Pharmacogenetic determinants of cyclophosphamide pharmacokinetics in pediatric cancer patients.

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A thesis

submitted in partial fulfillment of the

requirements for the degree of

Master of Public Health

University of Washington

2015

Committee:

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Program Authorized to Offer Degree:

Public Health Genetics

University of Washington

Abstract

Purpose: Cyclophosphamide (CY) is an integral component of many combination chemotherapy regimens to treat pediatric cancer patients. Numerous metabolic and transport pathways are involved in the formation and elimination of 4-hydroxycyclophosphamide (4HCY), the principal precursor to CY's cytotoxic metabolite phosphoramide mustard. We sought to characterize the pharmacogenomic– pharmacokinetic association in pediatric cancer patients.

Methods: Seventy-two children less than 21 years old receiving one of six CY-based combination chemotherapy regimens were included. The primary phenotypic endpoint was 4HCY/CY area under the curve (AUC) ratio, which was estimated using noncompartmental analysis after the first CY dose of a cycle. Numerous (N=323) single nucleotide polymorphisms (SNPs) were selected from literature review. DNA was genotyped using Illumina's GoldenGate genotyping. An additive model for the effect of minor (derived) alleles was fitted for each SNP, adjusting for age and ancestry via principal component decomposition of 31 ancestry informative markers (AIMs) that also were genotyped.

Results: The 4HCY/CY AUC ratio was evaluable in 49 children; the ratio decreased as age increased (R^2=0.148, p=0.0064). The most significant p-values for association were found for SNPs in *ABCC3* (p< 0.01), *ABBC4* (p=0.02) and *CYP2B6* (p<0.03). There was some suggestion that the association between outcome and the *CYP1A1* variant allele present in **2A* and **2B* might differ by ancestral background.

Conclusions: Considerable interpatient variability exists in CY pharmacokinetics, but this was not associated with genetic polymorphisms in pediatric cancer patients.

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Introduction

The prodrug cyclophosphamide (CY) is a widely used alkylating agent, used in over half of the regimens for newly diagnosed pediatric cancer patients.[1] There is substantial interpatient variability in the efficacy and toxicity of CY-based combination chemotherapy regimens. This variability in clinical outcomes may be partially attributed to the substantive pharmacokinetic variability of CY and its metabolites. CY is metabolized to 4-hydroxycyclophosphamide (4HCY), which is the precursor to CY's primary cytotoxic metabolite phosphoramide mustard.[2,3] Because phosphoramide mustard does not cross cell membranes easily, the transport of its precursor, 4HCY, into the cell is a key step of CY's cytotoxic activity.[2] Once 4HCY is transported into the cell, it exists in equilibrium with its tautomer, aldophosphamide (AldoCY). AldoCY rapidly undergoes β-elimination to acrolein and phosphoramide mustard, which in turn alkylates DNA via the formation of a reactive diazidironium species. The alkylation and formation of cross-links within DNA subsequently lead to apoptosis, thus producing the desired therapeutic effect.[4,5]

One of the most significant limitations to optimizing CY-based chemotherapy regimens, however, was the difficulty in quantifying plasma concentrations of 4-hydroxycyclophosphamide (4HCY) because this metabolite has an *in vitro* half-life of less than three minutes.[6,7] Personalizing CY doses using pharmacokinetics with the goal of improving clinical outcomes is feasible, but is highly labor intensive.[8] Alternatively, personalization with germline pharmacogenomics offers a more clinically-feasible method that could be generalizable to many pediatric cancer centers.

The association of germline polymorphisms with various CY endpoints – efficacy, toxicity or pharmacokinetics – have largely been conducted in adult populations (Table 1). However, children clear CY faster and have a different 4HCY and CY exposure than adults. Children have more rapid cyclophosphamide clearance normalized to standard body surface area (BSA, i.e., ml/min/m^2) compared to adults; the mechanism is unidentified.[9-13] There is a paucity of data regarding the effect of age upon the pharmacokinetics of CY metabolites, although the varying ontogeny of the various drug metabolizing enzymes and transporters involved in CY disposition are suggestive that the genotype – pharmacokinetic phenotype will differ based on age. Young children (i.e., < 10 years) have greater and more variable ratios of 4HCY/CY area under the plasma concentration – time curve (AUC) – a marker of 4HCY exposure – compared to adults (Figure 1). Approximately 25% (range 5-30%) of an intravenous CY dose is excreted unchanged in the urine, however a major fraction of a CY dose (70%) is metabolized to 4HCY by multiple cytochrome P450 enzymes (CYP, specifically CYP2B6, CYP2C9, CYP2C19, CYP3A4, and CYP3A5) and myeloperoxidase (MPO).[1,2,14,15] Exposure to 4HCY is further mediated by 4HCY elimination by glutathione S-transferases (GSTs), aldehyde dehydrogenases (ALDHs), and a subset of ATP-binding cassette transporters (ABCCs).[2] Studies focused on the pharmacogenomic – pharmacokinetic phenotype association predominantly in adult cancer patients have generated conflicting results (Table 1).[3,16,17] The results have been conflicting potentially due to small sample sizes, not accounting for the impact of concomitant drugs upon CY metabolism (e.g., thiotepa inhibiting CYP2B6[18] and affecting the association between CYP2B6 genotype and CY pharmacokinetics in thiotepa containing regimens[2,19]), and heterogeneous study populations. The majority of pharmacogenetic studies in children receiving CY (shaded blue in Table 1) were focused upon *glutathione S-transferase* (*GST)* polymorphisms,

with data suggesting they could influence 4HCY elimination.[20-24] Presently, candidate genes associated with CY pharmacodynamics in children have yet to be rigorously tested.[25] For these reasons, we focused upon the pharmacogenomics of 4HCY therapy in children to elucidate any relevant associations among pediatric patients that could be utilized in the clinical setting.

