Pharmacokinetic-Pharmacodynamic and Pharmacogenetic Studies of Flavopiridol and its Glucuronide Metabolite

DISSERTATION

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Abstract

Chronic lymphocytic leukemia (CLL) is the most common adult leukemia in Western world. Flavopiridol (Alvocidib, NSC 649890), as a pan cyclin-dependent kinases inhibitor (CDKI), initiates cell cycle arrest and p53-independent apoptosis through down-regulation of Mcl-1 and X-linked inactivator of apoptosis (XIAP). A novel pharmacokinetically (PK)-based dosing schedule with a 30-minute intravenous bolus loading dose (IVB) followed by a 4-hour continuous intravenous infusion (CIVI) in patients with refractory CLL produced an approximately 50% overall response rate. A major flavopiridol metabolite, flavopiridol glucuronide (flavo-G), was also evaluated in this study. In order to fully understand the between-subject variability (BSV) between patients, a phase 1 CLL patient data set (OSU0055) was evaluated and a two-compartment flavopiridol PK model followed by first-order elimination was developed by nonlinear mixed effects modeling. Bilirubin level was shown as a significant covariate, and OATP1B1 was first time discovered having a significant correlation with flavopiridol PK parameters. A functional analysis in vitro study was done to confirm that flavopiridol and flavo-G are substrates of OATP1B1. Since cellular redox status is important on cell survival and previous studies showed that flavopiridol can induce the decrease of intracellular GSH levels in transformed cells, glutathione (GSH) level was evaluated among leukemia cell models and patient’s CLL cells. The change in GSH level
compared to baseline after flavopiridol treatment varied among cell models and individual patient. In order to evaluate flavo-G with flavopiridol treatment, a linked parent-metabolite population PK model was developed. This model was expanded to include a pharmacodynamic logistic-regression component linked to flavopiridol exposure level. This developing PK-PD-PG model of flavopiridol will help to characterize the factors associated with inter-individual variability in drug disposition and outcomes, and provide us better understanding of the mechanisms and factors governing the balance between safety and efficacy.
Dedication

This document is dedicated to my family.
Acknowledgments

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Finally, my special thanks go to my family. I am deeply thankful to my parents for their everlasting love, support and encouragement.

“It's not what I am underneath, but what I do, that defines me.”
Vita

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Major Field: Pharmacy

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Table of Contents

Abstract ................................................................................................................................. ii
Dedication ............................................................................................................................ iv
Acknowledgments ............................................................................................................... v
Vita ...................................................................................................................................... vi
Publications ........................................................................................................................ vi
Fields of Study .................................................................................................................... viii
Table of Contents ................................................................................................................ ix
List of Tables ........................................................................................................................ xv
List of Figures ....................................................................................................................... xvi

Chapter 1: BACKGROUND AND INTRODUCTION ...................................................... 1

1.1. Background .................................................................................................................... 1

1.1.1. Flavopiridol .............................................................................................................. 1

1.1.2. Clinical Studies of Flavopiridol for Treatment of CLL ........................................... 2

1.1.3. Disposition of Flavopiridol and its Glucuronide Metabolits .................................... 3

1.1.4. Flavopiridol and Glutathione .................................................................................. 4

1.2. Significance .................................................................................................................... 5
3.1. Introduction ................................................................................................................. 39

3.2. Materials and Methods .............................................................................................. 41
  3.2.1. Materials ................................................................................................................. 41
  3.2.2. Cell Culture ............................................................................................................. 42
  3.2.3. Transporter Study .................................................................................................. 42
  3.2.4. Data Analysis and Statistics .................................................................................. 42

3.3. Results ........................................................................................................................ 44
  3.3.1. Flavopiridol Uptake Kinetics .................................................................................. 44
  3.3.2. Uptake of Flavopiridol and Flavo-G by OATP1B1-Expressing Cells ................. 45
  3.3.3. Uptake of Flavopiridol by Non-synonymous OATP1B1 SNPs. ....................... 45

3.4. Discussion ................................................................................................................... 46

Chapter 4: ROLES OF GLUTATHIONE IN FLAVOPIRIDOL MEDIATED CHRONIC
LYMPHOCYTIC LEUKEMIA CELL DEATH ....................................................................... 53

4.1. Introduction .................................................................................................................. 53

4.2. Materials and Methods .............................................................................................. 55
  4.2.1. Reagents ................................................................................................................ 55
  4.2.2. Cell Culture .......................................................................................................... 55
  4.2.3. Glutathione Assay ............................................................................................... 56
  4.2.4. Cytotoxicity Assays ............................................................................................ 57
4.2.5. Data Analysis ........................................................................................................... 58
4.2.6. Statistical Analysis ............................................................................................... 58

4.3. Results ....................................................................................................................... 59
4.3.1. Depletion of GSH by BSO, 4-S-CPG, or NAC and Flavopiridol Combination.
........................................................................................................................................ 59
4.3.2. GSH Mediates Flavopiridol Cytotoxicity in Leukemia Cell Models ............... 59
4.3.3. Baseline GSH and ΔGSH are Variable in Patient CLL cells. ......................... 60

4.4. Discussion .................................................................................................................. 61

Chapter 5: CLINICAL POPULATION PHARMACOKINETIC MODEL OF
FLAVOPIRIDOL AND FLAVO-G IN CHRONIC LYMPHOCYTIC LEUKEMIA

PATIENTS ......................................................................................................................... 68
5.1. Introduction ............................................................................................................... 68

5.2. Methods ..................................................................................................................... 69
5.2.1. Study Protocols and Subjects ........................................................................... 70
5.2.2. Pharmacokinetic Sampling and Analytical Assay ........................................... 70
5.2.3. Structural Model Development ......................................................................... 70
5.2.4. Full Model and Final Model Development ....................................................... 72
5.2.5. Evaluation of Associations Between PGx, PK and Outcomes ....................... 72
5.2.6. Model Performance and Stability ..................................................................... 73
7.2. Significance ................................................................................................................. 111
List of Tables

Table 2.1 Demographics and baseline labs of patients from OSU0055 and OSU0491...21
Table 2.2 Base model PK parameter estimates................................................................. 24
Table 2.3 Final model PK parameter estimates ................................................................. 29
Table 2.4 Population estimates from final model and bootstrap analysis....................... 2930
Table 2.5 Population mean parameters for flavopiridol under the index and validation
data set. .................................................................................................................................. 33
Table 4.1 Cytotoxicity of flavopiridol in the absence or presence of GSH-altering agents
after 72 hours ....................................................................................................................... 66
Table 5.1 Final model PK parameter estimates ................................................................. 90
Table 6.1 Comparison of different estimation methods used on base pharmacodynamic
model...................................................................................................................................... 103
Table 6.2 Summary of population pharmacodynamic model building runs................. 104
List of Figures

Figure 1.1. Structure of flavopiridol ................................................................. 7
Figure 2.1. Structure of two-compartment model of flavopiridol ...................... 23
Figure 2.2. Correlation between bilirubin level (mg/dL) and its random effects (ETA).. 25
Figure 2.3. Goodness of fit of the pharmacokinetic model for flavopiridol .......... 26
Figure 2.4. Conditional weighted residual (CWRES) versus population prediction, ID, Time (log scale) from final model. ................................................................. 27
Figure 2.5. The histogram of conditional weighted residual (CWRES).............. 28
Figure 2.6. Bootstrap results from final model ...................................................... 38
Figure 2.7 Association of PGx, PK and bilirubin from the phase 1 dataset .......... 34
Figure 2.8 Association of flavopiridol AUC and PGx from the phase 1 and phase 2 datasets .................................................................................................................. 38
Figure 3.1 Mixed model (Equation 5.2) nonlinear least squares fit of flavopiridol uptake data generated in HEK293 cell cultures .................................................. 49
Figure 3.2 Nonlinear least squares fits of flavopiridol uptake data generated in HEK293 cell cultures ........................................................................................................ 50
Figure 3.3 Uptake of flavopiridol, flavo-G, SN-38 and lenalidomide in OATP1B1 transfected cells ............................................................................................................ 51
Figure 3.4 Uptake of flavopiridol in nonsynonymous polymorphic variants of OATP1B1 ................................................................. 52
Figure 4.1 Flavopiridol demonstrates disruption of homeostasis of intracellular GSH in transformed leukemia cells ................................................................. 63
Figure 4.2 Comparison of residual viable cells after treatment with the absence or presence of GSH-altering agents ................................................................. 65
Figure 4.3 Normalized Intracellular baseline GSH and ΔGSH in patient CLL cells ...... 67
Figure 5.1 Two metabolite model structures of linked two-compartment model of parent (flavopiridol) and one-compartment of metabolite (flavo-G)................................. 81
Figure 5.2 Goodness-of-fit plots from two metabolite models................................. 82
Figure 5.3 CWRES versus dependent variables................................................... 84
Figure 5.4 CWRES versus independent variable.................................................. 86
Figure 6.1 PK Parameters versus covariates plot............................................... 89
Chapter 1: BACKGROUND AND INTRODUCTION

1.1 Background

1.1.1. Chronic Lymphocytic Leukemia

Chronic lymphocytic leukemia (CLL) is a lymphoid disease and most commonly occurs in the Western hemisphere (Lin 2010). B lymphocytes in patients with CLL are morphologically mature but immunologically incompetent (Keating, Chiorazzi et al. 2003). One major obstacle for CLL treatment is that CLL cells are resistant to apoptosis which causes prolonged cell survival and drug resistance in CLL patients (Keating, Chiorazzi et al. 2003). Although currently available agents such as fludarabine, cyclophosphamide, and rituximab are effective in inducing overall response (OR) rates, complete response (CR) rates and progression free survival (PFS) initially, most patients eventually develop drug resistance leading to disease relapse by unfavorable cytogenetic alterations (Robak, Jamroziak et al. 2009).

1.1.2. Flavopiridol

Flavopiridol (NSC 649890, alvocidib) is an N-methylpiperidinyl, chlorophenyl flavone, and its chemical structure is identical to a product obtained from *Dysoxylum binectariferum*, an indigenous plant found in India (Fig. 1.1). It inhibits cyclin-dependent kinase (CDK) by
alteration of tyrosine phosphorylation (Carlson, Dubay et al. 1996; Sausville, Johnson et al. 2000; Senderowicz and Sausville 2000) and competitive inhibition with ATP (Worland, Kaur et al. 1993). Through the inhibition of CDK7 and CDK9, flavopiridol inactivated positive transcription elongation factor b (pTEF-b), a kinase that phosphorylates and activates RNA polymerase II (RNAP II). Flavopiridol can down-modulate a variety of anti-apoptotic proteins such as Mcl-1 and X-linked inactivator of apoptosis (XIAP) via a p53-independent mechanism in CLL. Chromosomal aberrations in CLL patients have been defined into five categories: 17p deletion, 11q deletion, 12q trisomy, normal karyotype, and 13q deletion (Dohner, Stilgenbauer et al. 2000). Previous studies have shown that patients with 17p deletions had the worst prognosis, followed by patients with 11q deletions (Dohner, Stilgenbauer et al. 2000). Flavopiridol has shown promising clinical activity in CLL patients with high-risk genetic abnormalities such as del(17p13) and del(11q22) (Byrd, Lin et al. 2007).

1.1.3. Clinical studies of flavopiridol for treatment of CLL

Previously, a variety of different schedules of administration including a 72-hour hour continuous intravenous infusion (CIVI) (Byrd, Peterson et al. 2005), 24-hour CIVI (Schwartz, O'Reilly et al. 2002; Flinn, Byrd et al. 2005) and 1-hour intravenous bolus (Tan, Headlee et al. 2002) have been explored with flavopiridol in solid tumor and hematologic malignancies. Single agent flavopiridol using the 24- or 72-hour infusion in phase 2 clinical trial did not show significant clinical activity (Stadler, Vogelzang et al. 2000; Schwartz, Ilson et al. 2001; Shapiro, Supko et al. 2001; Aklilu, Kindler et al. 2003).
Modest activity was noted in CLL (Byrd, Peterson et al. 2005) and mantle cell NHL (Kouroukis, Belch et al. 2003) with a 1-hour bolus at 50mg/m² daily for three days.

A pharmacokinetically-based dosing schedule was developed by our group thereafter, which is 30 minutes intravenous bolus followed by 4 hours CIVI. We accomplished a phase 1 study (OSU0055) by using that dosing schedule for treatment in patients with refractory CLL (Byrd, Lin et al. 2007). In that study, overall response rate was more than 50% (Byrd, Lin et al. 2007; Phelps, Lin et al. 2009). Tumor lysis syndrome (TLS), which was caused by rapid tumor cell death, was the most serious toxicities after flavopiridol treatment (Christian, Grever et al. 2009).

1.1.4. Disposition of flavopiridol and its glucuronide metabolite

Previous studies have shown that flavopiridol elimination occurs through extraction and metabolism and is influenced by the multi-drug resistance protein-2 (MRP2, ABCC2) (Jager, Gehring et al. 2003; Jager, Gehring et al. 2003) and the breast cancer resistance protein (BCRP, ABCG2) (Robey, Medina-Perez et al. 2001; Kodaira, Kusuhara et al. 2010) which contribute to biliary excretion of both parent and glucuronide metabolite. Since sinusoidal efflux transporters, multidrug resistance-associated protein 1, 3 and 4 (MRP1/ABCC1, MRP3/ABCC3, and MRP4/ABCC4) accept glucuronide conjugates as substrates (Zhou, Wang et al. 2008; Hirouchi, Kusuhara et al. 2009), flavopiridol glucuronide (flavo-G) may also be exported into plasma from hepatocytes or excreted into urine from kidney by those efflux transporters.
1.1.5. *Flavopiridol and glutathione*

Glutathione (GSH) is an abundant natural tripeptide and synthesized in every cell of higher eukaryotes (Klaunig and Kamendulis 2004). Antioxidant action is one of the important roles of GSH, which helps maintenance the redox state of the cells (Lillig, Berndt et al. 2008). GSH reduces cytoplasmic protein disulfide bonds to cysteines, and GSH itself is oxidized to form glutathione disulfide (GSSG) in the process (Kemp, Go et al. 2008). In cells GSH is synthesized from its constituent amino acids, glutamic acid, cysteine and glycine, via two sequential ATP-consuming steps, which are catalyzed by γ-glutamylcysteine synthetase (γ-GCS) and GSH synthase. The synthesis of the di-peptide γ-glutamylcysteine by γ-GCS is the rate-limiting step in *de novo* GSH synthesis.

Previous studies showed that flavopiridol can induce a moderate decrease of intracellular GSH levels in transformed cells, such as K562, HeLa, RPMI-8226, PBMCs, and Jurkat cells, the later representing a significant change in levels (Ge, Byun et al. 2008). However, the mechanisms of flavopiridol-induced GSH level decreases in those cells are still unknown.

Trachootham *et al* showed that CLL cells display an elevated level of ROS associated with mitochondrial DNA (mtDNA) mutations and oxidative DNA damage compared to normal lymphocytes (Zhou, Hileman et al. 2003; Trachootham, Zhang et al. 2008). Mitochondria are essential cellular organelles that play important roles in energy metabolism and generation of ROS (Szewczyk and Wojtczak 2002). Under physiological
conditions, intracellular ROS is important in keeping redox balance and cellular proliferation (Pelicano, Feng et al. 2003). However, high levels of ROS can also cause cellular damage, depending on the levels and duration of ROS stress (Pelicano, Carney et al. 2004). Many cancer cells are in general metabolically active, exhibit increased production of ROS associated with mitochondrial dysfunction, which make them more vulnerable to exogenous ROS-generating agents through further oxidative stress (Szatrowski and Nathan 1991; Devi, Prasad et al. 2000; Pelicano, Carney et al. 2004).

