Population Pharmacokinetics of Cyclophosphamide and Metabolites in Children with

Neuroblastoma: a Report from the Children's Oncology Group

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ABSTRACT

Cyclophosphamide-based regimens are front-line treatment for numerous pediatric malignancies, however current dosing methods result in considerable interpatient variability in tumor response and toxicity. In this pediatric population, our objectives were to 1. quantify and explain the pharmacokinetic variability of cyclophosphamide, and two of its metabolites, hydroxycyclophosphamide (HCY) and carboxyethylphosphoramide mustard (CEPM); 2. apply a population pharmacokinetic model to describe the disposition of cyclophosphamide and these metabolites. A total of 196 blood samples were obtained from 22 children with neuroblastoma receiving intravenous (IV) cyclophosphamide (400 mg/m^2/day) and topotecan. Blood samples were quantitated for concentrations of cyclophosphamide, HCY and CEPM using liquid chromatography-mass spectrometry and analyzed using nonlinear mixed effects modeling with NONMEM software system. After model building was complete, the area under the concentration-time curve (AUC) was computed using NONMEM. Cyclophosphamide elimination was described by noninducible and inducible routes with the latter producing HCY. Glomerular filtration rate (GFR) was a covariate for the fractional elimination of HCY and its conversion to CEPM. Considerable interpatient variability was observed in the AUC of cyclophosphamide, HCY and CEPM. These results represent a critical first step in developing pharmacokinetic-linked pharmacodynamic studies in children receiving cyclophosphamide to determine the clinical relevance of the pharmacokinetic variability in cyclophosphamide and its metabolites.

INTRODUCTION

Cyclophosphamide is the cornerstone of curative chemotherapy regimens in over 50% of newly diagnosed pediatric cancer patients. Dose intensification of cyclophosphamide is associated with improved outcome in many of these patients, but is also associated with increased adverse drug reactions (ADRs). The pressing need for improving the safety of cyclophosphamide is highlighted by the impact of a minor dosing modification to the VAC (vincristine/dactinomycin/cyclophosphamide) regimen leading to unexpected increase in ADRs in children less than 3 years old.[1] Thus, there is a need to delineate factors in cyclophosphamide metabolism that may impact both response and ADRs to cyclophosphamide-based combination chemotherapy regimens. In adults, there is substantial interpatient variability in the cyclophosphamide area under the plasma concentration-time curve (AUC), and an even greater variability in the AUC of its metabolites when cyclophosphamide is dosed based on body surface area (BSA) or weight.[2, 3] Children have more rapid cyclophosphamide clearance normalized to BSA (i.e., ml/min/m^2) relative to adults, although the mechanism is unidentified.[4-8]

Cyclophosphamide predominantly undergoes hepatic elimination, with 25% (range 5-30%) of an IV dose excreted unchanged in the urine.[3] A major fraction of cyclophosphamide dose (70%) is metabolized to hydroxycyclophosphamide (HCY) by multiple cytochrome P450 enzymes.[3] A minor fraction of cyclophosphamide dose (5%) is metabolized to dechloroethylcyclophosphamide (DCCY) by CYP3A4/5.[9] The metabolite HCY exists in equilibrium with its tautomer–aldophosphamide, and the concentrations of these two metabolites are assayed together.[10] HCY undergoes intracellular transport, subsequently releasing phosphoramide mustard which covalently cross-links DNA. Phosphoramide mustard does not readily cross cell membranes and thus makes the transport of HCY critical.[3] Hydroxycyclophosphamide is metabolized by aldehyde dehydrogenase 1A1 (ALDH1A1) to

nontoxic carboxyethylphosphoramide mustard (CEPM).[3] CEPM is the predominant urinary

metabolite of cyclophosphamide.[3]

A substantial challenge in seeking a better understanding of cyclophosphamide

pharmacokinetics and pharmacodynamics has been the difficulty in quantitating HCY plasma

concentrations.[10] HCY is extremely unstable in biologic fluids (in vitro half-life in plasma < 3

minutes) and only recently have more clinically applicable methods been developed.[11-14] These

methods are accurate, sensitive and specific and have since provided reliable tools to advance

our understanding of the pharmacokinetic/dynamics of cyclophosphamide and its metabolites in

predominantly adult patients receiving myeloablative dose cyclophosphamide prior to

hematopoietic cell transplantation.[2, 15-19] In this study, we sought to be the first to characterize

HCY pharmacokinetics and to develop a population pharmacokinetic model of

cyclophosphamide, HCY, and CEPM in children receiving multiple daily doses of

cyclophosphamide.

METHODS

Patient population. Twenty-two children (17 male and 5 female) were included in this study.

