of the nadir neutrophil counts and platelet counts was used as dependent variables due to following two reasons. One of our assumptions was based on logarithmic transformation of the equation:

$SF = e^{[-k \times AUC]}$ which yields a linear relationship, e.g.

ln (nadir/platelet counts) = ln (pretreatment count)-k×AUC, where SF is the survival fraction of cells and k is the rate constant that determines the slope of the decay curve, AUC is the area under the curve of the antineoplastic agent under study, and ln is the natural logarithm. The other reason was that we assumed that the logarithm transformation of neutrophil/platelet nadir counts could result in a better normalization which made the data more predictable. [59]

Our results showed that AUC of dFdCTP correlated with hematological toxicity like thrombocytopenia only in arm B (Fig 5.4). The possible reason might be that gemcitabine is a highly hydrophilic compound and enters the cell through active human nucleotide transportation. When infusion rate (e.g. 75 minute infusion) was low, gemcitabine might be more efficiently transported into the cells. The percentage of drug remaining in blood circulation system would be low. However, when the infusion rate (e.g. 30 minute infusion) was high, potential saturation on human nucleoside transporters could result in a greater percentage of gemcitabine in blood circulation system during infusion. Different concentrations of gemcitabine in blood circulation system might produce a different dFdCTP accumulation velocity in the white blood cells. According to Michaelis_Menten

equation, the velocity of dCK would highly depend on the concentration of gemcitabine, the substrate of dCK. If the peak concentrations of gemcitabine were at about 10 μM in case of 75 min infusion, the enzyme velocity would vary sharply since the concentration of substrate was the same magnitude as Km (Km was reported as being in the range of 5-10 μM).[152, 153] If the peak gemcitabine concentrations were at relatively high value in case of 30 min standard infusion (33 mg/m^2 per minute), the enzyme velocity would approach maximum value at the plateau phase to saturate dCK. [154] Only in this situation, the formation rate of dFdCTP could reach its maximal value and the resultant values from different subjects could represent their dCK activities. Thus, the differences among individuals in toxicities could be differentiated. Hence, only for arm B, the difference of dFdCTP accumulation between individuals could be compared each other since their enzyme velocities were at the ceiling status and dFdCTP accumulation was highly dependent on enzyme's activity for each patient. Therefore, this would account for the fact that there was a good correlation between dFdCTP exposure and myelosuppression, especially for thrombocytopenia only in arm B. If we analyzed all the subjects from two arms, the data from arm A (75 min infusion) would become a confounding factor. Thus, we could not find any correlation in the combined dataset.

5.5.3. Early phase progression marker for non-responders to gemcitabine treatment in NSCLC

Individualization of chemotherapy for cancer patients is currently becoming more and more important in minimizing adverse effects without compromising efficacy. However, the biggest challenge is to identify and validate sensitive and specific markers for each

cancer treatment. One of the most important markers is early phase tumor shrinkage maker which is useful for oncologists to evaluate the therapeutic efficacy so as to decide if alternative agents should be sorted for non-responders to current treatment instead.

The effect of chemotherapeutic agents on solid tumors is most commonly categorized and reported based on the RECIST. This classification divides both target and non-target lesions' response into four categories-complete response (CR), partial response (PR), progressive disease (PD) and stable disease (SD) in all treatment cycles. It offers a simple criterion that standardizes the measurement and interpretation of tumor responses across clinical trials, allowing cross comparison between trials and anticancer drugs. However, this all cycle based evaluation assay may finally result in a treatment failure for non-responders who could miss the best opportunities for other anti-cancer drugs in early treatment phase. Hence, early detection of non-responders is a big challenge in achieving higher successful chemotherapeutic rates. In our study, a novel tumor shrinkage marker (ratio of dFdU:gemcitabine at the 120 min sample point) was identified to predict non-responders to gemcitabine treatment in NSCLC with as high as 95% prediction probability. Furthermore, no difference on demographic parameters was detected between responders and non-responders (section 5.4.7). In addition, there was no significant difference on the ratios of metabolite/parent for other sampling times except for 120 min. Responders generally had a smaller the ratio of dFdU/gemcitabine at 120 min. With a cutoff value of 500, the ratio of dFdU/gemcitabine at 120 min could be used to predict non-responders to gemcitabine treatment combined with carboplatin in NSCLC. As long as this marker could be validated with larger datasets in clinical setting, it would be easily accessible and useful in evaluating tumor progression in the early phase.

5.6. Conclusions

Based on our pharmacokinetic study, by using a 25% lower dose of gemcitabine at an infusion rate of 10 mg/m^2/min in combination with carboplatin in NSCLC, an equivalent clinical efficacy and safety profile was achieved compared to standard 30-min infusion regimen. In addition, our pharmacokinetic-pharmacodynamic association analysis showed that intracellular dFdCTP exposure could be a potential tool to predict thrombocytopenia due to gemcitabine based chemotherapy in 30 min infusion regimen. Due to the difficulty in measuring intracellular dFdCTP, other convenient surrogate markers in pharmacokinetics and pharmacogenetics should be further explored in order to better predict the efficacy of chemotherapy by optimizing individualized treatment. Moreover, our results showed that the early phase (after 2nd cycle) tumor shrinkage was highly related to plasma concentration ratio of dFdU/gemcitabine (metabolite:parent). The average ratio of non-responders at the 120 min sample point was significantly ($p = 0.011$; Mann Whitney test) higher than that of the responders. However, this surrogate marker is not correlated to patient survival time, one of the most important endpoints. A large prospective clinical trial would be designed to further validate our finding that the ratio of dFdU/gemcitabine is a good and valuable marker to predict non-responders in early phase tumor shrinkage through gemcitabine based chemotherapy in NSCLC patients.