It has been shown that human CLL cells have higher basal ROS levels, lower basal GSH levels than normal lymphocytes (Trachootham, Zhang et al. 2008), and high sensitivity to ROS stress induced by 2-methoxyestradiol and arsenic trioxide shown to effectively kill human CLL cells (Pelicano, Feng et al. 2003). After flavopiridol treatment, there was a significant increase of ROS and significant decrease of GSH in Jurkat cells (Decker, Dai et al. 2001). Studies showed defected oxygen consumption because of early mitochondrial damage in CLL cells after flavopiridol treatment (Kitada, Zapata et al. 2000). Because mitochondria are the major source of ROS production and rely on GSH as a major antioxidant (Fernandez-Checa, Kaplowitz et al. 1998), it is possible that the rapid redox imbalance and ROS mediated mitochondrial damage are caused by a rapid loss of cellular GSH (Trachootham, Zhang et al. 2008).

1.2. Significance
Chemoimmunotherapy which combines alkylating agents and purine nucleoside analogs with monoclonal antibodies has improved initial overall response (OR) rates, complete response (CR) rates and progression free survival (PFS). However, standard therapies still not able to cure CLL (Maddocks and Lin 2009). Flavopiridol has been used as a single agent for CLL treatment and has been shown 40-50% response in refractory or relapsed CLL patients (Byrd, Lin et al. 2007; Phelps, Lin et al. 2009; Lin 2010). However, since its narrow therapeutic window, the balance between safety and efficacy is a primary challenge for this drug. A thorough understanding of the factors influencing the onset of hyper-acute tumor lysis syndrome and inter-individual variability in response is a priority in order to maximize the benefit and minimize toxicities experienced by patients receiving therapy with this promising agent. In addition, by understanding the mechanisms of this drug will help us to design equally potent drugs with better safety profiles.
Figure 1.1. Structure of flavopiridol
Chapter 2: CLINICAL POPULATION PHARMACOKINETICS OF FLAVOPIRIDOL IN PATIENTS WITH CHRONIC LYMPHOCYTIC LEUKEMIA

2.1. Introduction

Flavopiridol, the first clinically evaluated CDK inhibitor, has shown promising results in patients with refractory CLL as discussed in chapter 1. However, like many other cytotoxic anti-cancer agents, it has a narrow therapeutic window, reducing the incidence of adverse events and increasing the probability of beneficial outcomes is a primary goal. Population PK analysis allows us to evaluate multiple factors, such as demographic, patient, and disease characteristics, and pharmacogenetic factors, etc, simultaneously. It is an efficient way to screen large number of individuals involved in our clinical trials and help us to understand factors leading to variability in PK for appropriate use of flavopiridol. This chapter describes the clinical population pharmacokinetic (PK) of flavopiridol in CLL patients. In order to evaluate the different levels of variability, such as between-subject variability (BSV) and residual variability, nonlinear mixed effect modeling method was applied for this PK analysis.

The model building process was based on a phase 1 data set (OSU0055) with 52 subjects, for which safety and tolerability were reported previously (Byrd, Lin et al. 2007; Phelps,
Lin et al. 2009). The aim of this study was to evaluate potential covariates which may correlate with variability from population PK analysis. Another phase 2 data set (OSU0491) with 67 subjects was used as a validation data set. Univariate analysis was used to evaluate the correlation between pharmacokinetic parameters and polymorphisms in drug transporters and UGTs. The clinical significance of such a population pharmacokinetic model is that it may be used to prospectively individualized flavopiridol therapy to achieve a target systemic exposure.

2.2. Methods

2.2.1. Study Protocols and Clinical Design

A phase 1 study (OSU-0055) data set was used to build a population pharmacokinetic model, and a phase 2 study (OSU-0491) was used for validation purposes. OSU-0055 and OSU-0491 clinical details (Byrd, Lin et al. 2007; Lin, Ruppert et al. 2009; Phelps, Lin et al. 2009) and analytical methods for flavopiridol and flavo-G were published previously by our group (Phelps, Rozewski et al. 2008; Blum, Phelps et al. 2010; Ni, Ji et al. 2010).

2.2.2. Structural Model Development

A total of 577 plasma concentration-time values from 52 patients in a phase 1 study (OSU0055) were used. The flavopiridol concentrations were below the lower limit of quantification (LLOQ) in a few samples immediately before the next flavopiridol infusion. Only samples above the LLOQ were included in the analysis.
Data were analyzed using nonlinear mixed effects modeling in NONMEM (Version 7.1.2; ICON, Ellicott City, MD, USA) to characterize the pharmacokinetics of flavopiridol in CLL patients. A sequence of first order (FO) followed by first order conditional estimation with $\eta$-$\varepsilon$ interaction (FOCEI) was implemented within each using as initial parameters those defined by the structure parameters ($\theta$), the variance of inter-subject errors or the intra-subject error for an individual ($\omega$), and the variance of residual error ($\sigma$) statements. In the FO method, the random vector $\eta$ is approximated by its mean value 0. That estimation method is improved by the FOCE algorithm which approximates $\eta$ by an individualized estimate $\eta_i$. The FOCEI method is the FOCE method applied with intra-individual mean-variance models (Beal 1989-2010).

In our PK data set, flavopiridol concentration data were expressed as $\mu$g/mL; Actual doses were expressed in mg and time in hour. Flavopiridol data were fitted simultaneously using the NONMEM subroutine ADVAN3 TRANS4. BSV in the different pharmacokinetic parameters was estimated using a proportional error model. For instance, variability in CL was estimated using $CL_i = \theta_i \cdot \exp(\eta_i)$, in which $CL_i$ represents the CL of the ith individual, $\theta_i$ is the population value of CL, $\eta$ is the between-subject random effect with mean 0 and variance $\omega^2$. BSV was estimated on all parameters initially. The gradient algorithms use the information from the gradient of the objective function to estimate where and how far to make the next step in the iterative process. When gradient starts become zero, it means that particular BSV is not able to be calculated (Ene I. Ette 2007). During our model iterative process, if gradient on particular
BSV started become zero, that BSV would be removed. Residual variability was estimated with a combined proportional and additive error model assuming a constant coefficient of variation over the complete concentration range superimposed on a constant absolute error.

The performance of the model was judged using both statistical and graphical methods (Ette and Ludden 1995; Wahlby, Jonsson et al. 2001). Statistical methods included the likelihood ratio test (LRT) which evaluates the change of objective function value (OFV) for the data set. The OFV equals the negative of two times the log likelihood (-2LL) of the data set. The change in OFV between two hierarchical models is chi-squared distributed with \( p_2 - p_1 \) degrees of freedom (\( p_1 \) and \( p_2 \) are the numbers of estimable parameters for the two models). \( \Delta \text{OFV} \) greater than 3.84 (Type I error rate: \( p<0.05 \)) was determined. For graphical methods, goodness-of-fit plots (observed versus population prediction and individual prediction concentration), diagnostic plots (conditional weighted residual (CWRES) versus dependent variable (population prediction) and independent variable (ID and time), histograms of ETA distributions) were evaluated (Ette and Ludden 1995).

2.2.3. Final Model Development

The addition of a covariate should account for some unexplained BSV and lead to a reduction in the OFV, as well as a better fit of the model to the data. When a covariate reduces BSV significantly, it may be used to improve model performance. Visual
evaluation was used to evaluate scatter plots of covariates versus each PK parameter and its BSV: age (years), body surface area (BSA, m\(^2\)), body weight (kg), platelets (\(\times 10^9/L\)), white blood cell count (WBC \(\times 10^9/L\)), hemoglobin (mM), albumin (g/dL), serum total protein (g/dL), calcium (mg/dL), chloride (mg/dL), potassium (mM), magnesium (mg/dL), sodium (mM), phosphate (mg/dL), alkaline phosphatase (ALP, U/L), alanine aminotransferase (ALT, U/L), aspartate aminotransferase (AST, U/L), serum total bilirubine (mg/dL), serum creatinine (CRT, mg/dL), lactate dehydrogenase (LDH, U/L), uric acid (mg/dL). If multiple covariates shown correlated with PK parameters and BSV, generalized additive model (GAM) approach as implemented in the software program Xpose (version 4.0) was used.

Each individual covariate was fit to the structural model using the power (equation 1) or fractional change (equation 2) models.

\[
\theta_i = \theta_{pop} \times \left( \frac{CON_{ji}}{CON_{j-med}} \right)^{\theta_j} \times \exp(\eta_i) \quad (1)
\]

\[
\theta_i = \theta_{pop} \times (1 + CAT_{ji} \times \theta_j) \times \exp(\eta_i) \quad (2)
\]

where \(\theta_i\) is the individual PK parameter estimate, \(\theta_{pop}\) is the population parameter estimate for the typical individual, and \(\eta_i\) is the BSV of parameters approximately log-normally distributed with a mean of 0 with standard deviations of \(\omega^2\) as mentioned above. \(CON_{ji}\) is the value of the continuous covariate \(j\) for individual \(i\), \(CON_{j-med}\) is the
population median value of covariate $j$ for all individuals, $\text{CAT}_{ji}$ is the value of the categorical covariate $j$ for individual $i$, and $\theta_j$ is an estimated parameter.

2.2.4. Internal Model Validation

Non-parametric bootstrapping method was used for internal model validation since it allows the parameter values to converge independently and a reasonable estimate of the confidence intervals was generated. 800 bootstrapped data sets were created. The results of all runs were used to create the percentile bootstrap confidence intervals, which were constructed by taking the lower 2.5% and the upper 97.5% value of each parameter estimate. This interval would cover the true value of the parameter estimate approximately 95% of the time without imposing an assumption of symmetry on the distribution.

Visual prediction check (VPC) plots were used to assess the model performance by Perl-speaks-NONMEM (PsN v3.2.12) (Yano, Beal et al. 2001; Lindbom, Ribbing et al. 2004). A total of 500 replicates were simulated using the final model to simulate expected concentrations and the 95% prediction intervals (PIs) were generated. The observed data was compared with data simulated from the final model and 95% PIs were visualized as shaded area. Any potential model mis-specifications were visualized by discrepancies between the dots representing the observed data and the shaded area.

2.2.5. External Model Validation
A total of 813 plasma concentration-time values from 67 patients in a phase 2 study (OSU0491) were used as a validation data set. This data set was used to fit the current model from phase 1 study and estimate the model parameters. The difference of the population mean parameter estimates from the index and validation data sets were evaluated.

2.2.6. Evaluation of Associations between PGx, PK and Outcomes

Both index data set and validation data set were used to evaluation of associations between PGx, PK and outcomes. Genes known to specifically affect flavopiridol disposition from previous literature, such as UGT1A1, UGT1A9, ABCC2 and ABCG2 and additional set of 52 other genes that code for metabolic enzymes and transporters commonly involved in drug disposition were investigated in this study. Sequencing method and a complete list of the genes and single-nucleotide polymorphisms (SNPs) evaluated was presented previously (Dai, Papp et al. 2008).

2.2.7. Data analysis and Statistics

To identify associations with genetics, the means of flavopiridol PK parameters were compared based on SNP genotypes using Student’s t-test and analysis of variance (ANOVA).

2.3. Results

2.3.1. Base Model Identification
577 flavopiridol concentrations from 52 patients from OSU0055 were used for this model. The demographic characteristics baseline labs of the study are listed in Table 2.1. A two-compartment model of flavopiridol PK was the most satisfactory to describe the observations. The structure of the final model is shown in Fig 2.1. BSV was initially assumed on each parameter (CL, V1, Q and V2). Base model parameter estimates and random effects are presented in Table 2.2. The shrinkage of the random effects was less than 30%, except for the between occasion viability. The objective function value for this base model was -3024.48. Overall, the fits are unbiased, reflecting the adequacy of the two-compartment model for flavopiridol.

2.3.2. Final Model Identification
Demographic and lab covariates versus PK parameters and BSV were subsequently evaluated by visual evaluation in the base model as described in the Method section. Bilirubin was indicated as the most significant covariate with a direct positive effect on flavopiridol inter-compartmental clearance (Q). When bilirubin was added as a covariate for Q (OFV= -3032.33), ΔOFV decreased by 7.84 (p<0.005) with 1 degree of freedom, and the COV step successfully completed. Adding bilirubin as a covariate for Q reduced inter-individual random effects on Q when comparing the base model (Fig. 2.2 (A) and (B)). GAM method was not used for further covariate evaluation since none of the covariates shown to affect PK parameters except bilirubin. The goodness-of-fit plots of both base and final models were shown in figure 2.3. There were slightly over-predicted of population prediction and individual prediction on base model, whereas some
improvements were shown on final model. All the conditional weighted residual (CWRES) were randomly scattered around the zero reference line, which suggested the adequacy of the residual error model (Fig. 2.4). Left skewed distribution of CWRES was reduced in final model compared to the base model (Fig. 2.5).

2.3.3. Internal Model Validation

The bootstrap method was used to estimate the bias, standard errors, and confidence interval in the final covariate model. 800 replicate data sets were generated and the final model was fit to each data set and the distribution of the parameter estimates examined for bias and precision. Table 2.4 lists the results of the bootstrap procedure, presented as mean and 95% bootstrap confidence intervals of the parameter estimates and random effects of the final model. Frequency distributions of parameters and their BSV from bootstrapping were shown in Fig. 2.6. All parameters were fall in 95% bootstrap confidence intervals. VPC results showed that the model predicted the observed concentrations well and 95% prediction interval was overlaid with observation concentrations (Fig. 2.7).

2.3.4. External Model Validation

The population PK model developed from the phase 1 (OSU0055) index data set was used to fit the phase 2 (OSU0491) validation data set. Table 5 presents the population mean parameter estimates for the index and validation data sets. All model parameters from the validation data set were comparable to those from the index data set.
2.3.5. *Evaluation of pharmacogenetic effects on final model PK parameters*

SNPs which significantly associated with PK parameters were shown in Figure 2.6 and p-values were listed in Table 2.6. Univariate analysis was also completed on flavopiridol AUC in both the phase 1 and 2 data sets independently. There were significant correlations between flavopiridol AUC versus SLCO1B1_rs3829310 (p=0.08) and ABCG2_rs2231142 (p=0.076) (Figure 2.9). Although there were no significant correlations between flavopiridol AUC versus these two SNPs in phase 1, which were probably due to limited patient number in this study, the trend between flavopiridol AUC versus these two SNPs were consistent.

**2.4. Discussion**

For the novel anti-cancer agent flavopiridol, it has narrow therapeutic window and its PK profile was previously observed to be associated with clinical outcomes, including response, cytokine release syndrome (CRS) and tumor lysis syndrome (TLS) (Phelps, Lin et al. 2009). In previously published reports, the use of non-compartmental methods resulted in the estimation of clearance, half-lives, $C_{\text{max}}$ and AUC (Tan, Headlee et al. 2002; Thomas, Tutsch et al. 2002; Byrd, Lin et al. 2007). However, the more flexible population pharmacokinetic approach was needed for quantify BSV, and to identify the influence of patient characteristics (demographic and lab results, etc) on this variability.
In this study, in order to evaluate the different levels of variability (between-subject, within-subject, and residual, etc.), nonlinear mixed effects model was used and two-compartment model followed by first-order elimination was used according to previous publication (Phelps, Lin et al. 2009). The model provided accurate prediction of plasma concentrations and PK parameters according to bootstrapping and VPC results. All parameters were estimated with acceptable precision, and residual error was small.