The pharmacokinetics were evaluated after the first cycle of fractionated non-myeloablative

dose cyclophosphamide with topotecan, administered under a limited institution pilot protocol,

ANBL02P1 which was conducted by the Children's Oncology Group (Duarte, CA). Institutional

Review Board approval was obtained at each individual participating institution, specifically

Children's Hospital and Regional Medical Center (Seattle, WA), Children's Memorial Hospital

Medical Center at Chicago (Chicago, IL), St. Jude Children's Research Hospital (Memphis, TN)

and University of California San Francisco (San Francisco, CA). Children from the Children's

Hospital at Westmead could participate in the clinical trial but were excluded from the

cyclophosphamide pharmacokinetic study due to the extended time period (i.e., >48 hours (h))

necessary to transport the plasma samples from Australia to the United States. Prior to study conduct, parental written informed consent was obtained for all children and written assent for those children over 7 years of age was obtained.

All children were diagnosed with neuroblastoma or ganglioneuroblastoma and had no prior systemic therapy. All children had adequate renal (i.e., serum creatinine $\leq$ 1.5 mg/dL, and creatinine clearance or radioisotope glomerular filtration rate (GFR) $\geq$ 60 ml/min/1.73 m²) and liver (i.e., total bilirubin $\leq$ 1.5 mg/dL and alanine aminotransferase < 300 units/L) function. Prior to chemotherapy administration, radioisotope GFR was determined using each individual institution's procedures.

Chemotherapy regimen. Cyclophosphamide was infused through a central venous access catheter over 30 minutes at a dose of 400 mg/m²/day in those children weighing greater than 12 kilograms or 13.3 mg/kg/day in those children weighing 12 kilograms or less. The body weight and BSA used to calculate doses were based on individual institutional guidelines. Cyclophosphamide was administered daily for 5 days. Cyclophosphamide doses were not adjusted based on pharmacokinetic data. Immediately after each cyclophosphamide infusion was completed, a 30-minute topotecan infusion began and was repeated daily after each dose of cyclophosphamide. Topotecan doses were pharmacokinetically targeted after doses 1 and 3. The success of topotecan targeting and clinical response will be reported in a separate manuscript.

Cyclophosphamide pharmacokinetic sampling and analysis. The blood volume for cyclophosphamide pharmacokinetic analysis was limited by the young age of the patient population and the blood volume needed for pharmacokinetically targeting topotecan doses. Cyclophosphamide pharmacokinetic blood samples were drawn from the lumen not used to infuse the cyclophosphamide dose. Pharmacokinetic blood samples were drawn immediately before, upon completion of the 30-minute infusion, and at 3, 6, and 24 h after the start of the first

and fourth cyclophosphamide infusions. The volume of blood drawn was 1 mL for the first four samples and 2 mL for the fifth sample (i.e., 24 h post infusion).

The sample processing procedures were derived from those developed in hematopoietic cell transplant recipients.[13, 14] Aliquots of each sample were placed into tubes containing either phenylhydrazine for analysis of HCY or ethylenediaminetetraacetic acid for analysis of cyclophosphamide and CEPM. The tubes were inverted 3 to 4 times, stored at 4°C for a maximum of 1 h and centrifuged. The supernatant was then decanted and stored at -70°C within 1 h of sample collection.

All samples were shipped on dry ice to our laboratory within 3 months. Cyclophosphamide, HCY, and CEPM concentrations in plasma were quantitated separately using the methods of Kalhorn et al[13, 14] with minor modifications as described in Table 1. In the case of CEPM, negative ion detection at m/z 293 was used. This ion, although lesser in abundance, has a lower incidence of background interference, allowing for more sensitive detection of CEPM. The HCY-phenylhydrazine derivative was detected in the negative ion mode as the chloride adduct at m/z 401.[14]

Population Pharmacokinetic Modeling. Given the small number of blood samples available in each subject, nonlinear mixed effects modeling was used to describe this pharmacokinetic dataset. The nonlinear mixed effects approach explicitly models the time course of the data and the hierarchical statistical variability which is present among subjects and within subjects. Pharmacokinetic models of cyclophosphamide metabolism were fit to all pharmacokinetic data (i.e., cyclophosphamide, HCY and CEPM) from all patients simultaneously by use of the first-order method in the nonlinear mixed-effects modeling software NONMEM (version V, double precision).[20] Between-subject variability (BSV) of parameters was modeled by use of a lognormal model. Residual unknown variability (RUV) was estimated by use of additive models for cyclophosphamide and CEPM and a combination of proportional and additive errors for

HCY. Individual parameters were calculated by use of the POSTHOC option (empirical Bayes estimates) of NONMEM. A nominal significance level of 0.05 (linked to a difference in objective function value of at least 3.86 points for one additional parameter) was assumed to aid in covariate model selection.