This manuscript reports the germline pharmacogenomics – CY pharmacokinetic phenotype from pediatric cancer patients. This study focused on characterizing the role of previously implicated genetic loci with the pharmacokinetic phenotype *in vivo* amongst children that were receiving CY. The genes and enzymes of interest were those involved in 4HCY formation (i.e., *CYP2B6*, *CYP2C9*, *CYP2C19*, *CYP3A4*, *CYP3A5*, *MPO)* and 4HCY elimination (i.e., *ALDH1A1*, *ALDH3A1*, *GSTA1*, *GSTM1*, *GSTP1*, *GSTT1*, *ABCC2*, and *ABCC4)* and outcomes after administration of CY-based chemotherapy (i.e., ERCC1, XRCC1, TP53, TLR4, NAT1/2, NOS, CSF3R). Based on the previously published literature on the mechanism of CY and 4HCY activity, this study employed a candidate gene approach to examine the roles of the aforementioned variants.

Methods

Patient population. Seventy-two children receiving their first cycle of a CY-based combination chemotherapy regimen were enrolled into the pharmacokinetic study. The Seattle Children's Hospital Institutional Review Board approved the study, and all participants' parents gave informed consent prior to participation. In addition to written informed consent from their parents or guardians, children aged 14-17 years gave written assent.

Pharmacokinetic Analysis of CY and Metabolites. Pharmacokinetic samples were drawn immediately before CY administration, upon completion of the 30-minute infusion, and at 2.5, 4.5, 6.5, 22.5, and 24 h after the start of the first CY dose. The sample processing and analysis procedures have been previously published.[3] Briefly, blood was drawn into tubes containing either phenylhydrazine, for analysis of 4HCY, or ethylenediaminetetraacetic acid, for analysis of CY and carboxyethylphosphoramide mustard (CEPM). The tubes were inverted three to four times, stored at 4°C for a maximum of 1 h, and centrifuged. Plasma was removed and stored at -70°C within 1 h of sample collection. All samples were shipped on dry ice to our laboratory within three months of collection. Plasma concentrations were determined for CY and its metabolites using previously reported methodology; 4HCY concentrations were quantitated separately.[33] A noncompartmental model was used to calculate the AUC from time 0 to infinity after the first CY dose using WinNonlin Version 2.0 (Pharsight, Mountain View, CA).

DNA genotyping. DNA was genotyped using Illumina's GoldenGate Genotyping assay on the VeraCode Platform (Illumina, San Diego, CA),[26] following manufacturer recommendations (VeraCode Assay Guide 11312819 rev A1). In brief, 250 ng of genomic DNA was aliquoted into 96-well plates, processed accordingly and scanned on the BeadXpress reader, using GenomeStudio (v2011.1) software. The following quality control (QC) procedures were conducted. Prior to running the study samples, 90 samples representing 30 parent-parent-child CEPH trios (Utah residents with ancestry from northern and western Europe)[27] were genotyped to assess performance of the Illumina OPA. Assay accuracy was verified by comparing genotypes to publicly available genotype data for these samples from HapMap (http://www.hapmap.org/), 1000Genomes (http://www.1000genomes.org), dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP/), and by assessing inheritance errors. Two external

control samples from the HapMap project were included on each plate to confirm reliability and reproducibility of the genotyping across the study plates. Intra- and inter-plate duplicates comprised >10% of all samples. Laboratory personnel were blinded to all research information about the samples. Other quality control procedures included use of barcodes on samples and plates, dedicated materials and working space, and visual review of SNP cluster plots by two laboratory staff members. Samples with weak signals, discordant duplicates, and outliers were repeated at least once.

Any SNPs with less than 90% concordance with public data for CEPH controls were considered failed. Among successfully genotyped SNPs, sample and SNP call rates were >85%. All non-ancestry SNPs were in Hardy-Weinberg equilibrium ($p>0.001$).

Statistical analysis. The primary endpoint of interest was the ratio of 4HCY/CY AUC after CY dose 1. This ratio is governed by the partial clearance of CY to 4HCY (mediated by CYP2B6, CYP2C9, CYP2C19, CYP3A4, CYP3A5, MPO) and the elimination of 4HCY (mediated by ALDH1A1, ALDH3A1, GSTA1, GSTM1, GSTP1, GSTT1, ABCC1, ABCC2, ABCC3 and ABCC4 transporters).[2,28,29] Secondary analyses was conducted on the other pharmacokinetic endpoints (i.e., AUC of CY, 4HCY, CEPM, deschloroethyl-cyclophosphamide (DCCY) corrected for the CY dose administered) and the pharmacokinetics-based candidate genes.[30] Since the mean ratio of 4HCY/CY AUC differs based on age and CY dose (Figure 1), an exploratory genotype-pharmacokinetic analysis was conducted using two-way analysis of covariance with age and CY dose as covariates. We used the Arimitage test for trend in a univariate analysis and linear regression in an analysis adjusted for age and CY dose. The entire set of candidate SNPs was tested in this manner to obtain nominal significance levels.

Adjustments for multiple testing were done by permuting the observed genotypes (or residual genotypes after adjustment) and repeating the testing procedure 10,000 times to obtain the joint null distribution of the test statistics, particularly the minimum p-value under the observed correlation structure. This is important because the correlation structure of the SNPs (linkage disequilibrium) produces clusters of correlated statistics for which a Bonferroni correction is expected to be quite conservative in assessment of the effect of the multiple testing. As an exploratory analysis, we grouped individuals with genotypes for which previous data suggested a similar phenotype (e.g., *CYP2C9* polymorphisms associated with lower enzyme activity, specifically *CYP2C9*1/*2, *2/*2, *1/*3, *3/*3*[31] were all categorized together as low 4HCY genotype compared to *CYP2C9*1/*1* with normal activity being categorized a high 4HCY genotype). This approach has the potential to increase power and provide additional evidence for some of the assumed phenotypic impacts. Power could also have be reduced by grouping genotypes with and without association with the actual outcomes together; we believed the unbiased approach to the phenotypic association in the primary analysis above was the most scientifically sound and justified at that stage of knowledge. Analysis was done using R/Bioconductor (www.r-project.org) and Microsoft Excel.