In this study, twenty-four demographic and lab data were investigated for their influence on the variability of pharmacokinetic parameters. Total bilirubin level on inter-compartment clearance (Q) was the only covariate kept in final model. Interestingly, a recent genome-wide meta-analysis for total bilirubin level study stated that variance of total bilirubin level can be explained SNPs of UGT1A1 and SLCO1B1 (Johnson, Kavousi et al. 2009), two genes which also metabolite and transport flavopiridol (detailed discussion on SLCO1B1 see Chapter 3), which may explain why total bilirubin level correlated with flavopiridol PK since both UGT1A1 and SLCO1B1 have shown to affect flavopiridol PK from this and previous studies (Phelps, Lin et al. 2009; Ni, Ji et al. 2010). However, the reason why bilirubin affects Q is not clear. In order to address that question in future, we need to confirm the finding of bilirubin affecting Q and UGT1A1/SLCO1B1 affecting flavopiridol PK from larger data set.

In order to validate our model, internal validation and external validation were performed. Internal validation included bootstrapping method and VPC in our study. The
The advantage of using bootstrap is that it allows us using the entire data set for model development. Because our samples are limited, it can be especially useful for evaluating the performance of our population model. All the PK parameters were falling into 95% CIs generated by bootstrapping (Table 2.4). VPC was also used to evaluate model performance in this study. VPC is a graphic method which is used for subjective evaluation of the pattern of discrepancies between model predictions and observations. It can be used to directly compare model prediction and observation (Wang and Zhang 2011). 95% PIs generated from the simulation covered majority of observe plasma concentrations (Figure 2.7). A phase 2 (OSU0491) data set was used for external validation. Typical values of PK parameters from both index data set and validation data set were close (Table 2.5), which suggested our model is stable on different data sets.

The multidrug resistance protein-2 (MRP2, ABCC2) (Jager, Gehring et al. 2003; Jager, Gehring et al. 2003) and the breast cancer resistance protein (BCRP, ABCG2) (Robey, Medina-Perez et al. 2001; Doyle and Ross 2003; Nakanishi, Doyle et al. 2003; Nakanishi, Karp et al. 2003; Sugimoto, Tsukahara et al. 2005) have been shown to influence flavopiridol elimination via biliary excretion. Results of our analysis from the phase 1 data indicated polymorphisms in MRP2 and OATP1B1 correlate with reduced central volume of distribution (V1) (Fig.2.8). These observations are consistent with the anticipated attenuated function of these transporters, which would be expected to cause higher substrate plasma concentrations relative to the fully functioning polymorphism (Grover and Benet 2009). Our phase 2 clinical trial data indicated the BCRP
polymorphism significantly correlated with reduced flavopiridol clearance and volume of
distribution, again suggesting attenuated efflux through BCRP (Fig. 2.9). Interestingly,
the associations between BCRP polymorphism and PK parameter did not appear from
phase 1 data. This may be due to different patient’s dataset used from this phase 2 study.

The phase 2 data set showed significant correlation between flavopiridol AUC versus
SLCO1B1_rs3829310 (p=0.08) and ABCG2_rs2231142 (p=0.076) (Figure 2.7 C, D). Although
there were no significant correlations between flavopiridol AUC versus these
two SNPs shown in phase 1 data set, the trend between flavopiridol AUC versus these
two SNPs were consistent (Figure 2.7 A, B). These findings suggested
SLCO1B1_rs3829310 and ABCG2_rs2231142 may affect flavopiridol exposure in
patients with CLL. Since drug exposure highly correlated with drug effects and safety
profile, we may need to consider individualized dosing for patients with those
polymorphisms.

In summary, these findings suggested polymorphisms of drug transporter genes may be
important factors contributing safety and efficacy for flavopiridol administration in clinic,
especially when flavopiridol is concomitant with other drug which is also substrate of
those transporters.
<table>
<thead>
<tr>
<th>Demographics</th>
<th>OSU0055</th>
<th>OSU0491</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>38</td>
<td>38</td>
</tr>
<tr>
<td>Female</td>
<td>14</td>
<td>19</td>
</tr>
<tr>
<td>Units</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean (Median)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>years</td>
<td>60 (60)</td>
</tr>
<tr>
<td></td>
<td>Range</td>
<td>38-84</td>
</tr>
<tr>
<td>Weight</td>
<td>kg</td>
<td>82.6 (83.0)</td>
</tr>
<tr>
<td>Body Surface Area</td>
<td>m²</td>
<td>1.96 (1.96)</td>
</tr>
<tr>
<td>Baseline CBC and Labs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Platelets</td>
<td>x10⁹/L</td>
<td>105 (106)</td>
</tr>
<tr>
<td>White Blood Cells</td>
<td>x10⁹/L</td>
<td>60.2 (16.9)</td>
</tr>
<tr>
<td>Hemoglobin</td>
<td>mM</td>
<td>10.5 (10.2)</td>
</tr>
<tr>
<td>Albumin</td>
<td>g/dL</td>
<td>3.5 (3.4)</td>
</tr>
<tr>
<td>Protein</td>
<td>g/dL</td>
<td>5.7 (5.6)</td>
</tr>
<tr>
<td>Calcium</td>
<td>mg/dL</td>
<td>8.3 (8.4)</td>
</tr>
<tr>
<td>Chloride</td>
<td>mM</td>
<td>103 (104)</td>
</tr>
<tr>
<td>Potassium</td>
<td>mM</td>
<td>3.7 (3.6)</td>
</tr>
<tr>
<td>Magnesium</td>
<td>mg/dL</td>
<td>2.0 (2.0)</td>
</tr>
<tr>
<td>Sodium</td>
<td>mM</td>
<td>139 (139)</td>
</tr>
<tr>
<td>Phosphate</td>
<td>mg/dL</td>
<td>5.7 (3.6)</td>
</tr>
</tbody>
</table>

Table 2.1. Demographics and baseline labs of patients from OSU0055 (n = 52) and OSU0491 (n = 67) included in pharmacogenetic analyses.
### Table 2.1. Continued

<table>
<thead>
<tr>
<th>Demographics</th>
<th>OSU0055</th>
<th>OSU0491</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Units</td>
<td>Mean (Median)</td>
</tr>
<tr>
<td><strong>Alanine Aminotransferase (ALT)</strong></td>
<td>U/L</td>
<td>22 (20)</td>
</tr>
<tr>
<td><strong>Aspartate Aminotransferase (AST)</strong></td>
<td>U/L</td>
<td>57 (33)</td>
</tr>
<tr>
<td><strong>Bilirubin</strong></td>
<td>mg/dL</td>
<td>0.8 (0.7)</td>
</tr>
<tr>
<td><strong>Creatinine</strong></td>
<td>mg/dL</td>
<td>1.1 (1.1)</td>
</tr>
<tr>
<td><strong>Lactate Dehydrogenase (LDH)</strong></td>
<td>U/L</td>
<td>223.6 (206.5)</td>
</tr>
<tr>
<td><strong>Uric Acid</strong></td>
<td>mg/dL</td>
<td>5.0 (5.2)</td>
</tr>
<tr>
<td><strong>Blood Urea Nitrogen</strong></td>
<td>mg/dL</td>
<td>14 (13)</td>
</tr>
<tr>
<td><strong>Glucose</strong></td>
<td>mg/dL</td>
<td>123 (114.5)</td>
</tr>
<tr>
<td><strong>Carbon Dioxide</strong></td>
<td>meq/L</td>
<td>29 (29)</td>
</tr>
</tbody>
</table>
Figure 2.1. Structure of two-compartment model of flavopiridol. 1, the central compartment of flavopiridol; 2, peripheral compartment of flavopiridol; K12, transfer from central to peripheral compartment of flavopiridol; K21, transfer from peripheral to central compartment of flavopiridol; K10, first-order elimination rate of flavopiridol from central compartment.
<table>
<thead>
<tr>
<th>Model Term</th>
<th>Parameter</th>
<th>Population Mean (RSE%)</th>
<th>%CV BSV (RSE%)</th>
<th>ETA shrinkage%</th>
</tr>
</thead>
<tbody>
<tr>
<td>CL = $\theta_{CL} \cdot \exp(\text{BSV}_{CL})$</td>
<td>$\theta_{CL}$ (L/h)</td>
<td>31.9 (4.1)</td>
<td>18.8 (47.2)</td>
<td>32.0</td>
</tr>
<tr>
<td>V1 = $\theta_{V1} \cdot \exp(\text{BSV}_{V1})$</td>
<td>$\theta_{V1}$ (L)</td>
<td>66.7 (3.0)</td>
<td>NE</td>
<td>NE</td>
</tr>
<tr>
<td>Q = $\theta_{Q} \cdot \exp(\text{BSV}_{Q})$</td>
<td>$\theta_{Q}$ (L/h)</td>
<td>7.82 (9.7)</td>
<td>58.05 (29.1)</td>
<td>13.28</td>
</tr>
<tr>
<td>V2 = $\theta_{V2} \cdot \exp(\text{BSV}_{v2})$</td>
<td>$\theta_{V2}$ (L)</td>
<td>149 (11.5)</td>
<td>65.1 (29.9)</td>
<td>17.28</td>
</tr>
<tr>
<td>BOV1</td>
<td>NE</td>
<td>21.7 (39.3)</td>
<td>25.46</td>
<td></td>
</tr>
<tr>
<td>BOV2</td>
<td>NE</td>
<td>22.9 (43.6)</td>
<td>42.09</td>
<td></td>
</tr>
<tr>
<td><strong>Residual Error</strong></td>
<td></td>
<td></td>
<td>ESP</td>
<td>Shrinkage%</td>
</tr>
<tr>
<td><strong>Proportional Error, as %CV</strong></td>
<td>38.6 (7.9)</td>
<td></td>
<td>10.25</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.2. Base model PK parameter estimates. The full (52 subjects, 577 plasma concentrations) dataset from OSU0055 was used. Parameters: CL, clearance of flavopiridol; V1, volume of central compartment of flavopiridol; Q, inter-compartmental clearance of flavopiridol; V2, volume of peripheral compartment of flavopiridol (units are noted in parenthesis); BSV and BOV are listed as %CV. $\Theta$, typical value of the PK parameters; BSV, between-subject variability; RSE, Relative standard error; NE, Not Estimated. (Objective Function Value: -3024.48)
Figure 2.2. Correlation between bilirubin level (mg/dL) and its random effects

(A) Correlation between bilirubin level and random effect for Q in base model; (B) Correlation between bilirubin level and random effect for Q in final model.
Figure 2.3 Goodness of fit of the pharmacokinetic model for flavopiridol. (A) Observed concentration versus population and individual prediction from base model. (B) Observed concentration versus population and individual prediction from final model.
Figure 2.4. Conditional weighted residual (CWRES) versus population prediction (A), individual prediction (B), ID (C), Time (D) from final model.
Figure 2.5. The histogram of conditional weighted residual (CWRES) (A) base model. (B) final model.
<table>
<thead>
<tr>
<th>Model Term</th>
<th>Parameter</th>
<th>Population Mean</th>
<th>%CV BSV</th>
<th>ETA shrinkage%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(RSE%)</td>
<td>(RSE%)</td>
<td></td>
</tr>
<tr>
<td>$CL = \theta_{CL} \cdot \exp(BSV_{CL})$</td>
<td>$\theta_{CL}$ (L/h)</td>
<td>32 (4.0)</td>
<td>19.5 (45.6)</td>
<td>31.1</td>
</tr>
<tr>
<td>$V1 = \theta_{V1} \cdot \exp(BSV_{V1})$</td>
<td>$\theta_{V1}$ (L)</td>
<td>68 (3.4)</td>
<td>8.4 (128)</td>
<td>64.5</td>
</tr>
<tr>
<td>$Q = \theta_0 \cdot \exp(BSV_Q)$</td>
<td>$\theta_0$ (L/h)</td>
<td>7.64 (7.5)</td>
<td>54.6 (30.5)</td>
<td>14.1</td>
</tr>
<tr>
<td>$V2 = \theta_{V2} \cdot \exp(BSV_{V2})$</td>
<td>$\theta_{V2}$ (L)</td>
<td>157 (9.8)</td>
<td>65.1 (30.0)</td>
<td>17.8</td>
</tr>
<tr>
<td>$\Theta_{BIL}$ on $Q$</td>
<td>$\Theta_{BIL}$ (mg/dL)</td>
<td>0.6 (41.7)</td>
<td>NE</td>
<td>NE</td>
</tr>
<tr>
<td>BOV1</td>
<td>NE</td>
<td>21.8 (39.7)</td>
<td>26.1</td>
<td></td>
</tr>
<tr>
<td>BOV2</td>
<td>NE</td>
<td>22.5 (44.9)</td>
<td>42.9</td>
<td></td>
</tr>
<tr>
<td>Residual Error</td>
<td></td>
<td></td>
<td></td>
<td>ESP Shrinkage%</td>
</tr>
<tr>
<td>Proportional Error, as %CV</td>
<td></td>
<td>38.2 (8.2)</td>
<td>10.75</td>
<td></td>
</tr>
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Table 2.3. Final model PK parameter estimates. The full (52 subjects, 577 plasma concentrations) dataset from OSU0055 was used. Parameters: $CL$, clearance of flavopiridol; $V1$, volume of central compartment of flavopiridol; $Q$, inter-compartmental clearance of flavopiridol; $V2$, volume of peripheral compartment of flavopiridol (units are noted in parenthesis); BSV and BOV are listed as %CV. $\Theta$, typical value of the PK parameters; BSV, between-subject variability; RSE, Relative standard error; NE, Not Estimated. (Objective Function Value: -3032.33).
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Final model estimate (%CV)</th>
<th>Bootstrap summary</th>
<th></th>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>( \theta_{CL} ) (L/h)</td>
<td>32 (4.0) 32.02 5 31.88 32.16</td>
<td>Mean Relative bias (%) Lower 95% Cl Upper 95% CI</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \theta_{V1} ) (L)</td>
<td>68 (3.4) 68.00 0.14 67.7 68.3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \theta_{Q} ) (L/h)</td>
<td>7.64 (7.5) 7.80 2.05 7.72 7.88</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \theta_{V2} ) (L)</td>
<td>157 (9.8) 159.21 1.41 156.54 161.88</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \Theta_{BIL} ) (mg/dL)</td>
<td>0.6 (41.7) 0.60 0.81 0.57 0.63</td>
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<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>OMEGA(_{CL})</td>
<td>19.5 (45.6) 18.52 -2.53 18.06 18.97</td>
<td></td>
<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>OMEGA(_{V1})</td>
<td>8.4 (128) 7.14 -15.0 6.73 7.55</td>
<td></td>
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<tr>
<td>OMEGA(_{Q})</td>
<td>54.6 (30.5) 50.28 -7.9 49.17 51.39</td>
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<tr>
<td>OMEGA(_{V2})</td>
<td>65.1 (30.0) 61.97 -4.8 60.99 62.96</td>
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<tr>
<td>OMEGA(_{BOL})</td>
<td>21.8 (39.7) 21.48 -1.45 21.12 21.84</td>
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<tr>
<td>OMEGA(_{BOV})</td>
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<td></td>
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</table>

Table 2.4. Population estimates from final model and bootstrap analysis

Relative bias, i(%) = (Pbs,i - Pest,i)/Pest,i x 100; Pbs,i: mean of parameter i estimate from bootstrap; Pest,i: final parameter i estimate.
Figure 2.6. Bootstrap results from final model.