The pharmacokinetic model framework we used is depicted in Figure 1 and its components have been previously described.[18, 21] The definition of the different parameters of this model is explained in Table 3. This integrated model simultaneously describes the pharmacokinetics of cyclophosphamide, HCY and CEPM and includes autoinduction of cyclophosphamide to HCY. The concentrations are expressed in µmol/L of cyclophosphamide (with molecular weight 261.1 g/mol), HCY (277.1 g/mol) and CEPM (293 g/mol). Because the model, expressed as a system of four differential equations, is by definition a "mass balance" equation, the modeling is performed in mass units (mg/m^2) and the amounts are converted to µmol/L for comparison with data. Similarly, the EC_{50} is reported in µmol/L of cyclophosphamide, but is converted within the model to mass units.

Elimination of cyclophosphamide is described via a noninducible route and an inducible route, with the latter leading to HCY formation. Autoinduction of HCY formation was modeled by use of a hypothetic enzyme compartment as described previously.[19, 21-23] The inducible clearance was proportional to the amount of enzyme in this compartment. The "initial" inducible clearance is defined as the inducible clearance at time 0. The enzyme followed a zero-order formation that was enhanced by cyclophosphamide with a maximum fold of induction (Emax)–type relationship and first-order degradation. The initial amount of enzyme was set to a dimensionless value of 1 at time 0.

The initial structural model (Figure 1), with three compartments for cyclophosphamide (CY) and metabolites HCY and CEPM as well as a fourth enzyme compartment to model the inducible clearance, was as described in Qiu and Yao et al[21] and in Salinger et al.[19]

$$\frac{dA_1(t)}{dt} = -\frac{CL_{NON}}{V_{CY}} A_1(t) - \frac{CL_{IND}}{V_{CY}} A_1(t) A_2(t)$$

$$\frac{dA_2(t)}{dt} = K_{ENZ}\left(1 + \frac{\dfrac{E_{MAX}}{V_{CY}} A_1(t)}{EC_{50} + \dfrac{A_1(t)}{V_{CY}}}\right) - K_{ENZ} \cdot A_2(t)$$

$$\frac{dA_3(t)}{dt} = \frac{CL_{IND}}{V_{CY}} A_1(t) A_2(t) - K_{HCY} \cdot A_3(t) - K_{34} \cdot A_3(t)$$

$$\frac{dA_4(t)}{dt} = K_{34} \cdot A_3(t) - K_{CEPM} \cdot A_4(t)$$

where the concentrations of cyclophosphamide, HCY and CEPM are $C_{CY}(t) = A_1(t)/V_{CY}$,

$C_{HCY}(t) = A_3(t)/V_{HCY}$ and $C_{CEPM}(t) = A_4(t)/V_{CEPM}$ respectively and where the model

parameters are described in Figure 1 and Table 3. The model departed from that of Qiu and

Yao et al[21] in the following ways. First, doses were based upon BSA rather than body weight

(see "Chemotherapy Regimen" section above). In those two children whose cyclophosphamide

dose was based upon weight, the actual dose (mg) administered was divided by that patient's

BSA. Weight and BSA are correlated in this pediatric population (r^2=0.97), and thus we would

expect similar results using weight-normalized dosing. Second, we made no assumption

regarding the influence of the covariate age upon the base model. Third, the volume of

distribution of HCY (V_{HCY}) and CEPM (V_{CEPM}) were both assumed based on our previous

experience with cyclophosphamide modeling in hematopoietic cell transplant recipients.[21, 23] In

our previous cyclophosphamide modeling, V_{HCY} and V_{CEPM} could not be estimated[23] and a value

of 1 L was assumed for adults.[21, 23] The average BSA in the original hematopoietic cell

transplant population cyclophosphamide pharmacokinetic model was 1.76 m^2,[21] and thus V_{HCY}

and V_{CEPM} were assumed equal to 1 L/1.76 m^2 (0.57 L/m^2).

As a validation of the model, we performed a visual predictive check wherein data were simulated for 4400 subjects (200 for each of the 22 measured covariate sets) using the final population pharmacokinetic model and the median and 90% prediction intervals plotted with the original data.

Statistical Analysis.

Generalized estimating equations were used to evaluate the association of AUC with independent variables that included age, sex, BSA, GFR and day of CY administration. Each analysis was done separately for the AUCs of CY, HCY, and CEPM. SAS Version 9.1 (Cary, NC) was used for all statistical analyses.

RESULTS

Patient Population. Of the 22 patients studied, 17 were male and 5 were female. At the time of diagnosis, the median age was 3.16 years (range: 1.30 – 9.37). At the time of cyclophosphamide infusion, the BSA was 0.62 m^2 (range: 0.45 – 1.47), actual body weight was 14.2 kg (range: 8.7 – 52.8), and GFR was 117 ml/min/1.73m^2 (range: 78 – 238).