Power calculation. Power calculations were based on the pharmacokinetic data from 147 pediatric and adult patients receiving a myeloablative dose of CY[32] and 22 children receiving a fractionated conventional dose of CY/topotecan.[3] The mean and standard deviation of each parameter of the ratio of 4HCY/CY AUC, 4HCY AUC and CEPM AUC were calculated. We sought to have evaluable pharmacokinetics on 90 children receiving standard dose CY, with a minimum of 8 patients per CY regimen. With a minimum of 8 patients per CY regimen, there is over 80% power to observe a 4-fold difference in the means of the ratio of 4HCY/CY AUC, 3-

fold difference in the means of 4HCY AUC and 2-fold difference in the means of CEPM AUC between two different groups. Unfortunately, the target accrual was not achieved for a multitude of reasons including increasing number of participants not finishing pharmacokinetic sampling after CY was more frequently administered in the outpatient clinic and a number of samples being below the limit of quantitation.

RESULTS

Patient population. In total, 72 pediatric cancer patients were included in the study analysis. Of these patients, 47% were female, with a median age of 5.5 years (range 0.6 – 17.5). Using NIH criteria, a large majority (75%) of the patients were identified as white. Included children were being treated for a range of diagnoses. These included 40 cases of acute lymphocytic leukemia (55.6%), 16 of Ewing's sarcoma (22.2%), 7 of rhabdomyosarcoma (9.7%), 3 of neuroblastoma (4.2%), and a total of 6 others, comprising roughly 8.4% of the patients. Patient characteristics are summarized in Table 2.

Pharmacokinetics. Of 72 patients, two received fractionated dosing (400 mg/m^2) while nearly all of the remaining children received conventional dosing (1000 – 1200 mg/m^2). The exception was one child that received a dosing of 600 mg/m^2. The median (range) AUC for CY, 4HCY, CEPM, DCCY, KetoCY, and the 4HCY/CY AUC ratio were summarized and compared. For the nine children with multiple measurements (e.g., at the first cycle with CY and a later cycle with CY), only the first dose of each cycle was used. Among the fractionated group, we found a median CY AUC of 599 μM×hr (376 – 1089 μM×hr), as compared to the conventional dosing group values of 1656 μM×hr (759 – 2546). Also, as expected, the 4HCY among the fractionated group was lower (median: 38 μM×hr, range: 31 – 47) than the conventional group (median: 73 μM×hr,

range: 24 – 154). Lastly, the AUC ratio was calculated for both the fractionated and conventional groups, giving an AUC ratio of 0.064 and 0.046, respectively. All of these measurements and calculations are summarized in Table 3.

Next, we compared the within patient variability in the 4HCY/CY AUC ratio in nine children. Of these patients, six of them had their measurements on day 1 of the first and second cycle of CY-based chemotherapy. The remaining three patients had the following measurements during either cycle 4 or 5. (See Figure 2) No patients in our study had more than three measurements. There was a strong correlation (R^2 = 0.710) of within-patient 4HCY formation clearances. Of the nine patients with repeat measurements, eight had both measurements of 4HCY formation clearances between 0.020 and 0.100. Only two patients within this group (58 and 32) saw a bigger change between the first and following cycles; the other individuals had consistent measurements. There was one subject of the nine that had both the highest 4HCY formation clearance and the largest difference between the first and following cycles.

Pharmacogenomic-pharmacokinetic phenotype association. An additive model for the effect of minor (derived) alleles was fitted for each SNP, adjusting for age and ancestry via principal component decomposition of 31 ancestry informative markers (AIMs) that also were genotyped for the study participants. The most significant p-values for association were found for SNPs in *ABCC3* ($p< 0.01$), *ABBC4* ($p=0.02$) and *CYP2B6* ($p<0.03$). There was some suggestion that the association between 4HCY/CY AUC ratio and the *CYP1A1* variant allele present in **2A*. *CYP1A1*2B* might differ by ancestral background.

DISCUSSION

Our study was the first to evaluate the pharmacogenomics – pharmacokinetic association for 4HCY in pediatric cancer patients receiving CY. The goal of this work was to improve overall survival in pediatric cancer patients receiving CY by optimizing its dosing. Key findings from the study include that CY pharmacokinetics cannot be predicted by various candidate gene SNPs chosen based on 4HCY formation enzymes (i.e., *CYP2B6, CYP2C9, CYP2C19, CYP3A4, CYP3A5, MPO*) or 4HCY elimination enzymes (i.e*., ALDH1A1, ALDH3A1, GSTA1, GSTM1, GSTP1, GSTTI, ABCC2, ABCC4*) or outcomes after administration of CY-based chemotherapy (i.e., *ERCC1, XRCC1, TP53, TLR4, NAT1/2, NOS, CSF3R*). Because of the labor intensity of pharmacokinetic sampling in the clinic, we conducted a single institution study to minimize costs, ensure consistent sample collection, and minimize variability in supportive care medications. Unfortunately, there was no association of 4HCY/CY AUC ratio with the candidate genes, suggesting that personalization with germline pharmacogenomics is not a viable method to improve outcomes in children receiving CY at this time.