OFV: objective function value; THETA1: CL; THETA2: V1; THETA3: Q; THETA4: V2; OMEGA1.1: BSV on CL; OMEGA4.4: BSV on Q; OMEGA5.5: BSV on V2.
Figure 2.7. Scatter plot of concentrations and 2.5\textsuperscript{th} and 97.5\textsuperscript{th} percentiles (dotted lines) from final model simulated data. Concentration data from phase 1 trial data set were simulated using final parameters estimates obtained with FOCEI. Solid line is the simulated mean concentration.
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Phase 1 data (Index data set)</th>
<th>Phase 2 data (Validation data set)</th>
<th>Difference (%)</th>
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<tbody>
<tr>
<td>$\theta_{CL}$ (L/h)</td>
<td>32</td>
<td>35.3</td>
<td>10.3</td>
</tr>
<tr>
<td>$\theta_{V1}$ (L)</td>
<td>68</td>
<td>69.5</td>
<td>2.2</td>
</tr>
<tr>
<td>$\theta_{Q}$ (L/h)</td>
<td>7.64</td>
<td>11</td>
<td>44.0</td>
</tr>
<tr>
<td>$\theta_{V2}$ (L)</td>
<td>157</td>
<td>120</td>
<td>-23.6</td>
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<tr>
<td>Residual variability</td>
<td>0.145</td>
<td>0.09</td>
<td>-37.9</td>
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Table 2.5. Population mean parameters for flavopiridol under the index and validation data set.
Figure 2.8. Association of PGx and PK from the phase 1 and phase 2 data sets. Flavopiridol PK parameter estimates were evaluated for relationships with PGx. P-values were shown in Table 2.6.
Figure 2.8. Continued

(E) SLCO1B1_rs4149056

(F) SLCO1B1_rs2306283

(G) ABCG2_rs2622624

(H) ABCG2_rs2622624

Continued
Figure 2.8. Continued

(I)  

(J)  

A/A or A/C  CC
ABCG2_rs3114018
CL (L/h)

A/A or A/C  CC
ABCG2_rs3114018
V1 (L)
<table>
<thead>
<tr>
<th>SNP Name</th>
<th>Associated PK parameters</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Phase 1</strong></td>
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<td></td>
</tr>
<tr>
<td>ABCC2_rs17222568</td>
<td>V1</td>
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</tr>
<tr>
<td>ABCC2_rs717620</td>
<td>Q</td>
<td>0.03</td>
</tr>
<tr>
<td>SLCO1B1_rs11045819</td>
<td>V1</td>
<td>0.0004</td>
</tr>
<tr>
<td>SLCO1B1_rs3829310</td>
<td>Q</td>
<td>0.03</td>
</tr>
<tr>
<td>SLCO1B1_rs4149056</td>
<td>CL</td>
<td>0.03</td>
</tr>
<tr>
<td><strong>Phase 2</strong></td>
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<td></td>
</tr>
<tr>
<td>SLCO1B1_rs2306283</td>
<td>Q</td>
<td>0.02</td>
</tr>
<tr>
<td>ABCG2_rs2622624</td>
<td>CL</td>
<td>0.008</td>
</tr>
<tr>
<td></td>
<td>V1</td>
<td>0.04</td>
</tr>
<tr>
<td>ABCG2_rs3114018</td>
<td>CL</td>
<td>0.004</td>
</tr>
<tr>
<td></td>
<td>V1</td>
<td>0.006</td>
</tr>
</tbody>
</table>

Table 2.6. SNPs significantly associated with PK parameters from Phase 1 and Phase 2 data sets.
Figure 2.9. Association of flavopiridol AUC and PGx from the phase 1 and phase 2 data sets. Flavopiridol AUC was evaluated for relationships with PGx. The correlation between flavopiridol AUC and ABCG2_rs2231142, SLCO1B1_rs3829310 showed the same trends in both studies. (A) and (B) were from phase 1 study; (C) and (D) were from phase 2 study.
Chapter 3: ROLE OF OATP1B1 IN THE DISPOSITION OF FLAVOPIRIDOL AND FLAVO-G

3.1 Introduction

Previous in vitro and in vivo studies have suggested that drug transporters are important for drug disposition (distribution and elimination), therapeutic efficacy and adverse event (AE) (Niemi, Pasanen et al. 2011). There are two major drug transporter superfamilies: ATP-binding cassette (ABC) transporter superfamily and solute carrier (SLC) transporter superfamily. OATP1B1 belongs to SLC transporter superfamily and it mediates the sodium-independent transport of a diverse range of amphiphilic organic compounds, such as bile acids, sulphate and glucuronate conjugates, steroid conjugates, thyroid hormones, and drugs such as valsartan, statins, and cyclosporine (Niemi, Pasanen et al. 2011). The international transporter consortium (ITC) recently published a paper which thoroughly discussed the key transporters from ABC and SLC superfamilies and their mechanisms in drug disposition and response. OATP1B1 effects on drug disposition were first time discussed in that paper, and a guideline for consideration its effects during drug development was also proposed (Giacomini, Huang et al. 2010).
The excretion of flavopiridol in laboratory animals was assessed by receiving intravenous doses of $[^{14}\text{C}]$-labeled drug. Greater than 90% of the administered radioactivity was recovered in feces and 5% to 6% in the urine (Flavopiridol Investigator’s Brochure 2002). Therefore, when considering which transporters affect flavopiridol, we should focus on the transporter located on hepatocytes. So far, two transporters have been identified to influence flavopiridol elimination from in vitro studies: MRP2 (Jager, Gehring et al. 2003; Jager, Gehring et al. 2003) and BCRP (Robey, Medina-Perez et al. 2001; Doyle and Ross 2003; Nakanishi, Doyle et al. 2003; Nakanishi, Karp et al. 2003; Sugimoto, Tsukahara et al. 2005) which contribute to biliary excretion of both flavopiridol and its glucuronide metabolites, flavo-G.

A common SLCO1B1 SNP is rs4149056 (c.521T>C, p.Val174Ala, V174A). It decreases the transporting activity of OATP1B1, resulting in markedly increased plasma concentrations of many statins, therefore increase the risk of statin-induced myopathy. However, the effects of this polymorphism are different on different statins, and also affect several other drugs PK profile (Niemi, Pasanen et al. 2011). Drug-drug interaction (DDI) studies involving OATPs have been primarily focus on OATP1B1(Giacomini, Huang et al. 2010). Cyclosporine, one of OATP1B1 inhibitors, can significantly increase statin exposure (Niemi 2009). Thus, understanding the OATP1B1 genetic polymorphisms is very important for DDIs studies and important for monitoring individual drug response.
Several clinical trials for flavopiridol have been shown the narrow therapeutic window and severe adverse events for this drug (Christian, Grever et al. 2009; Kristie A. Blum 2010). Understanding if there are more transporters affecting flavopiridol disposition in addition to MRP2 and BCRP would be critical. Our population PK model for flavopiridol in Chapter 2 showed that SLCO1B1/OATP1B1 polymorphisms associated with flavopiridol clearance and V1. These findings suggested flavopiridol might also be the substrate for OATP1B1, an uptake transporter located on the sinusoidal side of hepatocytes (Shimizu, Fuse et al. 2005). In this chapter, we implemented in vitro study as functional analysis to evaluate wild-type OAPT1B1 vector and three non-synonymous OATP1B1 SNP vectors: rs11045819 (p.Thr155Pro, T155P), rs2306283 (c.388A>G, p.Asn130Asp, D130N), and rs4149056 (c.521T>C, p.Val174Ala, V174A) effects on flavopiridol and flavo-G. By comparing intracellular levels of flavopiridol, flavo-G and SN-38, a known substrate of OATP1B1, in transient transfected cells with different vectors, we first time evaluated uptake function of OATP1B1 and its three SNPS for flavopiridol and flavo-G. LC-MS/MS methods were used to measure the concentrations for all drugs.

3. 2 Materials and Methods

3.2.1 Materials
Flavopiridol was obtained from NCI’s Developmental Therapeutics Program. Flavopiridol-glucuronide (flavo-G) was extracted from patient urine and purified as
previously reported (Lin, Ruppert et al. 2009). 7-ethyl-10-hydroxycamptothecin (SN-38) was purchased from Sigma (Sigma, St Louis, MO). Lenalidomide was obtained from Dr. Ching-Shih Chen in the OSU College of Pharmacy (Chen, Lau et al. 2007). All other chemicals were of analytical grade and commercially available. Cloning OATP1B1 and its non-synonymous SNP containing vectors was done by one of my colleagues and the methods were described in our previous paper (Ni, Ji et al. 2010). Gene orientation and homology of reference, rs11045819 (T155P), rs2306283 (D130N), and rs4149056 (V174A) SLCO1B1 SNPs were confirmed through direct full length sequencing of clones prior to experimentation.

3.2.2 Cell Culture
Madin-Darby canine kidney (MDCK-II) and human embryonic kidney (HEK-293) cells, purchased from ATCC (Manassas, VA), were cultured in 5% CO₂ at 37°C in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS, 100 units/ml penicillin, and 100 µg/ml streptomycin.

3.2.3 Transport Study
24-well plates were seeded with 2 x 10⁵ cells/well and transfected with the OATP1B1 and its three non-synonymous SNP containing vectors using FuGENE® 6 Transfection Reagent per the manufacturer’s protocols (Roche). Forty-eight hours post-transfection, cells were dosed with 10 µM flavopiridol or flavo-G in OptiMEM® I (Invitrogen, Carlsbad, California) incubation media containing 4% bovine serum albumin (BSA) for
10 and 30 minutes, respectively, at 37°C. After incubation, cells were washed with 4°C versene, trypsinized, and resuspended in 37°C versene at a total volume of 350 ul. A 150 μL aliquot of the cell suspension was lysed with 30 μl 6% Triton X-100 in PBS, and protein concentration was determined using Pierce® BCA protein assay (Thermo Scientific, Rockford, IL). The remaining 200μL cell suspensions were precipitated with 1mL, 4°C acetonitrile containing 200nM genistein, followed by vortex mixing and centrifugation at 16,000g for 10 min. The supernatant (1mL) was removed and dried in a vacuum concentrator then samples were resuspended in 150μL 95:5 water:acetonitrile plus 0.1% acetic acid, vortexed, and centrifugation at 16,000g for 10 min. Supernatants (100 μL) from flavopiridol and flavo-G treatment were analyzed using liquid chromatography and mass spec conditions as described previously (Phelps, Rozewski et al. 2008). Analytical LC/MS-MS method for SN38 and lenalidomide were used as previously published (Khan, Ahmad et al. 2005; Liu, Farley et al. 2008).

3.2.4 Data analysis and Statistics

Kinetic uptake parameters were calculated in SigmaPlot (v11.0, Systat Software, Inc., San Jose, CA) using non-linear least-squares regression analysis of the obtained data to the general expression:

\[
J = \frac{J_{\text{max}} \times C}{K_T + C}
\]  
(3.1)

\[
J = \frac{J_{\text{max}} \times C}{K_T + C} + P_{\text{m}} \times C
\]  
(3.2)
Both of the standard Michaelis-Menten model shown in equation 3.1 and the mixed model containing the saturable component along with a linear passive component as shown in equation 3.2 were evaluated to fit the uptake data. \( J \) is total substrate flux, \( J_{\text{max}} \) is maximum flux, \( K_T \) is the substrate concentration at half maximal flux, \( P_m \) is the membrane permeability coefficient, and \( C \) is the substrate concentration. All results are expressed as means ± standard deviation (SD). Uptake velocities were normalized to total protein in each well, and results were compared against empty vector controls using Student’s t-test. Fits to initial data were reviewed using both models (with or without the passive component). Weighted least-squares nonlinear regression was used in WinNonlin v5.2 (Pharsight, Mountain View, CA) to fit curves to the data generated.

In flavopiridol and flavo-G uptake studies, uptake velocities were normalized to total protein in each well and compared against empty vector controls using Student’s t-test.

3.3 Results

3.3.1 Flavopiridol uptake kinetics

The Michaelis-Menten model alone and the mixed model were evaluated to fit the uptake data generated from HEK293 cells. The residual plots show the differences in prediction accuracy between the two models (Fig 3.1). The mixed model gave lower residuals about the predicted values, which suggested that the mixed model provided a better fit compared to the Michaelis-Menten model alone. The model fitting from the mixed model
with the saturable and passive components was displayed and the calculated $J_{\text{max}}$, $K_T$, and $P_m$ were also included in Figure 3.2.

3.3.2 Uptake of flavopiridol and flavo-G by OATP1B1-expressing Cells.

To determine if SLCO1B1 was functionally relevant for flavopiridol and flavo-G disposition, uptake velocities of flavopiridol and flavo-G were calculated from HEK293 and MDCKII cells transfected with SLCO1B1 after the drug treatments. Transfection efficiencies were estimated at approximately 60% using GFP-containing control vectors. Mean uptake velocities were $261 \pm 12$ fmol/mg protein/10 min and $38 \pm 10$ fmol/mg protein/30 min for flavopiridol and flavo-G, respectively, in MDCK-II cells. Flavopiridol transport rates in HEK-293 cells were approximately 2-3 fold higher than in MDCK-II cells suggesting different membrane and transporter compositions in those two cell lines. Flavo-G transport rates were similar in both cell lines. Figure 3.3 shows relative uptake velocities of flavopiridol and flavo-G in both HEK293 and MDCK-II cells transfected with either SLCO1B1 or empty vector (as 100%). Expression of OATP1B1 was verified by evaluating uptake of a positive control substrate, SN-38 (Nozawa, Minami et al. 2005). A second agent, lenalidomide, was used as a negative control substrate. Total intracellular accumulation and initial transport velocities of SN-38, flavopiridol, and flavo-G were significantly increased in HEK293 and MDCK-II cells transiently transfected with SLCO1B1, compared to empty control vectors, whereas no increased uptake was shown for lenalidomide.

3.3.3. Uptake of flavopiridol by non-synonymous OATP1B1 SNPs.
In addition to evaluating flavopiridol uptake in cells transiently transfected with OATP1B1 wild-type vector, we also evaluated flavopiridol uptake in MDCKII cells transiently transfected with three non-synonymous OATP1B1 SNPs: Thr155Pro (T155P, rs11045819), Asn130Asp (D130N, rs230628) and Val174Ala (V174A, rs4149056). The results indicated significant decreases in flavopiridol transport rates (t-test p-value < 0.05) for the rs11045819 and rs4149056 variants, but the transport rate of the rs2306283 polymorph was similar to that in the reference SLCO1B1 transporter. Figure 3.4 displays these results.

3.4. Discussion

OATP1B1/SLCO1B1 was recently highlighted by the ITC as one of the seven most relevant transporters for drug development due to its broad substrate specificity, potential for DDIs, and clinically relevant polymorphisms (Giacomini, Huang et al. 2010). Several studies have shown SLCO1B1 polymorphisms were associated with BSV in drug treatment in patients (Smith, Figg et al. 2005). Later studies also showed it was important for the disposition of statin drugs (Niemi 2009) and various anti-cancer agents including irinotecan (Takane, Kawamoto et al. 2009).

Flavopiridol and flavo-G kinetic uptake studies on HEK293 and MDCKII cells were applied before transient transfected studies. We wanted to have a better understanding if those cells alone have active transport effect on flavopiridol and flavo-G. Our studies
showed HEK293 has active transport uptake effect in addition to passive diffusion on flavopiridol (Fig 3.1.) while MDCKII does not (data not shown). No flavo-G was detected in similar studies. According to these results, we used empty vector as negative control for reducing that active uptake effects from the cells themselves. We also used SN-38, a known OATP1B1 substrate to evaluate if our OATP1B1 vector has uptake function, so the results we got from flavopiridol and flavo-G were not false positive.