Quantitation of cyclophosphamide and metabolites. Twenty two and twenty patients, respectively, had pharmacokinetic sampling after the first and fourth cyclophosphamide dose. Cyclophosphamide and CEPM concentrations were detectable in 167 (85%) and 128 (65%), respectively, of 196 samples. HCY required separate bedside processing, and was detectable in 113 of 196 (58%) samples. The majority of samples which were below the limit of detection were the 24 h samples, despite obtaining 2 ml of blood in an effort to improve our ability to quantitate cyclophosphamide, HCY and CEPM. Of the 29 samples drawn at 24 h, no analytes could be quantitated in 23 samples, 5 had one analyte that was detectable and 1 was drawn

after the subsequent dose was given (the latter of which was deleted from population pharmacokinetic modeling).

Population pharmacokinetic modeling. In Model 1 (see Table 2), we started by assuming a value for $V_{CEPM} = V_{HCY} = 0.57$ L/m^2 and allowed the remaining pharmacokinetic parameters (i.e., CL_{IND}, CL_{NON}, V_{CY}, K_{HCY}, K_{ENZ}, K_{34} and K_{CEPM}) to be estimated relative to this assumption. Estimates of most of the parameters, other than K_{34} and K_{HCY}, were unaffected by the choice of V_{CEPM} and V_{HCY} value. The reported values of K_{34} and K_{HCY} should be considered as "relative" to these volume assumptions.

Various alternative models were evaluated, and the results are summarized in Table 2. Model 2 evaluated the actual dose administered, not normalized for BSA – essentially testing removal of BSA as a covariate. In this model the fixed volumes ($V_{CEPM} = V_{HCY} = 0.57$ L/m^2) were multiplied by the mean subject BSA of 0.681 m^2 to convert to units of liters (L). While this conversion may not be optimal, the clear underperformance of Model 2, as measured by increased objective function value (OFV), did not warrant further investigation.

In Model 3, we replaced the fixed-effects model for parameter V_{CY} with a mixed-effects model with lognormal random effect. This resulted in a highly significant improvement in OFV (i.e., decrease of 73.2 points) versus Model 1.

Using Model 3, we performed a covariate analysis by visual assessment of plots and by regressions of the seven individually varying parameter values (CL_{IND}, CL_{NON}, V_{CY}, K_{HCY}, K_{ENZ}, K_{34} and K_{CEPM}) and the corresponding random effects with the available covariates (i.e., age, gender, weight, BSA, and pre treatment GFR). The three most-promising parameter-covariate relationships were K_{HCY} vs. GFR ($r^2=0.31$, $p=0.004$), K_{34} vs. GFR ($r^2=0.25$, $p=0.01$), and CL_{NON} vs. gender ($r^2=0.13$, $p=0.05$). We tested each of these by postulating a linear relationship between parameters and covariates in Models 4, 5 and 6, respectively. Models 4 and 5 both showed significant ($p < 0.05$) improvement in OFV versus Model 3, while Model 6 did not.

Model 7 included linear functions of GFR for both K_{HCY} and K_{34}. This resulted again in a significant improvement over both Models 4 and 5 (at $p < 0.05$), and provided the final model. The final parameter estimates are summarized in Table 3. Figure 2 shows plots of observed vs. population predicted (PRED) and individually predicted (IPRED) concentrations of cyclophosphamide, HCY and CEPM for the final model.

Figure 3 provides model validation in the form of a visual predictive check. The median and 90% prediction intervals were plotted with the original data for cyclophosphamide and its metabolites HCY and CEPM after the first and fourth dose. The visual predictive check shows generally good agreement between model and data. That fact that fewer than 10% of data points lie outside the 90% prediction interval might indicate a slight overestimation of the variability. While this may be a consequence of the relatively small dataset, it is more likely the result of employing only a diagonal model covariance matrix. However, the data did not support estimation of parameter covariances.

AUC of cyclophosphamide, HCY and CEPM in children. The final model (Model 7) was applied to compute the AUC of cyclophosphamide, HCY and CEPM after each of the five cyclophosphamide doses. The resulting AUCs for cyclophosphamide, HCY and CEPM are shown in Figure 4 and provided in Table 4 for the whole patient population. Figure 5 contains examples of the pharmacokinetic model fit for two patients to the cyclophosphamide, HCY and CEPM concentration-time data.