CHAPTER SIX

Genotypic and Phenotypic Association of Gemcitabine in NSCLC Patients

6.1. Introduction

Gemcitabine displays a good anticancer effect profile on many types of cancers, especially for solid tumors due to its special mechanisms of action. Although it possesses a minor difference of chemical structure compared to that of cytarabine, gemcitabine shows a superior anticancer activity through its active phosphorylated metabolites (gemcitabine diphosphate and triphosphate) after it enters the cells. This may be attributed to high accumulation and slow elimination of gemcitabine triphosphates which inhibits processes required for DNA synthesis and RRM. According to the previous clinical trials, gemcitabine shows a better tolerable toxicity profile compared to other commonly used anticancer drugs. However, a significant variability in its toxicity and efficacy resulted in difficulty in toxicity management and treatment optimization. In order to improve clinical treatment efficacy and minimize the toxicity of gemcitabine, it is essential for scientists to better understand its transportation, activation and metabolism pathway. In addition, those functional single-nucleotide polymorphisms in gemcitabine disposition pathway will be identified through analyzing genotypic and phenotypic association. Membrane transporters are important in drug response as they are major determinants of drug absorption, distribution, and elimination. There are two major families of NTs: CNT and ENT. Although there are different specificities for these transmembrane transporters, gemcitabine has been proven to be the substrate to several of them including hENT1, hCNT1 and hCNT2. [107-112]

Hematological toxicities are the major adverse effects of gemcitabine even though this widely used anticancer agent has been thought to be tolerable in most cases. Severe neutropenia is usually dose limiting toxicity in clinical setting. [155-157] Gemcitabine

triphosphate concentration in the white cell has been used as a surrogate marker to evaluate the efficacy and toxicities of gemcitabine due to the difficulty in quantifying concentrations of dFdCTP inside tumor tissues. In this chapter, we screened 25 loci involved in gemcitabine disposition pathway and further analyzed the association between genetic variants and pharmacokinetic parameters of gemcitabine in Asian NSCLC patients.

6.2. Objectives

The main objectives are to comprehensively screen gene SNPs which are thought to play an important role on intracellular transportation, metabolism and activation of gemcitabine; to correlate the pharmacokinetic parameters of gemcitabine and its metabolites with polymorphism of genes involved in pathways of gemcitabine transportation, metabolism and activity and to identify genotypic variants associated with hematological toxicity, response and survival in Asian patients with advanced non-small cell lung cancer (NSCLC) receiving gemcitabine based chemotherapy.

6.3. Patients and Methods

6.3.1. Study population

In this clinical trial, the study population consisted of 94 healthy volunteers recruited at the blood donation clinic and 53 patients with NSCLC receiving treatment for their disease in the National University Hospital in Singapore. All patients were newly diagnosed cases treated with first-line gemcitabine at 750-1000 mg/m^2 on days 1 and 8 and carboplatin at AUC = 5 mg/ml x min on day 1 every 3 weeks. Among them, 43 (81%) were Chinese and 10 (19%) were Malays, 36 (68%) were male and the median age was 59 years (range 39-74 years). Some 44 (83%) had stage IV disease and 42 (79%) had a Kanorfsky Performance Score of ≥ 90. The patients' eligibility criteria were described in detail in previous chapter five. The institutional review board of the National University Hospital approved the study and informed consent forms were obtained from all subjects. Toxicities were evaluated every cycle using the National Cancer Institute Common Toxicity Criteria, version 2.0.

6.3.2. Blood Sampling

In pharmacokinetic analysis of dFdCTP, blood samples were collected from 33 patients as per described in chapter five. In pharmacogenetic analysis, 8ml of peripheral blood was drawn into heparinised vacutainer tubes (Becton Dickinson) from each subject before drug treatment and mononuclear cells isolated by Ficoll-Hypaque density gradient centrifugation according to manufacturer's instructions (GE Healthcare, Chalfont St. Giles, United Kingdom). DNA was extracted from the mononuclear cells using the Puregene DNA purification kit (Gentra Systems, Minneapolis, MN).

6.3.3. Quantitation of dFdCTP and Pharmacokinetic Analysis

The quantitation and pharmacokinetic analysis have been described in Chapter two and five, respectively.

6.3.4. Selection of SNP loci

SNP loci for analysis were identified from publications on genotypes of genes involved in gemcitabine transport, metabolism and activity [158-161] and a comprehensive search of various public databases: Genecards; (http://www.genecards.org), pharmGKB; (http://www.pharmgkb.org), NCBI; (http://www.ncbi.nlm.nih.gov). Candidates were selected based on the following priorities (1) their presence in coding regions (2) non-synonymous coding and (3) reported allele frequencies of greater than 5%. The designation of which genotypes were wild type was according to the NCBI database and nucleotide numbering began at the translational start site ATG.

6.3.5. Pharmacogenetic analysis

SNP detection was done by TI lab of Oncology Research Institute. Briefly, 50 ng of DNA was amplified in a 25µl reaction containing 1 x FastStart Reaction Buffer, 2mM magnesium chloride, 10µM deoxynucleotide mix and 1 unit FastStart Taq Polymerase (Roche Diagnostics, Mannheim, Germany) and 5µM each of forward and reverse primers obtained either from previous reports or using PSQ Assay design software (Biotage AB, Uppsala, Sweden). PCR cycling comprised 4 minutes at 95^{o}C, followed by 40 cycles of 30 seconds at 95^{o}C, 30secs at the appropriate annealing temperature and 1 min at 72^{o}C, before conclusion with a 1 minute at 72^{o}C in a Master Cycler (Eppendorf, Hamburg, Germany).

PCR products were incubated with 3µl of streptavidin magnetic beads (Amersham Pharmacia Biotech, Uppsala, Sweden) and 1x binding buffer (10mM Tris–HCl, 2M NaCl, 1mM EDTA, 0.1% Tween 20) and thoroughly mixed for 10min at 37^{o}C. The product mix was then denatured by 5 seconds incubation in 0.2M NaOH solution and washed in annealing buffer (20mM Tris–acetate, 2mM magnesium acetate) for 10 seconds. The single-stranded products were transferred to an annealing buffer containing 15 pmol of the sequencing primer and incubated for 2 min at 80^{o}C in a Hybaid Maxi 14 hybridization oven (Thermo Electron, USA). Pyrosequencing was then performed on a PSQ96MA pyrosequencer instrument (Biotage AB, Uppsala, Sweden) with optimized nucleotide dispensation orders.