Our functional transport data indicated this gene may play a role in hepatic uptake of both flavopiridol and flavo-G (Fig.3.4), which suggests SLCO1B1 polymorphisms may affect PK and associate with BSV in flavopiridol treatment. Interestingly, our flavopiridol PK studies in Chapter 2 suggested SLCO1B1 rs11045819 was associated with V1 (p=0.0004), rs3829310 was associated with Q (p=0.03) and rs4149056 was associated with CL (p=0.03) (Fig. 2.8 and Table 2.6.); SLCO1B1_rs3829310 was associated with AUC in both phase 1 and phase 2 studies (Fig. 2.9). In our previous flavopiridol PK study, SLCO1B1_rs11045819 was shown as a covariate on central volume of distribution(V1) and significantly improved our model (Ni, Ji et al. 2010). However, for that particular data set, BSV on V1 was not able to be estimated in base model, so more data sets are needed in order to estimate BSV on V1. By comparing the changing of BSV before and after adding SNPs of SLCO1B1 as covariates, we can have a better understanding if SLCO1B1 polymorphisms affect BSV in flavopiridol treatment or not.
Further *in vitro* evaluations of these and other SNPs are necessary in order to characterize their functional impact with respect to flavopiridol and flavo-G disposition. Additionally, the different uptake velocities presented from flavopiridol and SN-38 (Fig 3.3) suggests that flavopiridol may be a weak substrate of SLCO1B1 under the evaluated experimental conditions. However, characterization of the kinetics of flavopiridol and flavo-G transport in the cells transfected with SLCO1B1 will be necessary for understanding the full impact this gene has on overall flavopiridol disposition. Since our kinetic uptake study showed active transport in addition to passive transport in HEK293 cells alone, we should always use non-transfected cells as control for future studies.

In summary, this *in vitro* study combine with our PK findings for OATP1B1 in chapter 2 suggested we should be very cautious when concomitantly administering flavopiridol and drugs that are substrates of OATP1B1. We should monitor patients closely for signs and symptoms of excessive exposure to flavopiridol. In clinical trials, further DDI studies need to be done and the decision about dose reduction need to be made from those studies.
Figure 3.1. Mixed model (Equation 5.2) nonlinear least squares fit of flavopiridol uptake data generated in HEK293 cell cultures. Curves are also shown for the individual saturable (---) and passive (・・・) components. Data expressed as mean ± S.D., n = 3. $J_{\text{max}}=1408.68\text{pmol/mg protein/min}$; $K_T=19.86\mu\text{M}$; $P_m=20.85\mu\text{L/mg protein/min}$. 
Figure 3.2. Nonlinear least squares fits of flavopiridol uptake data generated in HEK293 cell cultures. Residual plots for fits correspond to the use of only a saturable Michaelis-Menten component (A) or both a saturable and passive component (B) are shown.
Figure 3.3 Uptake of flavopiridol, flavo-G, SN-38 and lenalidomide in OATP1B1 transfected cells. The bar graph indicates means +SD for triplicate determinations of 10 \( \mu M \) flavopiridol, flavo-G, SN-38 and lenalidomide uptake rates in cell lines (HEK-293 or MDCK-II) transfected with empty control vector or vector cloned with the OATP1B1 gene (SCLO1B1). Incubations with flavo-G were for 30 min., and all other drugs were for 10 min. Transport rates are expressed as percentages normalized to empty vector control. * \( p < .05 \), ** \( p<0.001 \), Student’s t-test.
Figure 3.4. Uptake of flavopiridol in nonsynonymous polymorphic variants of OATP1B1. The bar graph indicates means ± SD for triplicate determinations of 10 μM flavopiridol uptake rates in MDCK-II cells transfected with empty control vector or vector cloned with the reference or polymorphic SLCO1B1 genes. All incubations were for 10 min. Variants are indicated with respect to their amino acid change; Asp130Asn = rs2306283, Thr155Pro = rs11045819, Val174Ala = rs4149056. Transport rates were normalized to empty vector control. Differences in uptake rates were compared to the reference OATP1B1 transporter. * p < .05, ** p<0.01, Student’s t-test.
4.1 Introduction

Glutathione (GSH) is an abundant natural tripeptide and synthesized in every cell of higher eukaryotes (Klaunig and Kamendulis 2004) and it has a direct antioxidant function in the elimination of ROS (Merad-Saidoune, Boitier et al. 1999). Previous studies showed that flavopiridol can moderately decrease intracellular GSH levels in transformed cells, such as K562, HeLa, RPMI-8226, and peripheral-blood mononuclear cells (PBMCs), and significantly decrease GSH levels in Jurkat cells (Ge, Byun et al. 2008). Studies have shown that loss of mitochondrial membrane potential ($\Delta \Psi_{m}$), a standard indicator of mitochondrial damage, in flavopiridol-treated cells was detected as early as 5 hours, prior to the onset of propidium iodide (PI) positivity (Hussain, Lucas et al. 2008). In addition, neither a pan-caspase inhibitor nor specific inhibitors of caspase-8 and caspase-9 can rescue the loss of $\Delta \Psi_{m}$ after flavopiridol treatment in primary CLL cells. These observations suggest that flavopiridol may induce very rapid cell death that is different from classical apoptosis (Hussain, Lucas et al. 2008). Compared to normal
lymphocytes, CLL cells have shown higher basal levels of ROS and lower basal levels of GSH (Trachootham, Zhang et al. 2008).

Various agents are in common use for modulation of GSH levels. L-buthionine-S, R-sulfoximine (BSO) induces cellular oxidative stress through specific inhibition of γ-glutamylcysteine synthetase, thus preventing formation of γ-glutamylphosphate and ultimately γ-glutamylcysteine (Griffith 1982). Efficacy of BSO-mediated chemosensitization has been demonstrated in melanoma, breast and ovarian tumor (Fruehauf, Zonis et al. 1997). (S)-4-carboxyphenylglycine (4-S-CPG) can selectively inhibit cysteine transport mediated cysteine-glutamine exchange, therefore reducing GSH levels (Huang, Dai et al. 2005). N-acetyl-L-cysteine (NAC), a conventional antioxidant, is a synthetic precursor of intracellular cysteine and GSH (van Zandwijk 1995). NAC therefore increases the intracellular GSH pool. Studies have shown GSH and NAC were able to protect against ATO/ascorbic acid, a ROS generator, cytotoxicity in primary CLL cells, while depletion of GSH levels by BSO increased the cytotoxicity mediated by ATO/ascorbic acid in those cells (Biswas, Zhao et al. 2010).

To characterize the effects of flavopiridol on GSH homeostasis in transformed cell lines, we either used flavopiridol single treatment or combined flavopiridol with GSH altering agents mentioned above. BSO and 4-S-CPG were used to evaluate if they were able to enhance cytotoxicity. NAC was used to test if it was able to protect against flavopiridol induced cell death. Primary CLL cells were also used in this study to determine if
different GSH base levels relate to the final GSH levels in those cells after flavopiridol treatment.

4.2 Materials and Methods

4.2.1 Reagents

The GSH-Glo™ Glutathione Assay (Promega, Madison, WI) was used to measure the cellular level of glutathione after flavopiridol treatment. The CellTiter Fluor™ cell viability assay (Promega, Madison, WI) was used for simultaneously measuring cell viability. For transformed cells and CLL patient cells, normalized GSH level was used in our experiment. Normalized GSH level was calculated by GSH level (from GSH-Glo™ Glutathione Assay) divided by live cell numbers (from CellTiter Fluor™ cell viability assay) from the same well.

4.2.2 Cell culture

Written informed consent to collect blood was obtained from CLL patients under protocol OSU0055 approved by the Ohio State University IRB. In vitro studies were carried out using CD19⁺ cells obtained from CLL patients with elevated leukocyte counts. CLL cells were isolated from peripheral blood using RosetteSep® B cell enrichment cocktail (StemCell Technologies, Vancouver, BC). Isolated cells, Jurkat cells, the 697-neo and 697-bcl2 cell lines (vector-transfected control and stably transfected Bcl-2-overexpressing 697 cell lines, respectively) were suspended in RPMI-1640 media
(Invitrogen, Carlsbad, CA) supplemented with 100 Units/mL penicillin and 100 µg/mL streptomycin, and 10% human serum (Invitrogen, Carlsbad, CA). Cells were maintained in a humidified incubator at 37°C, 5% CO₂.

4.2.3. Glutathione assay

Jurkat, 697-neo and 697-bcl2, and patient CLL cells were incubated with flavopiridol (1µM, 5µM and 10µM) for 4 hours in 96-well plates. Incubation time (4 hours) was chosen based on active dosing regimen currently used in clinical flavopiridol studies. Detection of GSH using the GSH-Glo™ Glutathione Assay was enabled from the combination of two chemical reactions. The first reaction involved the generation of luciferin from a luminogenic substrate, catalyzed by GST in the presence of GSH. The luciferin produced in the first reaction is detected as a luminescent signal generated by the luciferase enzyme. The second reaction is initiated by adding an equal volume of Luciferin Detection Reagent, which simultaneously stops the GSH-Glo™ reaction and initiates a luminescent signal that is directly proportional to the amount of luciferin formed in the first reaction (GSH-Glo™ Glutathione Assay Technical Bulletin, Promega, Madison, WI). The CellTiter Fluor™ cell viability assay was used for measuring cell viability and simultaneously normalizing GSH from each same sample well. The live cell protease activity from intact viable cells was measured by a fluorogenic, cell-permeant, peptide substrate glycyl-phenylalanyl-aminofluorocoumarin (GF-AFC). The substrate enters intact cells where it is cleaved by the live-cell protease activity to generate a fluorescent signal proportional to the number of living cells. This protease activity
becomes inactive upon loss of cell membrane integrity and leakage into the surrounding culture medium and does not contribute to the live-cell response (CellTiter Fluor™ cell viability assay Technical Bulletin, Promega, Madison, WI). The EnVision® Multilabel Plate Reader (PerkinElmer, MA) was used to read both luminescence and fluorescence. The normalized intracellular glutathione in individual cell before and after flavopiridol treatment was calculated by total GSH amount from each well divided by cell numbers which were calculated from the cell viability assay.

4.2.4. Cytotoxicity assays

Cytotoxicity (sulphorhodamine B, SRB) assays were conducted to estimate LC_{50} values for flavopiridol under the various conditions. Briefly, cells were grown in standard culture conditions (see section 4.4.2) then placed in a 96-well plate (3,000 cells/well/190 µL) and pretreated with 50 µM of either BSO or CPG or with 3 mM N-Acetyl-L-cysteine (NAC) as a positive control agent for enhancing or maintaining GSH levels during treatment. After 24 hours, flavopiridol (10 µL) was added in a logarithmic dilution series (0.03-30µM) in triplicates. After 3 days, the medium was replaced with 50 µL 80% trichloroacetic acid (w/v) in PBS, cells were incubated at 4 °C for 2 hours, and plates were allowed to air dry after 5 washes with distilled water. Cells were stained 30 min at room temperature with 50 µL of 0.4% SRB (w/v) in 1% acetic acid. SRB solution was removed and cells were air dried after 5 washes with 1% v/v acetic acid. Tris Base (100 µl, 10 mM, pH 10.5) was added to each well followed by 5 min. orbital shaking. Buffer was removed, cells were again air-dried, and 10 mM Tris-HCl (pH 8.0) was
added. Absorbance at 570 nm was measured in each well, and background absorbance at 690 nm was subtracted. Based on previous flavopiridol cytotoxicity study results in PC3 and SUDHL4 cells (Parker, Kaur et al. 1998), 72-hour incubation time was used for evaluating different cytotoxicity levels at different concentrations (0.03-30µM).

4.2.5. Data analysis

Resulting data from SRB assays were fitted to an $L_{\text{max}}$ (maximum lethal concentration) model using WinNonlin v5.2 (Pharsight Corporation, Mountain View, CA), and $L_{C_{50}}$ (50% lethal concentration) values were estimated. The $L_{\text{max}}$ model was described by the following equation:

$$\% \text{Cell Viability} = L_{\text{max}} \cdot \left[1 - C/(C + L_{C_{50}})\right],$$

where $C$ is the concentration of substrate, and $L_{C_{50}}$ is the concentration to reach 50% maximum lethal effect.

4.2.6. Statistical analysis

Bar graphs and plots were generated using SigmaPlot (Systat Software Inc, San Jose, CA). Data were expressed as means plus or minus 95% confidence interval (CI). Student’s t-tests were used to compare the mean values of different experimental groups. One-way analysis of variance (ANOVA) was used to compare the mean values of GSH in the control samples with that of the tested samples with multiple time points. $P$-values less than .05 were considered statistically significant.
4.3. Results

4.3.1. Depletion of GSH by BSO, 4-S-CPG, or NAC and Flavopiridol Combinations

To evaluate the selective influence of flavopiridol on the redox properties of transformed cells, the intracellular levels of GSH were assessed in Jurkat, 697-neo, and 697-bcl2 cells treated with flavopiridol alone. GSH levels in Jurkat and 697-neo were significantly reduced after the treatment (p<0.005 and p<0.01, respectively). However, no significant changes were shown in 697-bcl2 cells (Fig 4.1A). In order to evaluate GSH altering agent effects during flavopiridol treatment, combinations of flavopiridol with BSO, 4-S-CPG or NAC were evaluated in Jurkat and 697-neo cells which GSH levels had shown sensitive to flavopiridol. When NAC was added alone, GSH levels were significantly increased in Jurkat cells (p<0.05) but no significant change in 697-neo cells. Both BSO and 4-S-CPG significantly reduced GSH levels in both Jurkat and 697-neo cells (p<0.005 in all treatments) (Fig.4.1B and 1C). Moreover, flavopiridol produced significant depletion of GSH in Jurkat cells even with the addition of NAC, even though in 697-neo cells this depletion was moderate (Fig. 4.1C). GSH levels in cells treated with 4-S-CPG or BSO were further depleted after flavopiridol treatment (Fig.4.1B and 1C).

4.3.2. GSH mediates flavopiridol cytotoxicity in leukemia cell models

To evaluate the effects of glutathione depletion on tumor cell sensitivity to flavopiridol, we evaluated flavopiridol cytotoxicity in the absence or presence GSH altering agents, BSO and 4-S-CPG, and NAC, in Jurkat, 697-bcl2, and 697-neo cells. All cell lines were
treated for 4 h with increasing concentrations of flavopiridol (0.03–30 µM) after 1 h pre-incubation with BSO (50µM), 4-S-CPG (50µM) or NAC (5mM) (Fig. 4.2). LC50s determined from these experiments by SRB assay were calculated by WinNonlin v5.2 and are listed in Table 4.1. Results indicated GSH depletion with BSO or 4-S-CPG increased the sensitivity of 697-neo and Jurkat cells to the cytotoxic effects of flavopiridol. In contrast, these agents had no measurable effects on 697-bcl2 cells. Notably, BSO had a dramatic effect on Jurkat cells, rendering an LC50 of 4.5nM compared to 87nM with flavopiridol alone. NAC did not significantly alter flavopiridol LC50 in these experiments. These data with BSO and 4-S-CPG suggest these leukemia cell models respond differently: they showed large effect in Jurkat cells, mild effect on 697-neo cells and no effect on 697-bcl2 cells (Table 4.1).

4.3.3. Baseline GSH and ΔGSH are variable in patient CLL cells

CLL cells from 11 patients were used in this study. Baseline GSH ranged from 70-420 fg/cell (Fig. 4.3) which was comparable to previous data produced by Trachootham and colleagues (Trachootham D, et al. Blood. 2008) using similar methods. In our studies of CLL cells from 11 patients, GSH levels in some patients were altered after flavopiridol treatment in a dose-dependent manner. Interestingly, GSH was significantly decreased in one patient and significantly increased in 2 other patients after flavopiridol treatment (Fig. 4.3.). These data indicate variability among CLL patient cells in GSH levels, both at baseline and after treatment with flavopiridol.
4.4. Discussion

Previous studies have shown in CLL flavopiridol cytotoxicity accompanies evidence of oxidative stress and mitochondrial membrane damage (Ge, Byun et al. 2008; Hussain, Lucas et al. 2008; Trachootham, Zhang et al. 2008). The timing of these events is early relative to the onset of apoptosis and suggests acute mitochondrial damage may represent the initial events leading to CLL cell death. Previous studies have shown that CLL cells display a decreased level of GSH (Trachootham, Zhang et al. 2008) and an increased level of ROS (Ge, Byun et al. 2008) compared to normal cells. With GSH as the primary defense against intracellular ROS, we hypothesized that flavopiridol induced cytotoxicity in CLL may be mediated by GSH levels prior to therapy.