The prodrug CY has a complex metabolite schema (Figure 3) with numerous drug-metabolizing enzymes (DMEs) and transporters. Initially, CY is activated to 4HCY by CYP2B6 (showing the greatest activity), CYP2C9, CYP2C19, CYP3A4, CYP3A5, and MPO.[2] Genetic polymorphisms in each of the genes encoding for these 4HCY formation enzymes, except for *CYP2C9* and *CYP3A5*, have been associated with either the efficacy, toxicity or pharmacokinetics after CY administration (Table 1). Once formed, 4HCY exists in equilibrium with its tautomer, aldophosphamide. Subsequently, 4HCY spontaneously decomposes to phosphoramide mustard, a DNA adduct that produces the desired cytotoxic effects in cancer cells, by ß-elimination of acrolein.

Additionally, attempts at modifying dosage based on pharmacogenetics, BSA or weight has yet to return reliable predictions of efficacy and toxicity, instead leading to considerable interpatient variability. To complicate matters further, there currently exists substantial variability in the expression, activation, and kinetics of the DMEs in children. Such heterogeneity in cancer pathology leads to differential responses to chemotherapeutic regimens and obfuscates the effect of concomitant chemotherapies. Notably, children receive different CY-based chemotherapy regimens than adults; however, our previous data indicated that D-actinomycin, cytarabine, doxorubicin, 6-mercaptopurine, topotecan, and vincristine had no effect upon 4HCY formation rate in human liver microsomes or CYP2B6 Supersomes®.[1]

In general, many studies investigating the pharmacogenomics and pharmacokinetics in pediatric cancer patients are limited by their small sample size, heterogeneity in disease and progression, inconsistency in CY dosing, and varying combination-based chemotherapy regimens. These challenges cannot be easily circumvented; adult data cannot be directly translated to children because the latter have faster CY clearance[2,3] and greater ratio of 4HCY/CY AUC (Figure 1A). In general, children have impaired hepatic metabolism over the first 6-12 months of life, followed by a period until adolescence when drug metabolism is more rapid relative to adults. The timing of when children attain the enzyme activity of adults is enzyme-specific, with hepatic CYP2B6, CYP2C9, CYP2C19, CYP3A4/5, and GST activity (normalized for body weight) being greater in children between the ages of 1–14 years than adults.[33-39] Data regarding the ontogeny of the other metabolizing enzymes and transporters relevant to 4HCY elimination in vivo are not available. Thus, the predominant cytochrome P450 catalyzing 4HCY formation may differ between children and adults, potentially influencing the genotype-phenotype relationship.

Previous studies, primarily *in vitro*, have investigated the relationships between genetic variants among CYP isozymes and CY metabolism and 4HCY formation.[1,19,40] As the principal oxidizing enzyme in the conversion of CY to 4HCY, for example, CYP2B6 has been a major focus of research to date.[15,41-51] We have previously demonstrated that the *CYP2B6* genotype is not consistently related to 4HCY formation *in vitro* or *in vivo*.[1] Nevertheless, we did observe an association between *CYP2B6* and the 4HCY/CY AUC ratio ($p<0.03$).

Associations have also been suggested for *CYP2C19* and the risk of adverse events, particularly those homozygous for the *CYP2C19*1* allele.[47,48] Specifically, Singh et al. (2007) estimated that, among patients with systemic lupus erythematosus receiving CY therapy, those homozygous and heterozygous for the **2* allele had a significantly lower risk of ovarian toxicity as compared to those that were *CYP2C19*1/*1*.[47] Similarly, Takada et al. (2004) estimated a 90% reduction in ovarian failure among lupus nephritis patients receiving CY that had at least one copy of the variant.[49] The *CYP2C19*2* allele produces an aberrant splice variant that leads to an alternative reading frame and premature stop codon, rending the enzyme non-functional.[52] For this reason, it has been hypothesized this variant might lead to reduced metabolic activation of CY and, hence, a lower risk of developing toxicity as well as decreased efficacy.[49] Our study findings, however, did not find a pharmacokinetic association between pediatric patients homozygous for the *CYP2C19*1* allele and the 4HCY/CY AUC ratio. We found this particularly surprising, as the aberrant splice variant results in a loss of function in CYP2C19 that would likely lead to inefficient conversion of CY to 4HCY and subsequent elimination.

On the other hand, data to suggest associations for *CYP3A4* and *CYP3A5* are scarce despite the enzyme products' prominent roles in 4HCY formation. In fact, the only positive

findings available were for the *CYP3A4*1B* polymorphism, which putatively results in higher CYP3A4 expression *in vitro*.[53] Interestingly, Gor et al. (2010) suggested that women with at least one *CYP3A4*1B* (rs2740574) variant had worse disease-free survival (DFS).[43] Our study found that the presence of at least a single *CYP3A4*1B* allele was not predictive of 4HCY pharmacokinetics, and thus would likely not be associated with clinical outcomes for these children. Similarly, the CYP3A5 enzyme has been investigated a number of times and has yet to present any clinically relevant association.[21,41-43,46,48-50] The *CYP3A5*3* (rs776746) variant was of interest as it results in decreased enzyme activity.[54] Yet, like with *CYP3A4*, no significant associations were identified. Like *CYP2B6* and *CYP2C19*, the difference in the 4HCY/CY AUC for the alleles of *CYP3A4* and *CYP3A5* were not statistically significant.

Lastly for 4HCY formation enzymes, to date only one study had been identified that assessed the effects of *MPO*, the product of which is important for oxidative activation, on CY outcomes. MPO is found in neutrophils and macrophages, both of which are known to invade tumors and kill cancer cells.[55] Ambrosone et al. presented data that patients with the homozygous GG polymorphism (rs 2333227) amongst those treated with a chemotherapeutic regimen including CY had an improved disease-free survival. This could be due to the fact that the G allele has been shown to result in heightened transcription.[55] However, the data collected in our investigation did not present evidence that *MPO* polymorphisms were associated with the 4HCY/CY AUC among pediatric cancer patients.