6.3.6. Statistics

Compliance with Hardy-Weinberg equilibrium was assessed using Clump software. Linkage disequilibrium between gene loci was calculated using exact test a Markov chain. The chi-squared test was used to assess differences in the genotype frequency between Asians and Caucasians healthy subjects [159-162] as well as associations between gene variants and tumor response in NSCLC patients. Associations between hematologic toxicity or pharmacokinetic parameters and gene variants were assessed using the Mann-Whitney test. Kaplan-Meier methods and log-rank test were used to compare overall survival and time to progression. Cox proportional hazards models were used to adjust for stage and performance status. All statistical tests were two-sided and SPSS software version 13.0 (SPSS Inc, Chicago, IL) was used. A p value of less than 0.05 was considered to indicate statistical significance.

6.4. Results

6.4.1. Distribution of gemcitabine pathway genotypes in healthy Caucasians and Asians

An extensive search of publications and genome databases on sequence variants in genes encoding proteins involved in pathways of gemcitabine transport, metabolism and activity identified 25 loci in 9 genes that qualified for analysis (see Figure 6.1.).

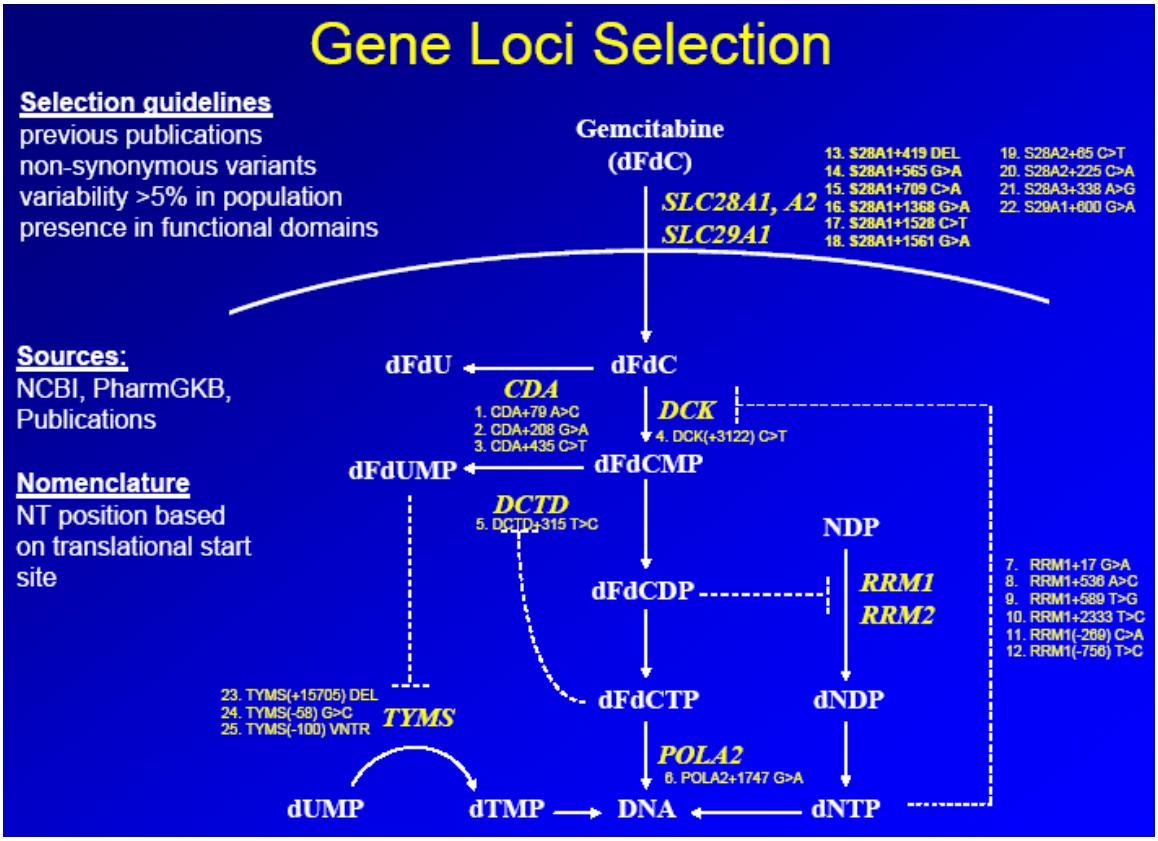


Figure 6.1 Selection of Gene loci involved in pathways of gemcitabine transport, metabolism and activity from an extensive search of publications and genome databases

Pyrosequencing enabled genotyping of all 25 loci in all 94 healthy Asian donors including 57 (61%) Chinese, 20 (21%) Malay and 17 (18%) Indian. The Primers, PCR annealing temperatures and dispensation sequences and used for genotyping in this study were listed as follows (Table 6.1).

Table 6.1 Primers, PCR annealing temperatures and dispensation sequences used for genotyping in this study