Here, we show that the ability of CLL cells to rapidly respond to acute GSH depletion may represent a measure of sensitivity to the cytotoxic effects of flavopiridol. This conclusion was based on several lines of evidence: (1) transformed leukemia cell lines have variable sensitivity to flavopiridol-induced GSH depletion; (2) flavopiridol potency significantly increased in transformed cells when combined with GSH depletion agents (BSO and 4-S-CPG); (3) primary CLL cells isolated from some patients exhibited a decrease in cellular GSH after flavopiridol treatment. In the bcl2 overexpressed cell line (697-bcl2), GSH level changes after flavopiridol or flavopiridol combined with GSH-altering agent treatment were much less than Jurkat or 697-neo cells, which may be due to overexpression of bcl-2 increases levels of GSH (Merad-Saidoune, Boitier et al. 1999).
There are two possible mechanisms which have been proposed in terms of overexpressing bcl-2 increasing levels of GSH: First, bcl-2 was able to inhibit the formation or action of ROS (Hockenbery, Oltvai et al. 1993; Kane, Sarafian et al. 1993). Second, bcl-2 increases intracellular GSH levels by inhibition methionine-dependent GSH efflux (Meredith, Cusick et al. 1998).

Our studies showed GSH levels in transformed cells were 10 to 100 times higher than those in patient CLL cells (Fig. 4.1 and 4.3). However, flavopiridol LC50s in transformed cells were lower than those from CLL patients from previous study. In that study, LC50 for flavopiridol ranges from 90-330nM for 24 hours incubation and 40-240nM for 96 hours incubation with flavopiridol (Flavopiridol Investigator’s Brochure. 2002). The reason may due to variability from patient cells since even in that study LC50 showed almost 6 fold difference between patients. One solution would be using the same patient CLL cells to measure both LC50 and GSH levels. However, it is always a challenge to get enough CLL cells from one patient for both experiments since how many CLL cells we can get from one patient depends on his lymphocyte count. For this present study, we were not able to get enough CLL cells for both experiments.

In summary, our study suggests that the intrinsic oxidative stress in transformed cell model makes those cells more sensitive to flavopiridol treatment. Changes of GSH levels in primary CLL cells and transformed cells after flavopiridol treatment are patient specific and cell-type specific, respectively.
Figure 4.1. Flavopiridol demonstrates disruption of homeostasis of intracellular GSH in transformed leukemia cells. (A) The profile of cellular GSH depletion in Jurkat, 697-neo, and 697-bcl2 cells after 4-h exposure to flavopiridol alone at 0, 1, and 10μM concentrations. (B) And (C) Profiles of cellular GSH depletion in Jurkat (B) and 697-neo (C) cells after 4-h exposure flavopiridol alone or pre-treatment with BSO, 4-S-CPG, and NAC at the concentrations indicated in each well in 96-well plate.
Figure 4.1. Continued

(B)

(C)
Figure 4.2. Comparison of residual viable cells after treatment with the absence or presence of GSH-altering agents. Representative cell viability curves of transformed leukemia cells treated with various concentrations of flavopiridol for 72 hours after 24 hr pretreatment with GSH altering agents. Cell viability was measured with the SRB assay. Top: Jurkat. Middle: 697-neo. Bottom: 697-bcl2. (●: BSO+Flavo; ○: 4-S-CPG+Flavo; ▼: Flavo; △: NAC+Flavo)
Table 4.1. Cytotoxicity of flavopiridol in the absence or presence of GSH-altering agents after 72 hours. LC$_{50}$s were determined by curve fitting of cytotoxicity profiles generated from SRB staining in Jurkat, 697-neo, and 697-bcl2 cell lines.

<table>
<thead>
<tr>
<th>Drug Treatment</th>
<th>LC$_{50}$(nM)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Jurkat</td>
<td>697-neo</td>
<td>697-bcl2</td>
</tr>
<tr>
<td>Flavo Alone</td>
<td>87</td>
<td>13</td>
<td>23</td>
</tr>
<tr>
<td>Flavo + BSO</td>
<td>4.5</td>
<td>6.0</td>
<td>26</td>
</tr>
<tr>
<td>Flavo + CPG</td>
<td>56</td>
<td>6.0</td>
<td>20</td>
</tr>
<tr>
<td>Flavo + NAC</td>
<td>85</td>
<td>15</td>
<td>20</td>
</tr>
</tbody>
</table>
Figure 4.3. Normalized Intracellular GSH level before and after flavopiridol treatment in patient CLL cells. Intracellular levels of GSH were measured in patient CLL cells after 4 hr incubations with flavopiridol at two different concentrations. The GSH level in Patient ID#3 were significantly reduced after flavopiridol treatment (p<0.005), while those in patients #9, 10, 11 were significantly increased (p<0.01).
Chapter 5: CLINICAL POPULATION PHARMACOKINETIC MODEL OF FLAVOPIRIDOL AND FLAVO-G IN CHRONIC LYMPHOCYTIC LEUKEMIA PATIENTS

5.1 Introduction

Phase 1 (OSU0055) and phase 2 (OSU0491) single agent studies have shown patients with durable remissions and a lack of prolonged immunosuppression in refractory and high risk CLL treated with flavopiridol indicated remarkable response rates of 41% and 53%, respectively (Lin, Ruppert et al. 2009; Phelps, Lin et al. 2009). Despite the impressive clinical activity, as discussed in previous chapters, the dose limiting toxicity (DLT), hyper-acute tumor lysis syndrome (TLS), resulted in a narrow therapeutic window for flavopiridol treatment.

The primary metabolic pathway for flavopiridol is glucuronidation (Phelps, Lin et al. 2009). Flavopiridol is conjugated by UGT1A1, 1A4 and 1A9 to form flavopiridol glucuronide (flavo-G) (Hagenauer, Salamon et al. 2001; Ramirez, Iyer et al. 2002). Previous in vitro study from other groups showed cytotoxicity of some flavonoid glucuronides had superior cytotoxicity compared to their parent compounds (Plochmann,
Korte et al. 2007; Wong, Zhang et al. 2009). In our studies, flavopiridol PK parameters were not significantly correlated with TLS, diarrhea, or neutropenia. However, a significant relationship was observed between flavo-G exposure and TLS grade in our previous study: patients experiencing no TLS had lower flavo-G/flavopiridol $AUC_{0-\infty}$ ratios ($3.0 \pm 1.8$) compared with patients experiencing TLS ($8.9 \pm 7.0$, $P = .005$) (Kristie A. Blum 2010).

While the role of flavo-G in clinical outcomes is not yet clear, expansion of our population PK model to include flavo-G is necessary to thoroughly characterize overall drug disposition and its contribution to outcomes. Previous studies examined the disposition of flavo-G by non-compartmental methods, describing area under the plasma concentration-time curve (AUC), clearance (CL), and terminal half-life ($T_{1/2}$) (Phelps, Lin et al. 2009). This study first proposed a linked parent-metabolite model: a two-compartment model for flavopiridol with first-order elimination and a one-compartment model for flavo-G with first-order input and first order elimination. The clinical significance of this population pharmacokinetic model is that it may be used to prospectively individualize flavopiridol therapy to achieve a target systemic exposure of parent compound and metabolite. The objectives of this study were to develop and validate a pharmacokinetic model for flavopiridol and flavo-G, and to identify the relationships between their pharmacokinetics and patient characteristics.

5.2. Methods
5.2.1 Study protocols and subjects

Study protocols and subjects were described in Chapter 2.

5.2.2 Pharmacokinetic sampling and analytical assay

PK sampling methods were described in Chapter 2. 577 flavopiridol concentrations from 52 patients (approximately 10 pharmacokinetic observations per patient) and 175 flavo-G concentrations from 27 patients (approximately 6 pharmacokinetic observations per patient) were analyzed by LC-MS/MS assay with 3 nM LLOQ of flavopiridol and flavo-G (Phelps, Rozewski et al. 2008; Ni, Ji et al. 2010).

5.2.3 Structural model development

Data were analyzed by using NONMEM to characterize the pharmacokinetics of flavopiridol and flavo-G in the patient population. Estimation methods were described in Chapter 3.

For the pharmacokinetic evaluation, a base model without covariates was developed first. The pharmacokinetic model used is schematically depicted in Figure 5.1. Flavopiridol and flavo-G concentration data were expressed as µg/mL, doses were expressed in mg and time in hour. Flavopiridol and flavo-G data were fitted simultaneously using the NONMEM subroutine ADVAN13, which makes use of differential equations to describe the rate of mass transport between the different compartments. ADVAN13 is a new routine introduced in NONMEM7. It is useful for stiff and non-stiff equations by introducing a new differential equation solver called LSODA, while ADVAN6 is only
useful for stiff equations. ADVAN6 was initially used for both metabolite models. However, ADVAN6 was not able to solve equations from model 2, while ADVAN13 successfully solved equations in both models. In order to compare model structures themselves, ADVAN13 was used during this study. Two different control files were used and for further comparison. The differential equations used for model 1 were described in equations 1-3:

\[
\frac{dA(1)}{dt} = -k_{12} \times A(1) + k_{21} \times A(2) - k_{10} \times A(1) - k_{13} \times A(1) \tag{1}
\]

\[
\frac{dA(2)}{dt} = k_{12} \times A(1) - k_{21} \times A(2) \tag{2}
\]

\[
\frac{dA(3)}{dt} = k_{13} \times A(1) - k_{30} \times A(3) \tag{3}
\]

The differential equations used for model 2 were described in equations 4-6:

\[
\frac{dA(1)}{dt} = -\frac{CL}{V_1} \times A(1) + k_{21} \times A(2) - k_{12} \times A(1) \tag{4}
\]

\[
\frac{dA(2)}{dt} = k_{12} \times A(1) - k_{21} \times A(2) \tag{5}
\]

\[
\frac{dA(3)}{dt} = fm \times \frac{CL}{V_1} \times A(1) - k_{30} \times A(3) \tag{6}
\]

In which \(A(n)\) and \(V_n\) describe the amount in and the volume of the nth compartment, respectively, \(k_{ij}\) represents the micro-constant rate describing the mass transport between the ith and jth compartment. \(Fm\) represents the fraction of flavopiridol clearance for the
formation of flavo-G. Since the volume of distribution of flavo-G is unknown, calculating with the amount of flavo-G is impossible. Therefore, flavo-G data were modeled with $V_3=1L$, and $k_{13}$ and $k_{30}$ can be calculated. Since the distribution volume of metabolite was fixed to 1 L, the fraction is interpreted as the ratio of the fraction flavopiridol converted to flavo-G.

BSV of the different pharmacokinetic parameters was estimated using proportional error models as described in Chapter 2. Residual variability was estimated with a proportional error model for flavopiridol and a combined proportional and additive error model for flavo-G assuming a constant coefficient of variation over the complete concentration range superimposed on a constant absolute error. The performance of the model was judged using both statistical and graphical methods as described in Chapter 2.

5.2.4 Full model and final model development

Full model and final model development methods were the same as described in Chapter 2.

5.2.5 Evaluation of associations between PGx, PK and outcomes.

To identify associations with genetics, the means of flavopiridol and flavo-G (Phelps, Lin et al. 2009) PK parameters were compared based on SNP genotypes using Student’s t-test and analysis of variance (ANOVA). For comparisons of PGx and clinical outcomes, SNP
genotypes and clinical response was evaluated using Fisher’s exact test. P-values were not further adjusted for multiple testing.

5.2.8. Model performance and stability
Conditional weighted residual (CWRES) was used to evaluate model misspecification (Hooker, Staatz et al. 2007). CWRES versus dependent variables (population prediction and individual predictions), independent variables (ID and time) and CWRES distribution frequency plots were used to evaluate model performance and to compare models.

Bootstrapping was used to evaluate model stability and to estimate confidence intervals for the PK parameters. 500 bootstrapped data sets were created and 456 runs were successful converged. The results of all successful runs were used to create the percentile bootstrap confidence intervals, which were constructed by taking the lower 2.5% and the upper 97.5% value of the set of estimates for each parameter. This interval should cover the true value of the parameter estimate approximately 95% of the time without imposing an assumption of symmetry on the distribution.

5.3. Results

5.3.1. Base model identification
577 flavopiridol concentrations from 52 patients (approximately 10 pharmacokinetic observations per patient) and 175 flavo-G concentrations from 27 patients (approximately 6 pharmacokinetic observations per patient) were available for this model. The
flavopiridol and flavo-G concentrations were below the LLOQ in a few samples immediately before the next flavopiridol infusion. Only samples above the LLOQ were included in the analysis.

All of the observed concentrations of flavopiridol and flavo-G were used in the pharmacokinetic analysis and their PK profiles were shown in Figure 5.1. Two-compartment model was used to describe flavopiridol PK profile in patients as described in Chapter 2. A one-compartment model was used and fit flavo-G PK profile. The structures of the two metabolite models are shown in Figure 5.2, illustrating the linkage of the parent and metabolite pharmacokinetics. BSV was initially assumed on each parameter in the model. However, gradients of BSV on V1 and V2 were negligible at the end of each run, so BSV on V1 and V2 were removed from final base model. PK parameters from model prediction and calculated K13 and K30 were listed in Table 5.1. Typical values of PK parameters and calculated K13 and K30 were similar from both models, except V2.

Fig 5.3 shows the goodness-of-fit plot of the parent–metabolite pharmacokinetic model for flavopiridol and flavo-G from both models. Observation versus individual prediction of flavo-G from model1 showed less scattered than model2, even though both models showed similar observation-versus-population prediction plots for parent and metabolite. CWRES versus population, individual prediction, ID and time from both models were similar for flavopiridol (Fig 5.4 and Fig 5.5). However, CWRES of flavo-G in model1
were equally distributed around zero reference line in all the CWRES diagnostic plots, which was not the case for CWRES of flavo-G form model2 (Fig 5.4 and Fig 5.5). Distributions of CWRES from these two models showed similar (Fig 5.6). Condition numbers for model1 and model2 were 2.45 and 155, respectively, which suggested model1 was more stable than model2. Based on the goodness-of-fit plots, diagnostic plots and condition numbers comparison, model1 was chosen as final structure model for further covariate model analysis.

5.3.2 Covariate model identification
Weight (WT) and white blood cell count (WBC) correlated with clearance of flavopiridol and flavo-G, respectively (Fig 5.7). However, the model did not show significant improvement (OFV changed less than 3.84, p<0.05) after adding either of these as covariate. Therefore, no covariate was included in the final model. Final model parameter estimates are presented in Table 5.2.

5.3.3. Model stability
The bootstrap method was used to estimate the bias, standard errors, and confidence intervals in the final covariate model. Five hundred (500) replicate data sets were generated. The final model was fit to each data set, and the distribution of the parameter estimates was examined for bias and precision. Frequency distributions of bootstrap parameters and their BSV showed that the original model prediction value were fall into
95% confidence interval and close to the mean of each parameter and its BSV calculated from bootstrapping results (Fig.5.8).

5.4. Discussion

The present study was the first population pharmacokinetic model for flavopiridol and its metabolite, flavo-G. Flavopiridol and flavo-G pharmacokinetics were best described by a two-compartment model for flavopiridol with first-order elimination and a one-compartment model for flavo-G with first-order input and first order elimination. Flavopiridol PK parameter estimations for CL, V1, and Q from this metabolite model were similar to results from the population flavopiridol PK model from chapter 2.