Another critical determinant of 4HCY is its elimination, which is influenced by ALDHs, GSTs and ABC transporters. High activity of these enzymes or transporters would be expected to lower the 4HCY/CY AUC ratio and potentially reduce the therapeutic efficacy of CY overall.

Other investigations have been conducted to assess the role of *GSTM1*, *GSTP1*, *GSTT1*, *ALDH1A1*, and *ALDH3A1* variants in the elimination of 4HCY.[20-24,46,48,56-60] The majority of literature regarding 4HCY elimination focused on the GST superfamily, which is responsible for phase II inactivation via conjugation with a thiol of sulfate group.[43] Associations between these loci and CY phenotypes have been very mixed.

GSTP1 currently has the most evidence in favor of pharmacogenetic associations. Research into *GSTP1* polymorphisms focused on a nonsynonomous SNP (rs1695) within codon 105 of the coding region, resulting in an amino acid change from isoleucine to valine. The consequence of this polymorphism includes a reduction in enzyme catalytic activity.[61] Many of the investigations to date have found positive associations between homozygosity of the variant allele (the enzyme possessing the valine residue) and measures of efficacy and toxicity after CY administration.[22-24,51,56,58-60,62-64] For example, Stanulla et al. suggested a 3-fold reduction in relapse with the homozygous Val105/Val105 genotype among pediatric acute lymphoblastic leukemia (ALL) patients, although the association was not statistically significant.[23] Other studies found an improvement in survival when comparing the homozygous variant with homozygous wildtype among female breast cancer patients.[58,59,62] Unfortunately, a number of studies have found this same genotype to confer a greater risk of toxicity – Zhong et al. found a greater risk of myelo- and GI-toxicity, while Allan et al. suggested an increased risk of chemotherapy-induced acute myeloid toxicity, although neither of these focused on children.[56,60] Despite all of these clinical associations presented in previous studies, there was no statistically significant difference in the outcomes of 4HCY formation clearance using *GSTP1* polymorphisms as a predictor. Despite lacking statistical significance, previous pharmacokinetic and pharmacodynamic research would lead us to believe that the difference in CYP metabolism

phenotypes is clinically relevant, and thus genotyping could eventually be used to dose CY more appropriately. Accordingly, we encourage the collection of more data to elucidate and identify a possible *GSTP1* variation – pharmacokinetic association with greater precision.

GSTM1 and *GSTT1* null genotypes have been associated with a reduction in risk of relapse[23,24] but also an increased likelihood of ≥Grade 3 toxicity[20] among pediatric cancer patients. Some studies, like Sharda et al., found synergistic effects between the GST polymorphisms.[22] Others failed to support any association between the *GSTM1* and *GSTT1* genotypes and phenotypic outcomes, although these studies are largely done with adults.[43,59,60] Unfortunately, the clinical outcomes supported by some previous research might not in fact be mediated by the 4HCY/CY AUC as we had originally suspected, as our data failed to show a significant association.

The polymorphic *GSTA1* enzyme product possesses the highest catalytic activity for glutathione conjugation of nitrogen mustard chemotherapy among the human GSTs.[65] The *GSTA1*B* allele (rs4715333) results in under-expression of the enzyme, yet previous studies have largely failed to demonstrate a significant association with pharmacokinetic outcomes.[46,50] However, Sweeney et al. did generate data to support that the *GSTA1*B/*B* genotype was associated with a reduction in a clinical outcome - the incidence of death during the five years following diagnosis among breast cancer patients.[66] Along with other pharmacokinetic studies, ours found no important association between the *GSTA1*B/*B* genotype and the 4HCY/CY AUC among pediatric cancer patients. Due to the lack of data generated to demonstrate an association, it might be that GSTA1 effects on health outcomes utilize a pathway distinct from

the possible effects on metabolism of prodrugs. We would suggest, therefore, that future studies draw direction from other potential biological mechanisms for GSTA1 enzymes.

There is scarce data regarding the influence of the ALDHs on CY; ALDH is responsible for the conversion of aldoCY to the inactive metabolite CEPM. Greater ALDH activity could lead to lower 4HCY AUC and worse clinical outcomes. To date, however, polymorphisms in the *ALDH1A1* locus have yet to be associated with CY pharmacokinetic phenotypes or clinical outcomes among adults, and no investigations were identified for *ALDH3A1* variation at all.[46] Once again, our study also showed no evidence that *ALDH* polymorphisms predict 4HCY pharmacokinetics.

Similarly, studies of the membrane transporters ABCC2 and ABCC4 have generated very little data in support of clinically relevant associations, despite being important for the transport of CY metabolites into bile. Previously, one polymorphism within *ABCC4* (G>T, rs9561778) was associated with an increased risk of CY-induced toxicity, including GI toxicity and leukopenia/neutropenia among breast cancer patients.[67] This could be due to a reduced function of ABCC4 and thus less efficient removal of metabolites. Prior to our study, no studies had attempted to uncover a possible pharmacokinetic relationship between ABCC activity and drug metabolism. However, our data suggests that SNPs for both *ABCC3* and *ABCC4,* but not *ABCC2,* polymorphisms that are predictive of 4HCY/CY AUC ratio in pediatric cancer patients. This result might be due to the fact that reduced biliary transport does not impact eventual conversion of 4HCY to 4-glutathionylcyclophosphamide (4-GSCY), which is mediated by the GSTs. Because it does not exist in equilibrium with its precursor, once the 4-GSCY is formed, it does not revert back and will remain until eventually being transported.