	PCR				Pyrosequencing	
Locus	Forward	Reverse	°C	bp	Primer	Dispensation
CDA 27	*GGTACCAACATGGCCCAGAA	CCTTTGAAGATTCTCCCCTCC	65	164	GGGCAGTAGGCTGACT	AGTGCTGCT
CDA 70	*CCTGCTTTGGAATGGGGC	CCTGGCTTTCCCACTCACCT	65	221	ACGGCCTTCTGGATA	AGCTCGTCG
CDA 145	GTGTACATGACCAAGCCGGATG	*GGGTGCTGACTCAGGCTGG	65	253	TGAGGACCTGCAGAA	CGACTCTAGT
DCK IVS1-1110	*GATTGATGGAGGACTTACCTGCTTC	AACTTATTGGGGCAAAGTGGG	65	206	GGGAGCTAGAAAATAAGCTA	CAGCTATAT
DCK 2190	*ATTAACCAGTCCAGACGCAC	TAGCTCCACTGTAGTTCATAAGGTT	65	179	AAGGTTTATACTTAAGAAAT	GATGATACA
DCTD 105	GTGCCATGCGGAGCTGAA	*TGAGCTACTGACGAGCAGGCA	65	176	AAAGGCTGTAGTATGTATGT	ATCTGCTGT
POLA2 583	AAGGGCAGGTGGGAGGCA	*AACAGCAGAGGACAGAAGCCTC	72	127	GGCCGGCAGCGGA	TCGAGTCAGA
RRM1 6	TTAGAGCCGCAGTCCAGTCTTG	*GAAGACAGAAGGCCGAGCG	60	233	ATGCATGTGATCAAGC	CGACGTGACGGAC
RRM1 179	CTATAGTGGCTGAAAGACCACAA	*TTTCCCTACTCAAAACACCTACCT	60	191	GAGAGTATCTGTTGGGATC	GCACACGAGAC
RRM1 197	*GTTGGGATCCACAAAGAAGACA	CAGTTTCTTCAGGTCAAGGCGTAT	60	170	CGAAGCATGAGTAAACC	GCACGTCTCA
RRM1 778	*ACCAGCGGCTAATCCAATC	ACATGCTGGTCTCTTCCAAGTC	60	181	TCTCTATTCTCCAAAGAGC	GACAGTCAT
RRM1RR37	GTCCCGAACTCGGCTTGC	*CACCCGTGACGCACAAAG	65	233	GCCTGTCAGTCTGTGAA	TGACGTACG
RRM1 RR524	ATACCCTGTCTCTGCCACCATC	*ACGCAAATCAGAGCCCTGAGA	65	96	CTGCCCTGCTTAAAAT	GTCGTCTCA
SLC28A1 521	*GTGCCAGCCATACCGTTGTG	ACACTCACGGAGATCCACTGCT	65	231	GATCCACTGCTTCCTGT	ACTAGCGAC
SLC28A1 510	GTGCCAGCCATACCGTTGTG	*ACACTCACGGAGATCCACTGCT	65	231	CTCTCCAAGTACAAGCAA	ACTAGCGCT
SLC28A1 456	*TATCAATGCTGCCCTCTCCTGG	CACAACGGTATGGCTGGCAC	65	179	TGGAAGCTGAGCCC	GCTCGATGT
SLC28A1 237	GCCCATCCCTCAAGGAAC	*GGCTTCTCCCCTACTCCC	60	225	CGAGTGGCTGGGCGA	TGCACGATC
SLC28A1 189	CCCGGGTGCTATTGTGTG	*CCCAGATCCAGCCTAGGG	60	287	GCAGGAATCTGCGTG	GTCGAGTCGTGC
SLC28A1 140	TGCAGGGTTCTGAAGAGG	*CATAACCCTGGAGCCAAC	60	319	CAAAGCTGAGGAGGTT	GTCTGTGCAG
SLC28A2 22	AGGCTTGGCCCTGAATTTGT	*GGGGATTGAGAGCAGCCAAATAC	60	207	CTGGCACAGTGAACC	ACTAGGCTGA
SLC28A2 75	CCTAACTTCCTCCTGGCTGAAGA	*ACCCAAACACAACAGGCCC	65	237	GCAAAACACACGCCA	TGCACTGTC
SLC28A3 113	TCAAAGAAGCCAGTAGCCATAT	*CCTAGGGTGTTTCTAGCTTCCA	60	469	GCTTCCTGGACAGGT	CTAGATCTG
SLC29A1 200	GTCCGGCTATCTACCTGCCC	*GCAGGCCCAGGTAACAGATG	65	172	CAGGTGGCTCGGA	TGAGCTATC
TYMS TSER	GTGGCTCCTGCGTTTCCCCC	GCTCCGAGCCGGCCACAGGCATGGCGCGG	63	Var.		
TYMS 1494del	CAATCTGAGGGAGCTGAGT	*CAGATAAGTGGCAGTACAGA	60	152	GAGTGTGGTTATGAACTTTA	CAGCTATAG

The frequency of each genotype with variation (19 SNPs) is depicted in Figure 6.2.

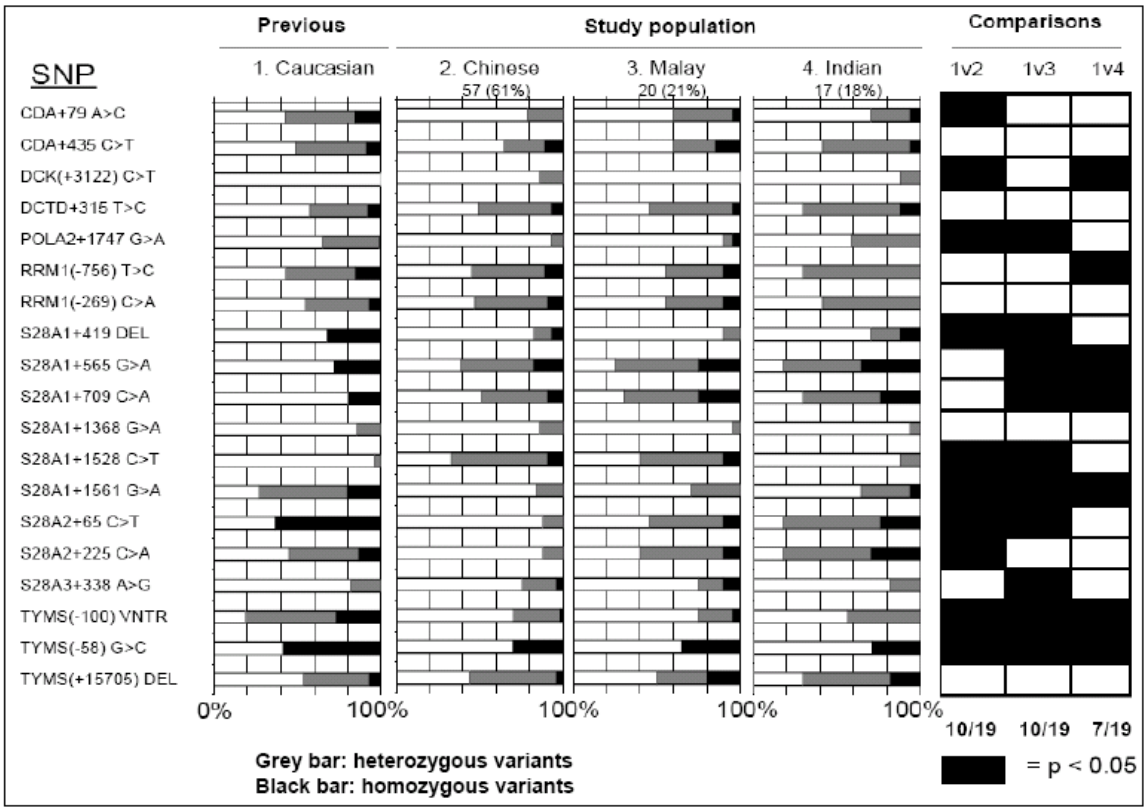


Figure 6.2 Difference in sequence variants distribution between 94 healthy Asians and Caucasians [158-161]