Identifiability is a major concern for metabolite modeling. The rate of conversion from parent to metabolite and metabolite volume of distribution are not simultaneously identifiable. Since the ratio of the rate of conversion to the metabolite volume is identifiable, if metabolite volume of distribution is fixed to a unit number (1L), then the rate of conversion can be estimated (Huitema, Mathot et al. 2001; Kerbusch, Mathot et al. 2001; Klein, Gupta et al. 2002). Because of the exact fraction of flavo-G formed from flavopiridol was not known in our study and there was no intravenous administered flavo-G PK data available, the modeling of flavopiridol and flavo-G was simplified by fixing flavo-G volume of distribution (V3) to 1L, so that flavo-G PK parameters can be estimated.
Two metabolite models were initially proposed for this study. Since those two models were not hierarchical models, OFV comparison was not used to compare model fitting since the change of OFV between two hierarchical models is calculated by likelihood ratio test (LRT), in which the more complex one can be transformed into the simpler model by fixing related components to zero (Wahlby, Jonsson et al. 2002). Instead, goodness-of-fit plots and CWRES were used to evaluate model misspecification. Observed versus population prediction plots describe inter- and intra-individual variability, whereas observed versus individual prediction plots only describe intra-individual variability (Beal 1989-2010). The plots from model2 were similar between population prediction and individual prediction plots versus observation, which suggested that the model had poor performance for individual prediction. However, individual prediction were predicted very well from model1(Fig.5.3.). In addition to intra-individual variability, there was still some inter-individual variability shown on the population prediction versus observation plot from model1 (Fig.5.3). The BSV may be due to polymorphisms from glucuronidase enzyme. Since flavopiridol was metabolized to flavo-G by UGT1A1 and UGT1A9 (Hagenauer, Salamon et al. 2001), single nucleotide polymorphisms (SNPs) of those genes in individual patients may contribute to those BSV in patients.

Typical value of PK parameters and calculated K13 and K30 were similar from both models, except V2. When V3 (fixed to 1L) and observed flavo-G concentrations were the same for both models, total amount of flavo-G in metabolite compartment were the same.
However, since different metabolite fractions were estimated from different models (0.225 and 0.118 for model1 and model2, respectively), the amount of flavopiridol which was converted to flavo-G would be different. In other words, the amount of flavopiridol would be different, which caused V2 predictions were different from the two models.

Unlike the flavopiridol PK model described in Chapter 2, bilirubin was not shown as a significant covariate in this metabolite model. However, weight still directly correlated with flavopiridol clearance. Also, WBC indirectly correlated with flavo-G clearance, which suggested higher WBC may be linked to higher flavo-G levels. This finding is consistent with previous clinical studies that showed WBC significantly correlated with TLS after flavopiridol treatment (Kristie A. Blum 2010) and higher flavo-G exposure level correlated with TLS(Phelps, Lin et al. 2009). However, more data set are need for further confirm this finding.

In conclusion, a population pharmacokinetic model for flavopiridol and its metabolite, flavo-G, was first time developed and validated. Flavo-G has been shown correlated with TLS(Kristie A. Blum 2010), the most severe adverse events after flavopiridol treatment. This model would help us predict flavo-G exposure by incorporating simulation steps in later clinical studies. Glucuronidase expression levels, physiology conditions, etc are all potential factors which would affect flavopiridol metabolite. Although we did not find any covariates which can reduce BSV, that may due to the patients recruited in this study.
If we can get more data set in future, this model would be useful to evaluate the covariates which contribute to the variants of flavo-G exposure in different patients.
Figure 5.1. Concentration and time profile (A) flavopiridol and (B) flavo-G.
Figure 5.2. Two metabolite model structures of linked two-compartment model of parent (flavopiridol) and one-compartment of metabolite (flavo-G). (A) model1 (B) model2. 1, the central compartment of flavopiridol; 2, peripheral compartment of flavopiridol; 3, central compartment of flavo-G; K12, transfer from central to peripheral compartment of flavopiridol; K21, transfer from peripheral to central compartment of flavopiridol; K13, transfer from central compartment of flavopiridol to central compartment of flavo-G; K10, first-order elimination rate of flavopiridol from central compartment; K30, first-order elimination rate of flavo-G from central compartment.
Figure 5.3. Goodness-of-fit plots from two metabolite models. Observed concentration from flavopiridol (left) and flavo-G (right) versus population prediction and individual prediction. (A) Model1. (B) Model2.
Figure 5.3. Continued

(B)
Figure 5.4. CWRES versus dependent variables.  (A) CWRES versus flavopiridol (left) and flavo-G (right) population prediction and individual prediction from Model 1. (B) CWRES versus flavopiridol (left) and flavo-G (right) population prediction and individual prediction from Model 2.
Figure 5.4. Continued

(B)
Figure 5.5. CWRES versus independent variable. (A) CWRES versus ID and Time from Model 1. (B) CWRES versus ID and Time from Model 2. (left: flavopiridol; right: flavo-
G)
Figure 5.5. Continued

(B)
Figure 5.6. CWRES distribution from both models. (A) Model 1. (B) Model 2.
Figure 5.7. PK Parameters versus covariates plot. (A) Flavopiridol clearance (L/hr) versus weight (WT; kg). (B) Flavo-G clearance (CLM; L/hr) versus white blood cell count (WBC; $10^9$/L).
<table>
<thead>
<tr>
<th>Model Term</th>
<th>Parameter</th>
<th>Population Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>CL = θ_{CL} \cdot \exp(BSV_{CL})</td>
<td>θ_{CL} (L/h)</td>
<td>Model1</td>
</tr>
<tr>
<td>V1 = θ_{V1} \cdot \exp(BSV_{V1})</td>
<td>θ_{V1} (L)</td>
<td>68.5</td>
</tr>
<tr>
<td>Q = θ_{Q} \cdot \exp(BSV_{Q})</td>
<td>θ_{Q} (L/h)</td>
<td>10.4</td>
</tr>
<tr>
<td>V2 = θ_{V2} \cdot \exp(BSV_{V2})</td>
<td>θ_{V2} (L)</td>
<td>140</td>
</tr>
<tr>
<td>K13</td>
<td>K13 (h⁻¹)</td>
<td>0.056</td>
</tr>
<tr>
<td>K30</td>
<td>K30 (h⁻¹)</td>
<td>0.61</td>
</tr>
</tbody>
</table>

Table 5.1. Final model PK parameter estimates. The full (56 subjects, 577 plasma concentrations) dataset was used. Parameters: CL, clearance of flavopiridol; V1, volume of central compartment of flavopiridol; Q, inter-compartmental clearance of flavopiridol; V2, volume of peripheral compartment of flavopiridol; V3, volume of central compartment of flavo-G; K13, first-order input rate from V1 to V3; K30, first-order elimination rate of flavo-G from V3. (Units are noted in parenthesis)
Figure 5.8. Bootstrap results from metabolite final model. OFV: objective function value; Parent.CL: clearance of flavopiridol; V1: central compartment volume of distribution for flavopiridol; Q: intercompartment clearance of flavopiridol; V2: peripheral compartment volume of distribution for flavopiridol; Fraction: ratio of flavopiridol been cleared as parent drug; Met.CL: clearance of flavo-G; ETA.Parent.CL: BSV on clearance of flavopiridol; ETA.Q: BSV on Q; ETA.Met.CL: BSV on clearance of flavo-G.
Figure 5.8. Continued
6.1. Introduction

CLL is the most prevalent adult leukemia in the Western hemisphere. Refractory CLL patients after fludarabine and chlorambucil treatment only have less than one year median survival time (Lin 2010; Wierda, Kipps et al. 2010). “Chemoimmunotherapy” which combine purine analogs and alkylating agents with monoclonal antibodies such as rituximab has shown improved initial overall response (OR) rates, complete response (CR) rates and progression-free survival (PFS). However, those treatments caused immunosuppressive in patients (Maddocks and Lin 2009). Thus, we need more effective therapies for CLL treatment which overcome classic drug resistance yet lack immunosuppressive properties.

Flavopiridol used as a single-agent on refractory or relapsed CLL patients has shown almost 50% OR in two clinical studies (Lin, Ruppert et al. 2009; Phelps, Lin et al. 2009). However, even high OR rates have been shown after the treatment, it is still important for
us to understand what cause other 50% patients not respond to the treatment and if drug exposure is involved.

In previous studies, we found flavopiridol area under the plasma concentration time curve \((AUC_{0-\infty})\) correlated with clinical response and cytokine release syndrome, and flavo-G \(AUC_{0-\infty}\) correlated with tumor lysis syndrome (Phelps, Lin et al. 2009). Those suggested that in addition to parent drug, flavo-G also might play a role in CLL treatment. In order to better understand the effects on clinical response, we evaluated both flavopiridol and flavo-G exposure in this study. For the best of our knowledge, there are no comprehensive analyses of flavopiridol or flavo-G exposure-response relationships have been done so far.

All CLL patients received flavopiridol treatment are evaluated for response including complete response (CR), partial response (PR), progressive disease (PD) and stable disease (SD) based on clinic, hematologic, and bone marrow features according to the Revised National Cancer Institute-sponsored Working Group Guidelines for response (Cheson, Bennett et al. 1996). Since our goal for the treatment is that patients gain CR or PR, a sound understanding of the key predictors of the response of flavopiridol would be important and will facilitate its optimal use.

The purpose of this study was to create appropriate population pharmacodynamic model to describe potential relationship between flavopiridol and flavo-G exposure \((AUC_{0-\infty}\) and \(C_{\text{max}}\)) and clinical response. We present a logistic regression model by using fixed effects
method to predict clinical response after flavopiridol treatment in patients with CLL, and flavopiridol AUC appeared to be a key predictor for the response.

6.2. Methods

6.2.1 Study protocols, subjects and clinical design

The Phase 1 clinical trial (OSU0055) data was used for this study. Protocols, subjects and clinical design were described in Chapter 2. There were 3 out of 52 patient responses which were not available, therefore only 49 subjects in this PD study.

6.2.2 Pharmacokinetic sampling and analytical assay

PK sampling and analytical assay were described in Chapter 2. Calculated parameters were obtained using standard non-compartmental methods with WinNonlin v5.2. (Pharsight, Mountain View, CA).

All patients were evaluated for response; criteria for response followed the Revised National Cancer Institute (NCI)-sponsored Working Group Guidelines for CLL (Cheson, Bennett et al. 1996). For patients not demonstrating disease progression, response status was evaluated utilizing the formal NCI criteria two months after completion of the last treatment of flavopiridol.

6.2.3. Pharmacokinetic-pharmacodynamic analysis

The pharmacokinetic-pharmacodynamic relationship between exposure and response was established with AUC$_{0-\infty}$ and C$_{max}$ of flavopiridol and its glucornide metabolite, flavo-G
which were obtained from the non-compartmental pharmacokinetic analysis. The reason we did not use the model predicted AUC and $C_{\text{max}}$ for flavopiridol and flavo-G from chapter 5 was that in that model we were unable to predict BSV on V1 and V2 which may affect the accuracy for calculating AUC and $C_{\text{max}}$.

25 patients (51%) had PR and 24 patients (49%) had either SD or PD. No patient had CR in this study. We combined SD and PD as one category (SD or PD = 0), and PR as a second category (PR = 1). In that case, the response of a particular patient was considered as binary. The probability of the response (SD/PD) with a DV value of 0 is denoted by $p = P(DV=0)$. The probability of the response (PR) with a DV of 1 is denoted by $P(DV=1)=1-p$. The probability distribution of DV can then be denoted by $P(DV=r)=p^r(1-p)^{1-r}$, $r=0,1$.

In our study, for a single patient, the individual binary responses of 49 patients can be described using the binomial distribution, with

$$P(Y=y)={n\choose y}p^y(1-p)^{n-y}, \quad n=49, \quad y=0, \ 1, \ \ldots, \ 49 \ (6.1)$$

Here, $y$ is the total number of the patients with successful outcomes (PR) and we assume that each patient’s response is independent of each other. The probability of a particular distribution of PR or SD/PD for those 49 patients is $p^y(1-p)^{n-y}$ ($n=49$) and the total number of ways in which a sequence of $y$ and $(n-y)$ can happen is

$$\binom{n}{y} = \frac{n!}{y!(n-y)!} \quad (n = 49) \ (6.2)$$
For our PD modeling purposes, we wanted to develop a model that was able to describe the relationship between binary endpoint(y) and predictor variables x(DV in our case), so we wanted to evaluate the conditional mean of y given x, or $E(Y|x)$. In our model, $E(Y|x)$ is only at values between 0 and 1. In order to allow the expected values from our data to be constrained between 0 and 1, we used the logit transformation method, which maps probabilities (p) onto a $-\infty$ to $+\infty$ scale.

Laplacian estimation methods with the likelihood and -2LL option were evaluated for this logistic regression model. Since there was only one single endpoint observation available from each patient, BSV was added to the logit with the estimation of OMEGA fixed to 0 ($\text{OMEGA 0 FIXED}$). See Discussion section for more details.

In this study, the base model to be specified in NONMEM is described in the Eq. (6.1) and (6.2):

$$Logit_j = \theta_1 + \eta_j \quad (6.3)$$
$$P_j = \frac{e^{Logit_j}}{1 + e^{Logit_j}} \quad (6.4)$$

where $\theta_1$ is the typical value of the logit; $\eta_j$ is the discrepancy between the typical value of the logit and the true logit in the $j$th patient; $\eta_j$ is independent and identically distributed random variables with mean 0 and variance $\omega^2$; and $P_j$ is the predicted probability of PR in the $j$th patient. The value of logit determines the rate of change in $P_j$, with higher values indicating a faster rate of change.
In order to evaluate the relationship between flavopiridol and flavo-G exposure and patient response, linear models included a centered effect of either AUC_{0-\infty} or C_{\text{max}} or both. The models are described as the following equations:

\[
\text{Logit}_j = \theta_1 + \theta_2 \times (AUC_j - 10.5) + \theta_3 \times (CMAX_j - 0.8) + \eta_j \quad (MOD1)
\]
\[
\text{Logit}_j = \theta_1 + \theta_2 \times (AUC_j - 37) + \theta_3 \times (MMAX_j - 1.97) + \eta_j \quad (MOD2)
\]
\[
\text{Logit}_j = \theta_1 + \theta_2 \times (AUC_j - 10.5) + \theta_3 \times (AUCM_j - 37) + \eta_j \quad (MOD3)
\]
\[
\text{Logit}_j = \theta_1 + \theta_2 \times (AUC_j - 10.5) + \eta_j \quad (MOD4)
\]
\[
\text{Logit}_j = \theta_1 + \theta_2 \times (AUCM_j - 37) + \eta_j \quad (MOD5)
\]
\[
\text{Logit}_j = \theta_1 + \theta_2 \times (CMAX_j - 0.8) + \theta_3 \times (MMAX_j - 1.97) + \eta_j \quad (MOD6)
\]
\[
\text{Logit}_j = \theta_1 + \theta_2 \times (MMAX_j - 1.97) + \eta_j \quad (MOD7)
\]
\[
\text{Logit}_j = \theta_1 + \theta_2 \times (CMAX_j - 0.8) + \eta_j \quad (MOD8)
\]

where \(\theta_1\) is the typical value of the logit at the median AUC_{0-\infty} or C_{\text{max}} value: 10.5 \(\mu\text{g}\cdot\text{h/mL}\) and 37 \(\mu\text{g}\cdot\text{h/mL}\) are median AUC_{0-\infty} values for flavopiridol (AUC) and flavo-G (AUCM), respectively; 0.8 \(\mu\text{g/mL}\) and 1.97 \(\mu\text{g/mL}\) are median C_{\text{max}} values for flavopiridol (CMAX) and flavo-G (MMAX), respectively. \(\theta_2\) and \(\theta_3\) are the typical value of the slope relating its associated covariate to the logit. \(\eta\) and \(P_j\) represented the same meaning as in equation (6.1) and (6.2).