In summary, we are the first to evaluate a possible relationship between the pharmacogenomics – pharmacokinetic phenotype of 4HCY in pediatric cancer patients. Of the variations investigated, three associations were seen between the CY metabolic phenotypes and variation in the genes controlling the disposition of CY and its active metabolite – *ABCC3*, *ABCC4*, and *CYB2B6*. A number of limitations might have contributed to the few significant associations, including sample size, differing CY dosing, and heterogeneity in chemotherapy regimens. First, due to the large number of loci investigated relative to the number of children enrolled, the study lacked power to detect potential associations for any given variant unless the effect size was substantial. Many previous studies also suffered from this limitation. Furthermore, it is conceivable that combinations of variants (for example, children predicted to have low 4HCY formation for all CYP variants as compared to other subjects) might produce the expected pharmacogenomic – pharmacokinetic association we anticipated, but again due to a small number of subjects, we were not able to investigate this possibility. Larger studies necessarily entail multi-center participation and will still require decades for sufficient accrual of rare genetic variants. Finally, the children enrolled varied with regards to concomitant medications and dosing of CY, which was a necessity due to the rarity of pediatric cancer diagnoses. This could have confounded the pharmacogenetic analysis. The sample size was too small to adjust for this potential variable. However, previous in vitro results suggested that the concomitant chemotherapy did not impact 4HCY formation in human liver microsomes.[1]

It has long been known that the variants explored in our study do have clear effects on enzyme kinetics, and as such likely impact 4HCY formation and elimination. Thus, we hope that further pharmacogenetic studies be carried out with larger populations receiving more comparable CY doses and regimens that can have the power to detect any associations that

would improve clinical practices and outcomes. Such future studies should incorporate the strengths of our investigation, including consistent sample collection methods and reducing variability in supportive drug medications. In conclusion, we explored the pharmacogenomic – pharmacokinetic relationship for 4HCY among pediatric cancer patients. Although previous studies have demonstrated, both *in vitro* and among adult cancer patients, that the pharmacokinetics of 4HCY is likely affected by common variants in CY drug metabolizing enzymes, we found no significant association between variants of these genes and the 4HCY/CY AUC among pediatric cancer patients. Nevertheless, we believe that the lack of evidence is at least partially an unfortunate byproduct of our sample size, as changes to enzyme functionality and efficiency that would be expected from these variants should alter drug metabolism. Alternative approaches to personalize CY therapy should be pursued to improve its efficacy and safety.

Non-standard abbreviations: cytochrome P450 (CYP), cytochrome P450 2B6 (CYP2B6), cyclophosphamide (CY), body surface area (BSA), area under the curve (AUC), 4-hydroxycyclophosphamide (4HCY), single nucleotide polymorphisms, (SNP), aldehyde dehydrogenases (ALDH), glutathione S-transferases (GST), aldophosphamide (AldoCY), human liver microsomes (HLM), analysis of variance (ANOVA), carboxyethylphosphoramide mustard (CEPM)

ACKNOWLEDGEMENTS

The participation of the children and their families in these studies is greatly appreciated. The expertise of the staff in the Pediatric Clinical Research Center at Seattle Children's Hospital, and in the Pharmacokinetics Laboratory at the University of Washington (Meagan Bemer, Brian Phillips and Linda Risler) are greatly appreciated.

Table 1: Summary of Candidate Genes in Adults Receiving Conventional Dose CY-based Chemotherapy, or Children Receiving Any Dose CY-based Chemotherapy (blue background)[a]

Candidate Gene	4HCY Genotype[b]		Pharmacogenetic Association[c]	
	High	Low	Positive	Not found
Genes Regulating Enzymes Involved in 4HCY Formation				
CYP2B6	*1/*1	*1/*4 *1/*5 *1/*6	Efficacy[42,44,49] ADRs[44,46] PK[15,45,46]	Efficacy[43,51] ADRs[47,48] PK[41,50]
CYP2C9	*1/*1	*1/*2 *2/*2 *1/*3 *3/*3	Not available (NA)	Efficacy[42,43,49] PK[15,41,45,50]
CYP2C19	*1/*1 *1/*2	*2*2	Efficacy[49] ADRs[47] PK (if CY dose <1 g/m^2)[41,50]	Efficacy[42] PK[15,46]
CYP3A4	*1	*1B	Efficacy[43] ADRs[48]	Efficacy[21] ADRs[51] PK[45,46]
CYP3A5	*1/*1 *1/*3	*3/*3 *1/*6 *1/*7	NA	Efficacy[21,42,43,49] ADRs[48] PK[41,46,50]
MPO[d]	GG	AG AA	Efficacy[55]	NA
Genes Regulating Enzymes or Transporters Involved in 4HCY Elimination				
ALDH1A1	*2	*1	ADRs[72]	PK[46]
ALDH3A1	*2	*1	PK[41]	NA
GSTA1	*B*B	*A*A *A*B	Efficacy[66] ADRs[41]	ADRs[48,51] PK[46,50]
GSTM1	*0 (Null)	Pres-ent	Efficacy[20–24,57] ADRs[20]	Efficacy[43,59] ADRs[48,56,60] PK[46]
GSTPI	V105	I105[e]	Efficacy[22–24,58,59,62,64,73] ADRs[51,56,60]	Efficacy[21,24,43,59] ADRs[48] PK[46]
GSTT1	*0 (Null)	Pres-ent	Efficacy[20,22,23,57] ADRs[20]	Efficacy[21,24,43,59] ADRs[48,60] PK[46]
ABCC2[d]	NA	NA	NA	ADRs[74]
ABCC4[d]	NA	NA	ADRs[74]	NA

[a]Excluded studies from adults receiving myeloablative dose CY in adults [35,19,75–79]
[b]Rare heterozygotes of variants (e.g., *CYP2C9*2/*3*) would be low 4HCY genotypes
[c]Efficacy, ADRs=adverse drug reactions, PK=pharmacokinetics
[d]Characterization of the allelic variants of MPO, ABCC2 and ABCC4 upon CY pharmacokinetics has yet to be reported.