All gene frequencies were in Hardy-Weinberg equilibrium. There was no sequence variation at 6 gene loci (CDA+208, RRM1+17, RRM1+5 36, RRM1+589, RRM1+2333, and SLC29A1+600), hence these loci were excluded from further analysis. The following gene loci pairs were in linkage disequilibrium: CDA+79 and CDA+435 (r^2=0.209), RRM1 (-756) and RRM1 (-269) (r^2=0.905), SLC28A2+65 and SLC28A2+225 (r^2=0.937), SLC28A1+565 and SLC28A1+709 (r^2=0.608), SLC28A1+1528 and SLC28A1+1561 (r^2=0.601).

Comparison of the genotype distributions of the Asian populations assessed in this study with those reported from Caucasians showed difference in 10/19 (53%) loci between Caucasians and Chinese, 10/19 (53%) between Caucasians and Malays and 7/19 (37%) between Caucasians and Indians (Figure 6.2.). There were no significant differences among genotype distributions for Asians (Chinese, Malays and Indians combined) in this study and those reported previously. [158-161]

6.4.2. Impact of hCNT2 Polymorphism on Neutropenia

Grade 3/4 neutropenia frequencies of gemcitabine in combination with carboplatin were found to associate strongly with sex in our previous phase I trial with 15 subjects. [15] In this current phase II study, we further tested if sex was still an important determinant on hematological toxicity with a much larger sample size (n = 58) using nonparametric test (Mann Whitney test). The results showed that there was a strong association between sex and neutrophil nadir (Figure 6.3).

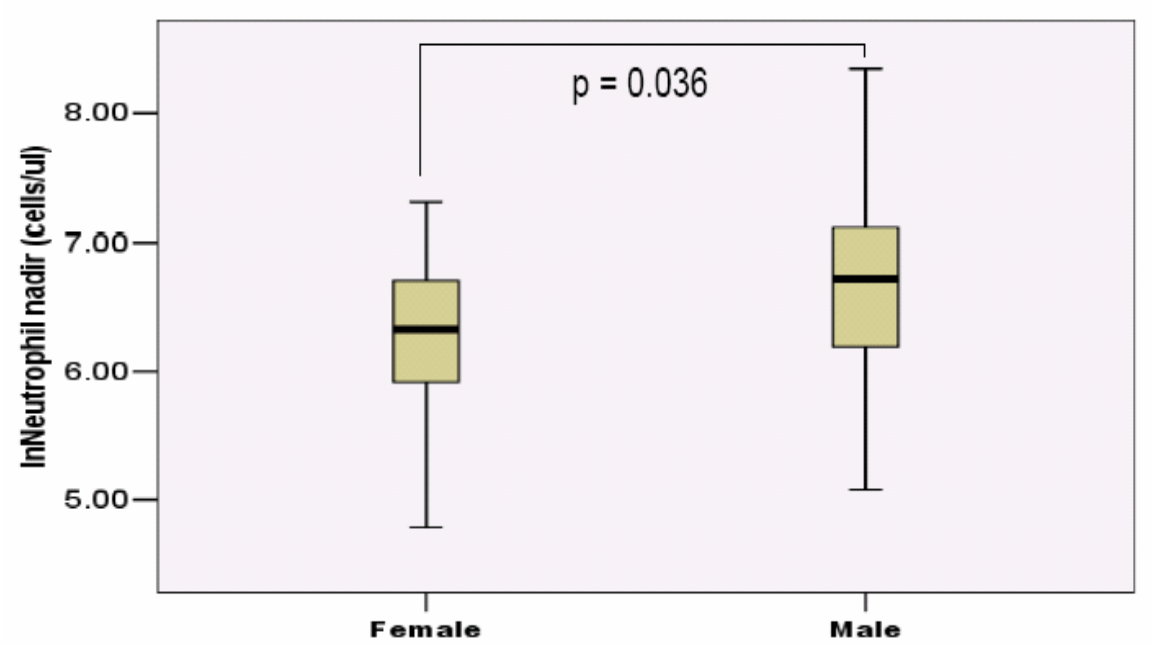


Figure 6.3 Effect of sex on neutrophil nadir to gemcitabine treatment

Baseline neutrophil counts between male and female subjects were comparable. The average counts for males and females were 6.60 ± 2.75 and 6.09 ± 3.84 respectively. No significant difference was detected by using t-test ($p = 0.63$) as well as Mann Whitney test ($p = 0.78$). However, female subjects had significant lower nadir neutrophil counts than those of male subjects after chemotherapy.

In order to answer why sex could result in a significant difference on neutropenia, pharmacokinetic and pharmacogenetic data analyses were conducted.

6.4.2.1. The Effect of Sex on Pharmacokinetics of Gemcitabine

Pharmacokinetic analysis was performed using WinNonLin version 5.2 (Pharsight Inc., Mountain View, California, USA). Non compartmental analysis was used to estimate the pharmacokinetic parameters. The terminal portion of the curve was determined by extrapolation of the log –linear concentration –time curve to infinity and regression of the last three points of the curve without weighting.

The important pharmacokinetic parameters like AUC, Cmax and Cl of gemcitabine and dFdCTP as well as AUC ratio of dFdU/gemcitabine were compared between males and females but no significant difference was detected [e.g. the average $AUC_{0\text{-}inf}$ of gemcitabine (1287.60 ± 541.22 μM*min) for females vs males (1437.94 ± 981.83 μM*min) was not significantly different ($p = 0.56$, t-test)].