Considerable interpatient variability was observed in cyclophosphamide AUC (i.e., 2.9 fold variability after dose 1 and 3.0 fold variability after dose 4). For the AUC of cyclophosphamide, the median was 431 µM*h (range: 258-746) on day 1 and 313 µM*h (range: 171-510) on day 4. The cyclophosphamide AUC was associated with age (p=0.03), BSA (p=0.028), and day of administration (p=0.0001). The cyclophosphamide clearance, calculated based on the AUCs in Table 4, was 3.58 L/h/m^2 (range: 1.73-5.53) after dose 1 and 4.86 L/h/m^2

(range: 1.93-7.37) after dose 4 (Table 5). Model-predicted initial inducible clearance (i.e., the clearance value extrapolated to before the first dose, without circulating cyclophosphamide) was 1.77 L/h/m^2.

In comparison to cyclophosphamide AUC, there was greater variability in the AUC of HCY and CEPM (i.e., 3.8-6.0 fold variability). For the AUC of HCY, the median was 26.8 µM*h (range: 11.4-63.8) on day 1 and 37.0 µM*h (range: 17.3-66.4) on day 4. The HCY AUC was associated with GFR (p=0.031), and day of administration (p<0.0001). For the CEPM AUC, the median was 16.5 µM*h (range: 9.3-38) on day 1 and 23.3 µM*h (12.7-76.6) on day 4. The CEPM AUC was associated with day of administration (p=0.0032). There was negligible correlation (i.e., all R^2<0.039) between AUC$_{CY}$ and AUC$_{HCY}$ after dose 1 or after dose 4 and between AUC$_{CY}$ and AUC$_{CEPM}$ after dose 1 or after dose 4.

DISCUSSION

This manuscript is the first to report considerable pharmacokinetic variability of the plasma concentrations of HCY in children receiving daily nonmyeloablative cyclophosphamide. Hydroxycyclophosphamide is transported intracellularly to release the active metabolite phosphoramide mustard, and thus may be a key determinant in the efficacy and toxicity of cyclophosphamide-containing regimens. Quantitation of the other cyclophosphamide metabolites was not feasible because of the limited blood volume per sample. This manuscript is also the first describing a population pharmacokinetic model of cyclophosphamide, HCY, and CEPM in children. The key findings of this pediatric study include the presence of considerable interpatient variability of cyclophosphamide AUC and its metabolites and the development of a population pharmacokinetic model with covariates to predict these AUCs. The population pharmacokinetic model identified baseline GFR as a covariate of HCY elimination rate and CEPM formation rate. By reducing unexplained variability, covariate identification can reduce

the need for pharmacokinetic sampling, thereby facilitating the development of a limited sampling schedule feasible for adequately sized pharmacokinetic and pharmacodynamic studies. This work represents an essential step in advancing our understanding of risk factors for poor response or severe ADRs to cyclophosphamide in pediatric cancer patients.

Cyclophosphamide-based combination chemotherapy regimens are used to treat the majority of children with cancer. Traditional dosing methods based on single covariates, such as BSA, do not necessarily address the presence of variability sufficiently well.[25, 26] The natural first step in obtaining an improved understanding of pharmacodynamic variability is to evaluate the interpatient variability in the systemic exposure of the parent drug and relevant metabolites. This is the main contribution of these results; however there are additional potential benefits. Specifically, pharmacokinetic-linked pharmacodynamic studies are hindered by the need for intense blood sampling schedules, and in the case of HCY, bedside processing. A population pharmacokinetic model provides a rigorously quantitative approach to incorporate population information (i.e., typical values and variability of drug disposition, together with explanatory covariates) in the determination of an individual's systemic exposure and can reduce the need for individual-specific information. Population pharmacokinetics can potentially be used to simultaneously describe the concomitant role of demographic covariates (e.g., age), and clinical covariates (e.g., GFR). Improved understanding of the determinants of variability can then lead to improved trial design. While the awareness of these issues has increased for drugs in the development phase, there is a still strong need to improve dosing for drugs that are already in use, such as cyclophosphamide. Understanding the sources of variability in young patients is of the utmost importance to optimize exposure to anti-tumor chemotherapy to ultimately achieve the maximum disease response with non-lethal toxicity. In addition, a population pharmacokinetic model facilitates development of an improved limited sampling schedule, which

could decrease the needed blood volume and resource intensity of pharmacokinetic-linked pharmacodynamic trials.[27]

Cyclophosphamide clearance agrees with the previously published values in pediatric populations (Table 5), suggesting that topotecan does not influence cyclophosphamide elimination.[4, 6-8] Similarly, the autoinduction of cyclophosphamide clearance (resulting in decreased AUC for subsequent doses) agreed with previous reports.[17, 28-30] In addition, the current convention of dosing children less than 12 kg on a mg/kg basis (i.e., patients 2 and 19) appears to yield a comparable AUC to those larger children (i.e., >12 kg) who receive cyclophosphamide 400 mg/m^2 (Table 4). The latter point is critically important, as children younger than 3 years of age experienced increased ADRs after what was anticipated to be a minor dosing modification to the VAC (vincristine/dactinomycin/cyclophosphamide) regimen.[1] These pharmacokinetic data, along with the accompanying population pharmacokinetic model, represent initial steps towards the goal of optimizing cyclophosphamide dosing in such young children.