Table 2. Patient characteristics (N=72)[a]

Age (yrs)	5.5 (0.6 - 17.5)
Height (cm)	110.5 (66.7 - 170.0)
Weight (kg)	20.3 (7.0 - 116.7)
BSA (m^2)	0.78 (0.35 - 2.33)
Sex (% female)	34 (47%)
Race	
White	54 (75.0%)
Hispanic/Latino	6 (8.3%)
Asian	6 (8.3%)
African American	2 (2.8%)
Other[b]	4 (5.6%)
Diagnosis	
Acute lymphoblastic leukemia	40 (55.6%)
Ewing's Sarcoma	16 (22.2%)
Rhabdomycosarcoma	8 (11.1%)
Neuroblastoma	3 (4.2%)
Lymphoblastic leukemia	2 (2.8%)
Renal	1 (1.4%)
T-cell Non-hodgkin's lymphoma	1 (1.4%)
Soft tissue sarcoma	1 (1.4%)
Regimen[c]	
CY/thioguanine/cytarabine	22 (30.6%)
CY, cytarabine	21 (29.2%)
vincristine, doxorubicin, CY	20 (27.8%)
vincristine, dactinomycin, CY	6 (8.3%)
Topotecan/CY	2 (2.8%)
CY/etoposide	1 (1.4%)

[a] Data as "number (%)" or "median (range)"

[b] Other was defined by NIH criteria

[c] D = delayed intensification of COG AALL0932 – CY thioguanine cytarabine; Con = consolidation CY, cytarabine, 6-mercaptopurine; VDC = vincristine, doxorubicin, CY; VAC = vincristine, dactinomycin, CY; CE = CY, etoposide of COG ANBL0531

Table 3. Pharmacokinetic results

Parameter	Number Evaluable	Median (Minimum-Maximum)
CY clearance (L/m^2/hr)	57	2.52 (1.41-6.26)
4HCY/CY AUC ratio	49	0.0458 (0.011-0.126)
HCY AUC (μM×h) divided by CY dose (mg/m^2)	49	0.070 (0.024-0.129)
CEPM AUC (μM×h) divided by CY dose (mg/m^2)	47	0.074 (0.044-0.159)
DCCY AUC (μM×h) divided by CY dose (mg/m^2)	18	0.122 (0.058-0.331)
KetoCY AUC (μM×h) divided by CY dose (mg/m^2)	20	0.056 (0.023 – 0.076)

Figure 1. Effect of age, CY regimen, and CY dose upon 4HCY/CY AUC ratio.

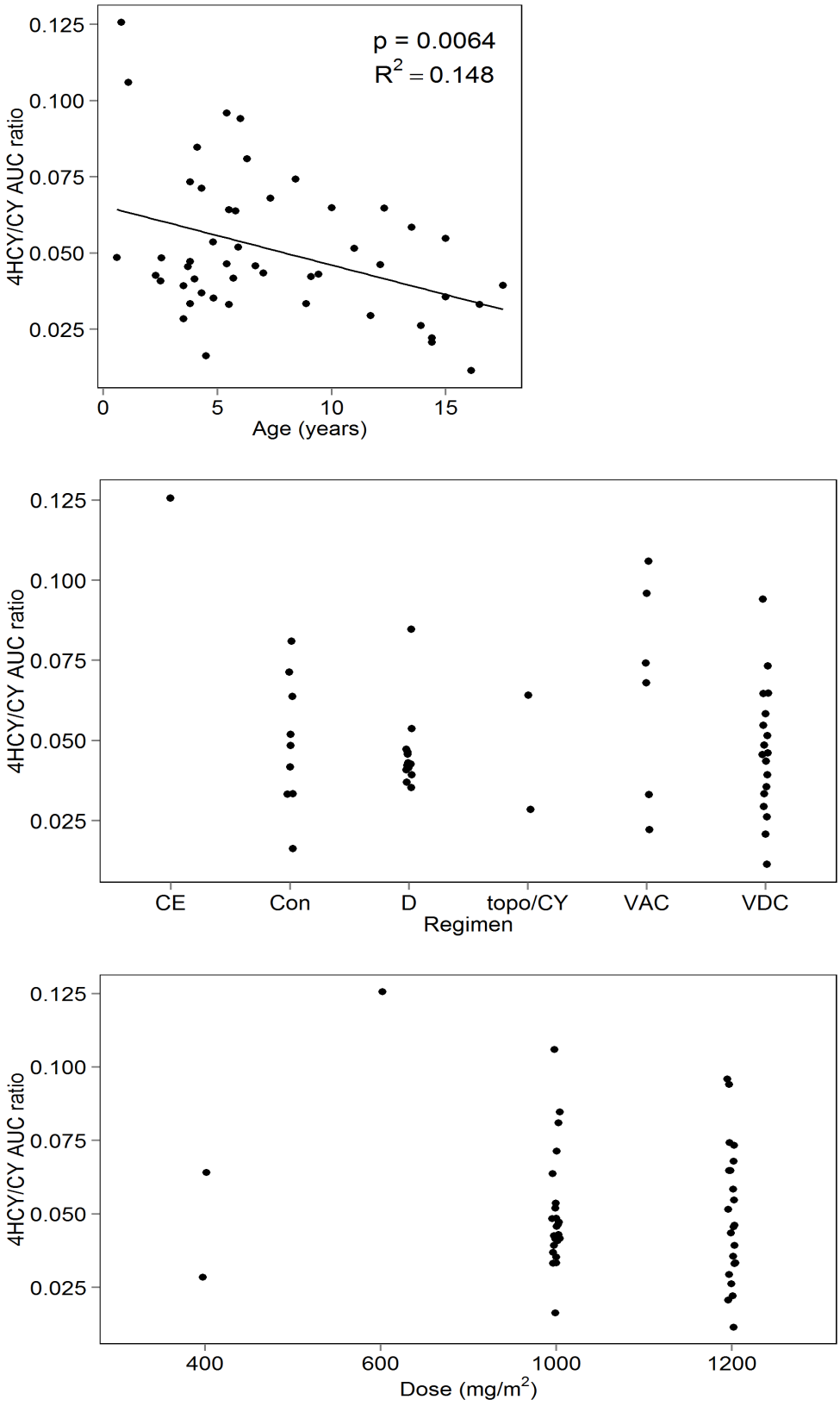


Figure 2. Comparison of 4HCY formation clearances (4HCY AUC/HCY AUC) between the initial ("First Cycle") and subsequent ("Following Cycle") doses[a]