6.4.2.2. Phenotypic and Genotypic analysis

Since neutropenia is a very common adverse effect during gemcitabine treatment, we only selected those SNPs which possess more than 10% frequency both for males and females. In addition, we focused on evaluating those SNPs with more than 2-fold difference between males and females. According to these two criteria, only two SNPs within one gene, SLC28A2+65 and SLC28A2+225, were selected as our study target SNPs (Figure 6.4).

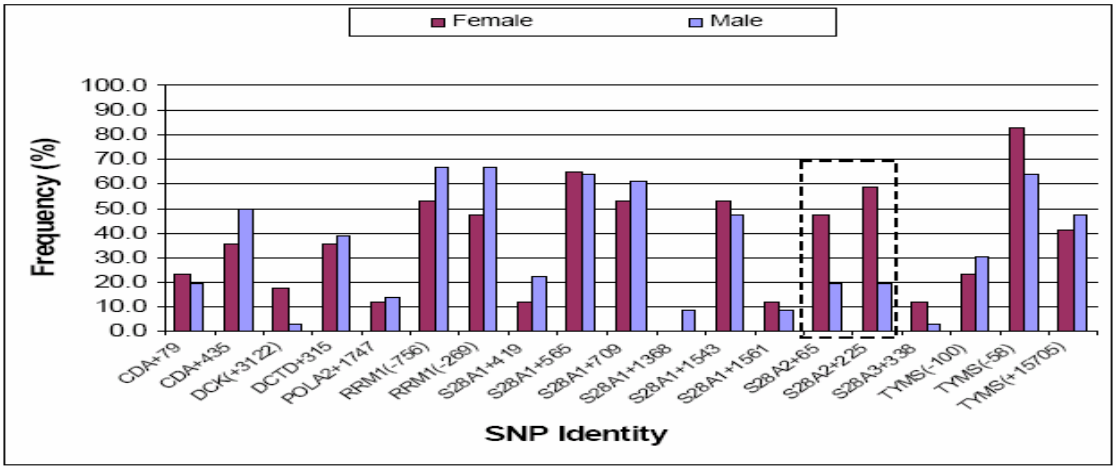


Figure 6.4 SNPs distribution between males and females

Both SLC28A2+65 C>T and SLC28A+225 C>A variants were associated with neutrophil nadir. For SLC28A2+65 C>T variants, patients with CC genotypes had a higher median neutrophil nadir than those with CT/TT genotypes (0.82 $x10^9$ cells/L and 0.46 $x10^9$ cells/L respectively, p=0.021) (Figure 6.5, left). For SLC28A+225 C>A variants, the median neutrophil nadir for genotypes CC vs CA/AA was 0.82 $x10^9$ cells/L and 0.46 $x10^9$ cells/L respectively (p=0.049) (Figure 6.5, right)

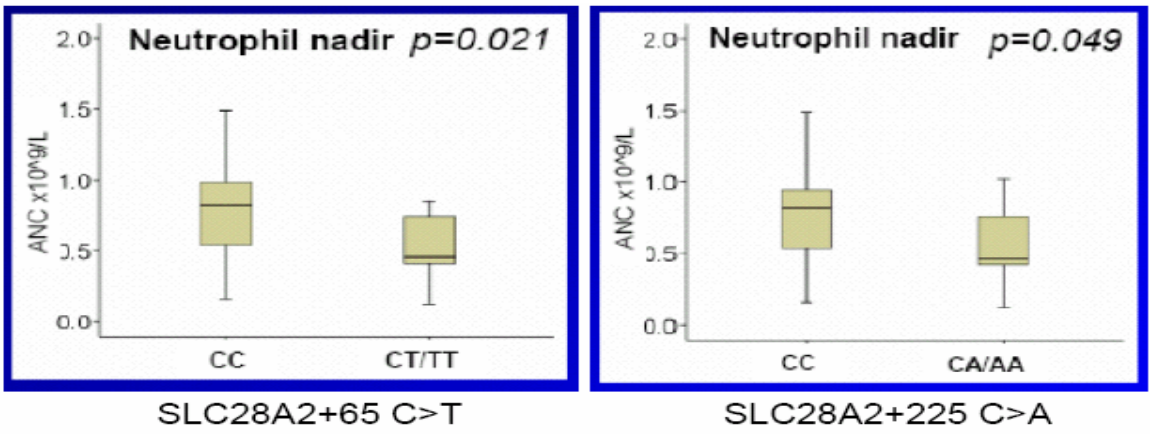


Figure 6.5 Association of SLC28A2+65 C>T (left) and SLC28A2+225 C>A (right) with neutrophil nadir

In addition, these two variants in SCL28 were also associated with patients' survival. The median overall survival of patients with the CC genotype was 8.2 (5.7-10.3) months and for CT/TT genotypes 18.3 (11.6-25.0) months (p=0.001) (Figure 6.6 left). The median overall survival in patients with CC and CA/AA genotypes was 8.5 (6.2-10.8) months and 18.3 (7.7-28.9) months respectively (p=0.003) (Figure 6.6 right).