Using analytical methods developed for pharmacodynamic studies in myeloablative hematopoietic cell transplant patients (Table 1), we are the first to describe the pharmacokinetics of HCY in children. The interpatient variability in the AUC of HCY was substantial, that is 5.6 fold after dose 1 and 3.8 fold after dose 4 (Table 4). A similar trend was found in the AUC of CEPM (Table 4). In contrast to the reports of Yule et al that some children had no detectable CEPM concentration,[4, 5, 7, 8] we were able to estimate the AUC of CEPM in all patients (Table 5) due to the increased sensitivity of our analytical method (Table 1) and the use of a population pharmacokinetic model (Figure 1). Notably, there was a weak correlation between the AUC of CY and the AUC of its metabolites, in agreement with previous reports.[12, 16, 17, 31, 32] This suggests that the cyclophosphamide clearance (as AUC = dose/clearance) cannot be used to predict metabolite exposure as has been suggested.[8, 33, 34]

This is the first population pharmacokinetic model of cyclophosphamide and metabolite disposition in young children, specifically less than 10 years of age (i.e., 1.30 – 9.37 years). The majority of the population pharmacokinetic models were presumably (i.e., age not reported)[35] or were exclusively from[36] populations older than 10 years of age. However, our initial population pharmacokinetic model of cyclophosphamide in hematopoietic cell transplant patients receiving myeloablative dose cyclophosphamide (60 mg/kg/day × 2 days) with total body irradiation (CY/TBI) did include eight children whose age were less than 10 years old.[21] In the CY/TBI population, age was the only covariate which improved model fitting and was inversely correlated with the noninducible clearance (CL_{NON}) of cyclophosphamide.[21] In agreement with this finding, the CL_{NON} in the neuroblastoma patients was higher (i.e., 1.13 L/hr calculated from 1.83 L/h/m^2 × 0.62 m^2 median patient BSA) than that observed in the CY/TBI conditioned patients (i.e., 0.665 L/hr calculated from 0.00899 L/hr/kg[21] × the mean patient body weight of 74 kg in that dataset). However, age was not a covariate for CL_{NON} (Tables 2 and 3) within the narrow age range (i.e., < 10 years) of this neuroblastoma population. Regarding the initial inducible clearance of cyclophosphamide (CL_{IND}), the CL_{IND} was 1.11 L/h (1.79L/h/m^2 × 0.62 m^2 median patient BSA). This is lower than previously reported values from adult populations, which range from 2.11 L/h[21] (i.e., 0.0286 L/h/kg × the mean patient body weight (74 kg) in that dataset) and 2.91 L/h.[15, 35] This suggests that the relative contribution of the CL_{IND} is lower in children, although further data are needed to confirm this finding. The remaining parameters compare favorably with population pharmacokinetic models of cyclophosphamide and its metabolites in adult populations. For example, the pediatric pharmacokinetic analysis provides a value of 46.5/h for K_{HCY}, which is the HCY elimination rate. This large value can be perceived as suggesting a very rapid elimination, however, it is less than previously published values for adults from our group (i.e., 147/h)[19, 21] and others (i.e., 118 to 169/h).[35, 37] The assumed values of V_{HCY} and V_{CEPM} are in accordance with previous reports, as we have remarked in the results section.

GFR was a covariate for the fractional elimination of HCY and its conversion to CEPM (Tables 2 and 3). Further studies are needed to confirm these findings, as a physiologic rationale for GFR being a covariate with these two parameters is currently elusive. CEPM is the predominant urinary metabolite after cyclophosphamide administration and its AUC was dramatically higher in an anephric child.[38] The conversion of HCY to CEPM is mediated by hepatic aldehyde dehydrogenase. This model predicted the individual and population concentrations well (Figure 2). It also allows for calculation of modeled AUCs of cyclophosphamide, HCY and CEPM after each of the five cyclophosphamide doses (Figures 3 and 4) while obtaining pharmacokinetic blood samples after dose 1 and 4 only. This population pharmacokinetic model needs to be validated in a separate pediatric population, especially due to our limited ability to quantitate all three analytes in many of the 24h samples. We plan to validate this model in a separate cohort of children receiving non-myeloablative dose cyclophosphamide and subsequently create an optimal limited sampling schedule to conduct pharmacokinetic and pharmacodynamic studies in children. Identifying pharmacodynamic relationships in future studies could lead to personalized cyclophosphamide dosing to achieve a target AUC to improve efficacy or decrease toxicity. We are currently employing this approach after myeloablative cyclophosphamide dosing in hematopoietic cell transplant recipients.