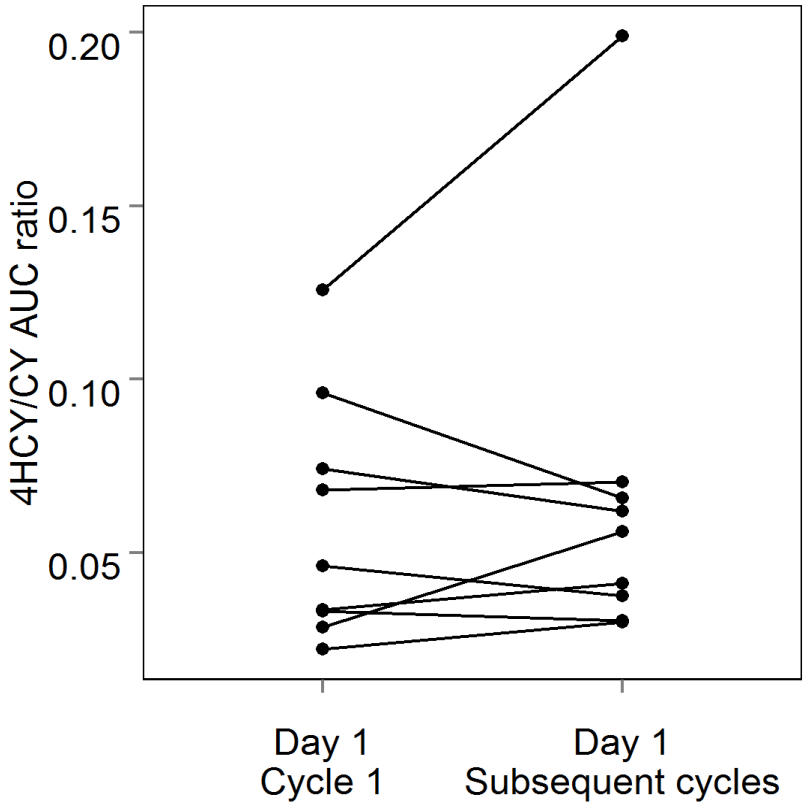
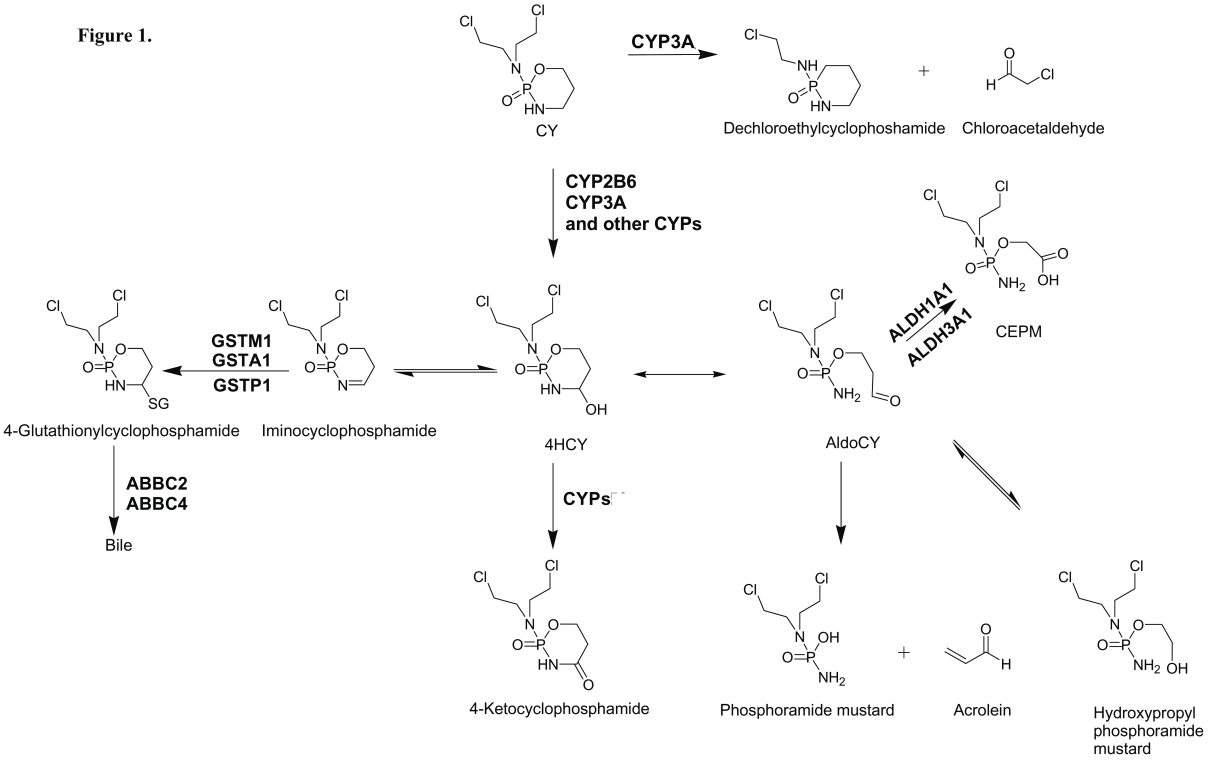


Figure 3: Schematic of CY metabolism[1]



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