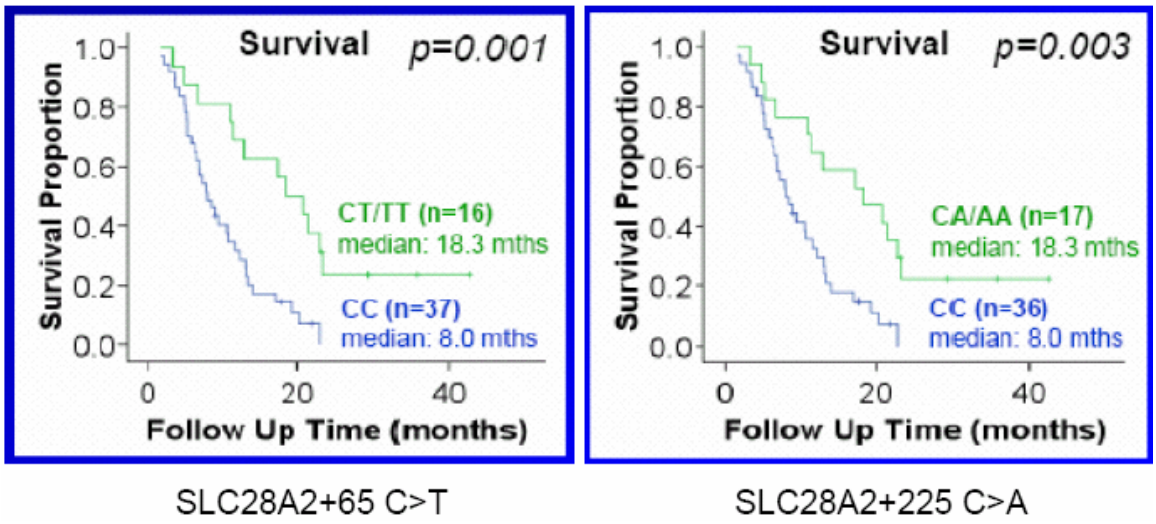


Figure 6.6 Association of SLC28A2+65 C>T (left) and SLC28A2+225 C>A (right) with survival

In separate multivariate analyses, both SLC28A2+65 CT/TT (Hazard Ratio = 0.31 [95% confidence interval = 0.15-0.65], p=0.002) and SLC28A+225 CA/AA (0.35 [0.17-0.71], p=0.004) genotypes were independently associated with improved overall survival after adjusting for stage and performance status. With both genotypes, stage and performance status entered, SLC28A2+65 CT/TT (0.09[0.01-0.84], p=0.035) was the only independent variable.

Neutropenia frenquency and survival of NSCLC patients in gemcitabine and carboplatin combination therapy are mainly dependent on genetic variants on human SLC28A2 (hCNT2) according to our systematic genetic screening on gemcitabine genetic pathways. Although sex seemed to have an important impact on neutropenia (Figure 6.7), with data showing significant differences in grade 0 and grade 4 neutropenia between males and females in 53 subjects, this influence could not be supported when the frequency analysis was done within mutant hCNT2 cohort (Figure 6.8).

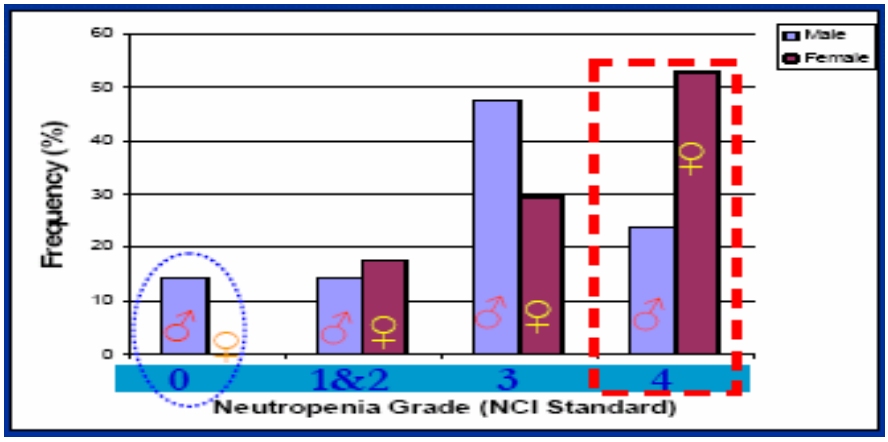


Figure 6.7 Comparison between neutropenia grade and frequency of all subjects (n = 53)

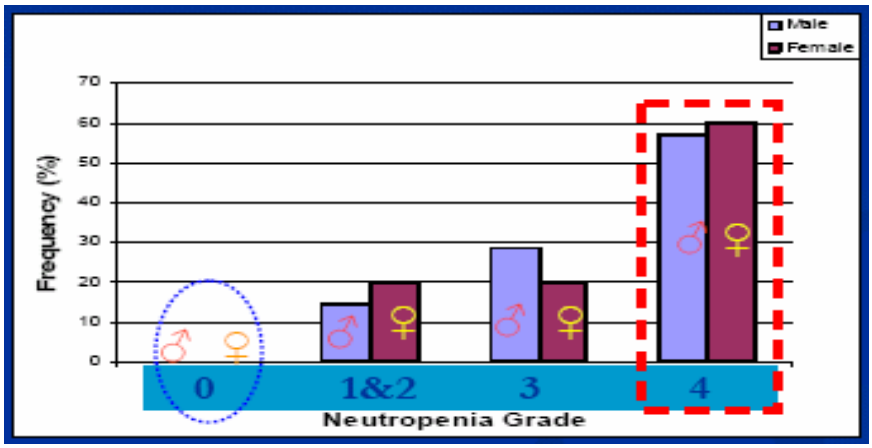


Figure 6.8 Comparison between neutropenia grade and frequency within the cohort expressing S28A2+225 (C>A) (n = 17)

6.5. Discussion

Studies have identified various gene polymorphisms that are associated with outcome and toxicity in patients with NSCLC. Recently, a polymorphism in the gene uridine diphosphate-glucuronosyltransferase 1A1 was associated with toxicity and survival in NSCLC patients treated with irinotecan-based chemotherapy has been reported.[163] To our knowledge, our study is the first to systematically assess the association between gemcitabine transport pathway gene polymorphisms with clinical outcome in NSCLC patients receiving gemcitabine-based chemotherapy.

The frequency of gene variants involved in the gemcitabine pathway has been previously reported in the healthy Caucasians [158] whereas data on Asian subjects have been limited to genes involved in the gemcitabine transport, SLC28A1, SLC28A2 [159, 161]and ribonucleotide reductase.[160] Our study contributes further to the literature by providing data on healthy Chinese, Indian and Malay subjects. No significant differences among the Asians with regards to genotypes distribution were found in our study. However we did find a significant difference in the distribution of up to 10 gene loci between healthy ethnic Asians and the Caucasian population.