Pharmacodynamic studies are needed to determine the clinical relevance of this interpatient variability in the pharmacokinetics. Quantitation of cyclophosphamide and CEPM concentrations allowed for comparison to historical cyclophosphamide pharmacokinetic data within pediatric patients. It is also important to quantitate CEPM concentrations because elevated CEPM AUC is associated with a higher risk of liver toxicity in hematopoietic cell transplant recipients receiving myeloablative dose CY/TBI.[2] Quantitation of HCY was critical, as its pharmacokinetics has been reported in only one anephric child to date.[38] The majority of pharmacodynamic studies with cyclophosphamide and its metabolites have been conducted in patients receiving myeloablative cyclophosphamide for conditioning prior to hematopoietic cell

transplantation.[2, 15, 31] In this population, the pharmacodynamics of cyclophosphamide and its

metabolites differ based on the conditioning regimen.[31] In those receiving

busulfan/cyclophosphamide, the AUC of cyclophosphamide, HCY and CEPM are not related to

toxicity or response.[31] However, in patients receiving CY/TBI, an elevated AUC of CEPM is

associated with a higher risk of liver toxicity.[2] This has led to ongoing studies of targeting

cyclophosphamide doses based on the AUC of CEPM in hopes of lowering toxicity while

maintaining the AUC of HCY in hopes of maintaining engraftment rates.[18] These results

suggest that the concentration – effect relationships may differ between combination

chemotherapy regimens and thus, future pharmacokinetic and pharmacodynamic studies

should be conducted in homogeneous disease populations treated with the same combination

chemotherapy regimen. The development to this population pharmacokinetic model of

cyclophosphamide and its metabolites is an integral step in improving the efficacy and toxicity of

cyclophosphamide -based regimens in pediatric cancer patients. Despite its widespread use for

over fifty years, there are still uncertainties regarding the optimal dose of cyclophosphamide as

shown by the increased rate of ADRs after a minor dosing modification to the VAC from mg/kg

to mg/m^2 dosing of vincristine/dactinomycin/cyclophosphamide in young children.[1] This

pharmacokinetic data, along with the accompanying population pharmacokinetic model, are first

steps in determining if there are select populations of children which may need different

cyclophosphamide doses. In addition, the creation of a limited sampling schedule – which is

essential for pharmacokinetic-pharmacodynamic children in infants and toddlers – may be

possible after validation of this population pharmacokinetic model. A limited sampling schedule

will greatly facilitate recruitment of enough children to conduct pharmacokinetic and

pharmacodynamic studies, which are desperately needed in hopes of improving the efficacy of

cyclophosphamide.

In conclusion, we have shown substantial interpatient variability in the pharmacokinetics

of cyclophosphamide and its metabolites in children. Topotecan administration did not appear

to affect the clearance of cyclophosphamide. The development of a population pharmacokinetic model with relevant covariates will provide a framework to evaluate the pharmacodynamics of cyclophosphamide, with the long-range goal of improved prediction of an individual child's response and/or ADRs of cyclophosphamide-based regimens in this population.

Table 1. Quantitation of cyclophosphamide and metabolites by liquid chromatography-mass spectrometry

Analyte	Matrix	Sample preparation	Charge/Ion	Limit of detection	
				FHCRC	Yule et al[4-8]
Cyclophosphamide	150 µL plasma	Protein precipitation 1.ml acetonitrile	Positive/261	0.6 µM[13]	1 µM
HCY	500 µL whole blood + 1 mL phenylhydrazine	Solid phase extraction	Negative/401	0.25 µM[14]	Not assessed
CEPM	100 µL plasma	Protein precipitation 150 µL acetonitrile	Negative/293	0.25 µM[14]	3 µM

Table 2. Alternative models evaluated for cyclophosphamide, HCY and CEPM

Model	Model description	Result	OFV
1	Initial model	Proper convergence and covariance estimation only with excellent starting values	1152.2
2	Dose not normalized by BSA	Inferior model	1394.0
3	Added random component to V_{CY} in Model 1	Significantly decreased OFV, decreased variability (both BSV and RUV) and improved precisions	1079.0
4	Model 3 but K_{HCY} a linear model in GFR	Decreased OFV and variability (both BSV and RUV) over Model 3	1058.6
5	Model 3 but K_{34} a linear model in GFR	Decreased OFV and variability (both BSV and RUV) over Model 3	1063.4
6	Model 3 but CL_{NON} a linear model in gender	No improvement over Model 3. Gender provides no predictive value	1079.0
7	Both K_{HCY} and K_{34} linear models in GFR	Significantly improved OFV (over Models 3, 4 and 5). Final model	1041.6