6.3. Results

6.3.1. Base pharmacodynamics model identification
Patient characteristics and treatment groups were described in Chapter 5. Patient response and toxicity were previously described (Byrd, Lin et al. 2007; Phelps, Lin et al. 2009). Forty-nine patients had scores recorded two months after completion of the last treatment of flavopiridol as follows: 0 = stable disease or progressive disease; 1 = partial response. No patients had complete responses in this study.

The report file from the base model run with Laplacian estimation with the likelihood or -2LL method was summarized in Table 6.1. Both estimation methods provided the same minimum value of the objective function and logit value. However, the standard error was 2.30 from likelihood method and 7.01 from -2LL method. Since both estimation methods had the same OFV and logit value, and the likelihood method had a significantly smaller standard error compared to that of the -2LL method, the likelihood method was used for further model building. By incorporating predict logit from the model (LOGODDS=0.04) into equation (6.4), the predicted probability of PR was 53%, which was similar to the probability of PR in our data set.

6.3.2. Final pharmacodynamics model identification

AUC$_{0-\infty}$ and maximum concentration (C$_{max}$) of flavopiridol and flavo-G were used as covariate candidates. From covariate screening, OFV was significantly changed ($\Delta$OFV=-7.69) in MOD4 when compared to the base model (Table 6.2). With additional covariate added to MOD4, OFV was not changed more than 3.84 (MOD1, 2, and 3). Table 6.3 summarized the final model (MOD4): the typical value of the logit at the
median AUC ($\theta_1$) (SE) is -2.62 (2.44); the typical value of the slope relating AUC to the logit ($\theta_2$)(SE) is 0.30 (0.40). Figure 6.1 showed observed and predicted clinical outcome with respect to flavopiridol AUC.

6.4. Discussion

In this study, an exposure–response model was developed using a population PK/PD approach. Single efficacy, which was PR (DV=1) versus SD/PD (DV=0), was used as the dependent variable. Logistic regression technique was used to model the binary endpoints.

Our previous population PK chapters (chapter 2 and chapter 5) used mixed-effects models due to the repeated sampling from each subject over time and the goal to estimate different variability including the variation from the same subject over time and the variation between each subject. However, for general binary endpoint modeling purposes, a fixed-effects model approach was applied and only one level of random effects (OMEGA) was added to the logit as $\eta_j$ (as shown in equation 6.3 in our study). As there was only one endpoint collected in each subject, the OMEGA was fixed to 0 in our population PD analysis study. Here we developed a logistic regression population PD model to describe the relationship between clinical outcome and drug exposure.

Our previous studies found that flavopiridol $\text{AUC}_{0-\infty}$ was correlated with response, and met the .05 level of significance when comparing means across response groups in the
same phase 1 trial. Significant differences in mean \( \text{AUC}_{0-\infty} \) are observed between PR and PD and SD groups (\( P < .004 \)) (Phelps, Lin et al. 2009).

Adding flavopiridol AUC as a covariate in our base model significantly reduced OFV (\( \Delta \text{OFV} < -7.69, p < 0.005 \), one degree of freedom), which suggested flavopiridol exposure was significantly correlated to clinical outcome. No significant correlation was found between response and flavo-G \( \text{AUC}_{0-\infty} \), \( C_{\text{max}} \) of flavopiridol and flavo-G (Table 6.2). Our final model suggests that when AUC of flavopiridol increase 1hr*μM, logit value will increase 0.3.

This model adequately predicted the clinical outcome for our phase 1 study since 53% patients in this study had partial response while our model predicted result was 51%, which was only 3.8% difference. The finding of flavopiridol exposure significantly correlate with clinical outcome was consistent with our previous ANOVA analysis. Observed and predicted clinical outcome with respect to flavopiridol AUC showed higher AUC correlated with higher probability of having PR (Fig 6.1). We also noticed that there was some overlap in AUC between patients had PR and SD/PD, which suggested there are other potential factors other than AUC also contribute to response. In addition to drug metabolite enzymes and transporters which affect flavopiridol PK and furthermore drug exposure and response, the sensitivity of CLL cells in individual patient may also affects response. For example, in Chapter 4 we discussed the results from our in vitro studies by using primary CLL cells that GSH levels in CLL cells response differently to
flavopiridol even at the same concentration. In the different transformed cell lines with different baseline GSH levels, the same flavopiridol concentration caused different cytotoxicity. Those findings suggested drug exposure level was not the only factor in terms of response.

In conclusion, a population PD model was developed and characterized the positive PK-PD relationship between flavopiridol AUC and clinical outcome. The population modeling described here continuous to refine our knowledge of this potent drug on CLL patients, and when appropriate, will lead to optimized dosing strategies.
Table 6.1. Comparison of different estimation methods used on base pharmacodynamic model. OFV: Objective function value; LGODDS: Typical value for logit; se: standard error

<table>
<thead>
<tr>
<th>Run</th>
<th>OFV</th>
<th>LGODDS</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>0055_PD_BASE_LIKELIHOOD</td>
<td>67.91</td>
<td>0.04</td>
<td>2.31</td>
</tr>
<tr>
<td>0055_PD_BASE_2LL</td>
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<td>0.04</td>
<td>7.01</td>
</tr>
<tr>
<td>Model Number</td>
<td>Run</td>
<td>OFV</td>
<td>ΔOFV</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------------</td>
<td>------</td>
<td>------</td>
</tr>
<tr>
<td>MOD1</td>
<td>0055_PD_AUC_CMAX</td>
<td>58.97</td>
<td>-8.94</td>
</tr>
<tr>
<td>MOD2</td>
<td>0055_PD_AUC_MMAX</td>
<td>59.21</td>
<td>-8.70</td>
</tr>
<tr>
<td>MOD3</td>
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</tr>
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<td>0055_PD_AUC</td>
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</tr>
<tr>
<td>MOD5</td>
<td>0055_PD_AUCM</td>
<td>65.75</td>
<td>-2.16</td>
</tr>
<tr>
<td>MOD6</td>
<td>0055_PD_CMAX_MMAX</td>
<td>65.97</td>
<td>-1.94</td>
</tr>
<tr>
<td>MOD7</td>
<td>0055_PD_MMAX</td>
<td>66.12</td>
<td>-1.79</td>
</tr>
<tr>
<td>MOD8</td>
<td>0055_PD_CMAX</td>
<td>67.63</td>
<td>-0.28</td>
</tr>
</tbody>
</table>

Table 6.2. Summary of population pharmacodynamic model building runs. MOD: Model; OFV: Objective function value; ΔOFV: the change of OFV from base model.
<table>
<thead>
<tr>
<th>THETA1</th>
<th>THETA2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(AUC_flavo)</td>
</tr>
<tr>
<td>Estimate</td>
<td>SE</td>
</tr>
<tr>
<td>-2.62</td>
<td>2.44</td>
</tr>
</tbody>
</table>

Objective function value: 60.22

Table 6.3. Summary of final population pharmacodynamic model estimates
Figure 6.1. Observed (dots) and predicted clinical outcome (line) with respect to flavopiridol AUC.
Chapter 7: CONCLUSIONS AND PERSPECTIVES

7.1. Conclusions

Despite advanced treatment such as “chemoimmunotherapy”, CLL remains incurable with standard therapies. Refractory or relapsed patients have less than one year median survival time (Lin 2010). Ofatumumab, a second generation anti-CD20 monoclonal antibody has shown 50% OR rate as single-agent treatment and its median PFS was 5.7 months for fludarabine- and alemtuzumab-refractory patients and 5.9 months for fludarabine-refractory CLL with bulky (>5cm) lymphadenopathy patients (Wierda, Kipps et al. 2010). Flavopiridol, a small molecule drug, used as a single-agent to treat refractory or relapsed CLL patients has shown OR rates similar to Ofatumumab in our clinical studies, but with longer PFS (8.6 months) (Lin, Ruppert et al. 2009; Phelps, Lin et al. 2009; Wierda, Kipps et al. 2010). It broadly inhibits CDKs, including CDKS 1, 2, 4, 7, 8 and 9. Its anti-cancer mechanisms act through a p53 independent pathway by activating caspase 3 in primary CLL cells and down-regulating anti-apoptosis proteins Mcl-1 and XIAP, which are overexpressed in patients with CLL. Flavopiridol also can decrease phosphorylation and transcriptional activity of RNA polymerase II (Byrd, Lin et al. 2006).
This study focused on a phase 1 flavopiridol clinical trial (OSU0055), using a population PK/PD approach to evaluate potential effects from demographics and genetics of drug transporters and metabolic enzymes which may contribute to BSV and residual variability in pharmacokinetics and outcomes. In vitro studies were also applied to functionally evaluate OATP1B1 and the impact of GSH depletion; two factors with potential effects on flavopiridol treatment in PK and outcomes, respectively.

Large BSV was observed in both the response and toxicity associated with flavopiridol treatment. To investigate the genetic factors contributing to this variability, we used a pharmacogenetic approach and evaluated the effects of gene polymorphisms on PK and clinical outcomes. The candidate gene approach in our study was based on the hypothesis that genetic variations in metabolism and transporter genes play an important role in the pharmacokinetics or pharmacodynamics of flavopiridol and would potentially affect the drug’s efficacy and/or toxicity.

Flavopiridol has a narrow therapeutic window, and it is therefore critical for us to understand the factors that influence drug exposure. In Chapter 2, we developed a population flavopiridol PK model based on OSU0055 by using non-linear mixed effects modeling methods. A two-compartment model followed by first-order elimination adequately fit our data. Bilirubin was shown as the only significant covariate, and it impacted inter-compartmental clearance in this model. We focused on SNPs of drug transporters (BCRP, MRP2 and OATP1B1), and UDP-glucuronosyltransferas. BCRP,
MRP2 and OATP1B1 all showed significant correlation with various PK parameters from our model. However, there was no correlation shown between UGTs and flavopiridol PK parameters. Results indicated polymorphisms of drug transporters (BCRP, MRP2 and OATP1B1) may significantly affect flavopiridol disposition, which DDI studies may be necessary if combination regimens are used and other drugs are substrates or inhibitors of those transporters. It is important to note that our study was relatively small, and included pharmacogenetic data from only 39 patients. Effects we observed therefore likely represented those SNPs with the largest effects. Larger studies would potentially reveal other SNPs that significantly contribute to variability but perhaps to a lesser or equal magnitude.

To validate our findings about the OATP1B1 transporter from the population flavopiridol PK model, in chapter 3 we evaluated the uptake function of OATP1B1 and its three nonsynonymous SNPs: Thr155Pro (T155P, rs11045819), Asn130Asp (D130N, rs230628) and Val174Ala (V174A, rs4149056). Our in vitro studies showed Thr155Pro and Val174Ala SNPs significantly reduced flavopiridol uptake velocity, while Asn130Asp SNP did not. Interestingly, our in vitro findings correlated with the clinical data, as SNPs in OATP1B1 (rs11045819 and rs4149056) that affected uptake velocities also had significant effects on PK parameters (V1 and CL, respectively).

Previous studies have shown that GSH acts as an antioxidant and plays an important role in cellular redox balance and protects cells from oxidative stress (Lopez-Lazaro 2010). In
chapter 4, we evaluated GSH levels before and after flavopiridol treatment in transformed cell lines and primary CLL cells. GSH levels were reduced in both transformed cells and CLL patient cells after flavopiridol treatment in our study. Results from our study and from previous studies (Ellerby, Ellerby et al. 1996; Meredith, Cusick et al. 1998) showed that overexpression of bcl-2 increases GSH levels (Fig. 4.1A). Since the overexpression of bcl-2 increased the baseline GSH level in 697-bcl2 compared to 697-neo cells, it may explain that the reduction of GSH in 697-bcl2 cells was less than in 697-neo cells (10% and 25%, respectively) (Fig.4.1A). GSH altering agents (BSO and 4-S-CPG) significantly reduced flavopiridol LC_{50} in Jurkat and 697-neo transformed cell lines. This supports a hypothesis that glutathione level mediates the cytotoxic effects of flavopiridol. In addition, flavopiridol treatment of CLL patient cells showed that the change of GSH level was variable among patients. Future studies will further evaluate the effects of GSH levels on flavopiridol by measuring LC_{50} and the change in GSH post-flavopiridol dose in primary CLL cells from the same patient.

Our previous studies showed that after flavopiridol treatment, flavo-G exposure highly correlated with TLS in CLL patients (Lin, Ruppert et al. 2009; Phelps, Lin et al. 2009; Kristie A. Blum 2010; Ni, Ji et al. 2010). Chapter 5 presented the development of a metabolite PK model that incorporated flavopiridol and its metabolite, flavo-G. This model may serve as a useful tool for simulating both flavopiridol and flavo-G exposure and predicting adverse events from flavopiridol treatment in future studies. As flavopiridol is metabolized by UGT1A1/1A4/1A9 in humans and we have the genetic
SNP data on UGT1A1 and 1A9, we evaluated if genetic polymorphism covariates in these two genes were able to reduce BSV in our model. The results showed trends for improvement with inclusion of these SNPs, but the results were not statistically justified. Expansion of our database and inclusion of additional patient data and additional UGT1A4 SNPs information will enable a more definitive assessment of the impact of these genes on PK and outcomes.

Chapter 6 presented a population PD approach to model clinical response with respect to flavopiridol and flavo-G exposure. Our resulting model sufficiently represented the previously observed clinical relationship between flavopiridol AUC and response (Phelps, Lin et al. 2009). However, the overlapping lower AUCs between responders and non-responders suggest other factors aside from parent drug PK are involved. Within our current data set, no other factors help to explain this overlap. In chapter 4, we showed that GSH levels affect CLL cells sensitivity to flavopiridol treatment, which may partially explain why even with the same exposure patients could have different response. This is another potential avenue for future investigation.

7.2. Significance

CLL is the most prevalent adult leukemia in the Western hemisphere, and 15,000 to 20,000 new cases appear in the United States every year (Lin, 2010). Refractory patients from chemotherapy or “chemoimmunotherapy” have less than one year median survival.
Flavopiridol, the first generation of cyclin-dependent kinase inhibitors, has shown remarkable anti-cancer activity in refractory CLL: 50% OR in two clinical trials.

This study highlights the complexity of the network of factors that contribute to drug disposition, drug activity and outcomes from therapy. The clinical development of flavopiridol and the data presented herein demonstrate that PK is one of the important components in this activity, ultimately affecting outcomes from treatment. We presented the first PK/PD/PG approach to evaluate possible factors that affect flavopiridol disposition and clinical outcomes. Three transporters (OATP1B1, BCRP, and MRP2) were shown to correlate with inter-individual variability in PK. A population PD model was developed and characterized the positive PK-PD relationship between flavopiridol AUC and clinical response. PD results also suggested more factors other than drug exposure can affect clinical response.

We and our collaborators continued to probe the pharmacological bases for the impressive activity of flavopiridol and other CDKIs. Dinaciclib (SCH 727965) is a second-generation CDKI under development and has shown clinical similarity to flavopiridol, both in response rates and in its ability to induce TLS (JM. Flynn 2010). As this class of agents continues in clinical development, better understanding of the mechanisms and factors governing the balance between safety and efficacy will be essential if flavopiridol and/or other CDKIs will achieve FDA approval and eventual use in the broader clinical setting.
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116