Gemcitabine is a prodrug and its efficacy is highly dependent on intracellular drug concentration of dFdCTP. Some pioneer work in gemcitabine study showed that dFdCTP concentration in the peripheral leucocytes might be used as a surrogate marker to evaluate the efficacy and toxicities to gemcitabine treatment since dFdCTP was the major intracellular active metabolite (85-90%). [12, 47] Due to the difficulty in harvesting, sample preparation and quantification of dFdCTP concentrations in clinical samples, very few results were available on clinical pharmacokinetic studies of intracellular dFdCTP. Our

PK analysis did not show significant relationship between intracellular dFdCTP PK and the selected transporter genetic variants studied.

Sex seemed to influence neutropenia in our 53 NSCLC patients during their chemotherapy with gemcitabine and carboplatin combination therapy but no difference on neutropenia severity and frequencies were formed between males and females in the hCNT2 cohort. This strongly suggested that genetic variants but not sex would be a determinant factor in neutropenia and survival for infusion gemcitabine based therapies. Our data showed that both SLC28A2+65 C>T and SLC28A+225 C>A variants were significantly associated with neutrophil nadir and survival as well. Two adjacent amino acid changes in 2 SNPs variants could change substrate specificity of hCNT2. Previously even though hCNT2 was not regarded to be selective for pyrimidine nucleoside substrates, it has been identified as one of gemcitabine transporters according to latest research findings. [113, 117] Herein, we proposed that hCNT2 mutations (SLC28A2+65 C>T and SLC28A+225 C>A) could be potential genetic markers for better survival and more severe neutropenia to gemcitabine treatment. The reasons for high impact of hCNT2 on gemcitabine neutropenia and survival may be due to the change of substrate specificity of hCNT2 when mutations take place. Under normal physiological conditions, gemcitabine is mainly transported through hCNT1 and hCNT3 due to their substrate specificities for pyrimidine nucleosides. Under this condition, the effect of hCNT2 on gemcitabine's intracellular transportation could be negligible. However, when hCNT2 mutates, its impact on gemcitabine intracellular accumulation would be dramatically increased. Thus, this could result in a significant increase of dFdCTP concentration in hCNT2 mutant subjects since both variants of SLC28A2+65 C>T and SLC28A2+225

C>A are located in coding region of this transport gene. [161] Therefore, this genetic marker on hCNT2 could be very useful for oncologists to individualize patient treatment as patients with SLC28A2+65 C>T and SLC28A2+225 C>A would be expected to have a significant benefit on survival. In the mean while, this group of patients may also experience higher riskes of severe neutropenia. This genetic variant-drug response marker would have a greater clinical implication if it could be validated with a larger population. SLC28A2 +65 C>T variants were significantly lower in Chinese (12%) and Malays (55%) compared with Caucasians (63%) whereas the frequency of SLC28A2 +225 C>A variants was lower in the Chinese (12%) compared with Caucasians (55%). Based on the differences in the distribution of these gene variants, we speculated that Asians may have a lower risk of hematological toxicity but also a lower overall survival through gemcitabine treatment. The effect of ethnicity on gemcitabine pharmacology should be further investigated in larger scale clinical trials.

6.6. Conclusions

Although the genetic distribution of gemcitabine activation pathway related genes have been studied intensivly in Caucasians, less information is available for Asians. A systemic screening of genetic polymorphism was processed in our study for understanding genetic distribution in healthy Asian population; ten gene loci were found to be significantly different in the distribution of genotypes between Asians and Caucasians among 25 SNPs.

Sex seemed to have some impact on hematological toxicities to gemcitabine treatment in our Phase I clinical trial and a larger scale clinical Phase II study. However, this influence of sex was found to be not important factor in our study. Instead, SNPs in hCNT2 were identified as a potential determinant on hematological toxicities and patient survival in gemcitabine treatment combined with carboplatin although no pharmacokinetic differences were detected between wild-type and variants of hCNT2. With current advanced gene screening technique, it would be very convenient and fast to analyze the SNPs in hCNT2 for NSCLC patients in gemcitabine based treatment. This could provide oncologists with valuable information for individual patient's treatment. Nevertheless, a larger clinical trial would be necessary to further validate our current findings.

CHAPTER SEVEN

Conclusions

In this thesis on Pharmacology of gemcitabine in the Asian Population, bioanalytical methods for accurate, reliable and precise quantitation of plasma gemcitabine and dFdU as well as intracellular dFdCTP formed the essence for the studies. These methods were successfully developed and validated.

The chapter on *in vitro* study demonstrated that exposure time above 2 μM gemcitabine was an important factor for achieving cytotoxic effects on HONE1 cells. In addition, gemcitabine resistance can be overcome by novel combination therapies, e.g. gemcitabine plus PXD101.

For the clinical studies, the initial dose finding trial for fixed rate infusion of 10 mg/m^2/min gemcitabine indicated that 75 min infusion (total dose of 750 mg/m^2) was tolerable for Asian patients. This fixed rate schedule was selected as arm A for comparison with arm B, the standard 30 min infusion of 1000mg mg/m^2 in the later phase II trial. The results showed no significant differences between the two arms with regards to pharmacokinetic parameters, patients' toxicities and clinical efficacy even though there was a 25% lower total dose of gemcitabine at the fixed rate infusion schedule. One interesting indication found was that the metabolite/parent (dFdU/gemcitabine) ratios at 120 min were highly related to early phase response rate according to RECIST criteria. The ratios of dFdU/gemcitabine at 120 min for non-responders were significantly higher than those of the responders. Ratios larger than 500 seemed to predict that the patient would be a non-responder in the early phase treatment cycle to gemcitabine.

Finally, the genetic status in hCNT2 was identified as a determinant of hematological toxicities and survival in treatment with gemcitabine in combination with carboplatin through pharmacogenetic and pharmacodynamic association. The patients with genetic

variants both in SLC28A2+65 C>T and SLC28A2+225 C>A would have a longer survival but also could experience more serious toxicities than those with wild-type. Genetic screening for hCNT2 could be a useful clinical disease progression marker.

Both the dFdU/gemcitabine ratios at 120 min as a potential predictor for early phase response as well as the patients' genetic status of hCNT2 as a useful marker for toxicities and survival need to be validated with a larger prospective trial.

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