OFV, minimum value of objective function. BSV, Between-subject variability. RUV, Residual unknown variability

Table 3. Estimates of population pharmacokinetic parameters of model for cyclophosphamide (CY), HCY and CEPM

Parameter	Designation (units)	Estimate (SE%)	BSV (%)	CY	HCY	CEPM
Volume of distribution of CY	V_{CY} (L/m^2)	13.1 (5.1)	17.8			
Noninducible clearance of CY	CL_{NON} (L·h^{-1}·m^{-2})	1.83 (30.6)	61.7			
Initial inducible clearance of CY	CL_{IND} (L·h^{-1}·m^{-2})	1.79 (28.2)	65.7			
Enzyme degradation rate	K_{ENZ} (h^{-1})	6.57E-3(55.3)	126			
Volume of distribution of HCY	V_{HCY} (L/m^2)	0.57, fixed				
HCY elimination rate	$K_{HCY} = \alpha + \beta \cdot (GFR-GFR^*)/GFR^*$ (h^{-1})		35.9			
- typical value at median GFR	α (h^{-1})	47.0 (20.9)				
- change in K_{HCY} per unit GFR from median	β (h^{-1})	-26.6 (36.8)				
K34, CEPM formation rate from HCY	$K_{34} = a + b \cdot (GFR-GFR^*)/GFR^*$ (h^{-1})		20.3			
- typical value at median GFR	a (h^{-1})	0.331 (9.2)				
- change in K_{34} per unit GFR from median	b (h^{-1})	-0.234 (14.1)				
Volume of distribution of CEPM	V_{CEPM} (L/m^2)	0.57, fixed				
CEPM elimination rate	K_{CEPM} (h^{-1})	0.447 (16.0)	38.1			
CY concentration of half-maximal induction	EC_{50} (µmol/L)	0.6, fixed				
Maximum induction	E_{MAX}	5, fixed				
Residual error						
Proportional error (%)					24.7	
Additive error (µmol/L)				6.2	0.295	0.389

GFR is glomerular filtration rate (ml/min/1.73 m^2). GFR* is the median GFR (i.e., 117 ml/min/1.73 m^2).

Table 4. The AUC of cyclophosphamide (CY) and its metabolites on day 1 and 4 of cyclophosphamide administration

ID	Age (Yr)	Sex	Weight (kg)	BSA (m^2)	GFR (ml/min/1.73 m^2)	Dose mg/kg	Dose mg/m^2	AUC of CY (μM*h) Day 1	Day 4	AUC of HCY (μM*h) Day 1	Day 4	AUC of CEPM (μM*h) Day 1	Day 4
1	1.93	M	15.6	0.67	125		400	357	254	18.4	27.4	12.4	18.5
2	1.30	M	8.7	0.45	238	13.3	257	569	510	36.5	50	9.3	13.4
3	3.22	M	14.7	0.63	145		400	461	291	24.9	37.6	11.3	17.1
4	5.51	F	13.3	0.61	148		400	487	286	43.8	56.1	21.8	28
5	3.53	F	13.6	0.63	109		400	582	393	22.4	36.8	10.5	17.3
6	1.83	F	12.8	0.54	78		400	591	348	29.5	34.6	23.9	28.3
7	3.10	M	15.1	0.63	116		400	643	380	25.8	38.5	18.1	27.9
8	3.43	M	12.4	0.58	112		400	746	404	40.7	51.7	20	25.4
9	3.48	M	15.3	0.68	190		400	468	344	36.8	61.6	16.8	28.3
10	9.02	M	52.8	1.47	141		400	277	209	27	33.9	14.5	18.2
11	1.89	M	14.4	0.57	78		400	317	246	11.4	17.3	9.8	14.9
12	8.36	F	24.3	0.91	99		400	611	316	27.3	34.3	13.9	17.6
13	2.26	M	12.7	0.57	101		400	282	234	40.2	42.3	38.8	40.9
14	2.49	M	12.5	0.57	158		400	541	441	25.5	37.6	16.1	24
15	9.37	M	32.9	1.14	100		400	399	278	17.9	23.8	13	17.4
16	2.46	M	18.1	0.73	161		400	400	224	26.6	32.8	10.3	12.7
17	3.24	M	18.6	0.74	173		400	280	209	47.7	56	19.2	22.6
18	2.41	M	14	0.6	115		400	363	309	19.4	24.2	23.6	29.9
19	1.44	F	9.7	0.48	110	13.3	269	258	171	20.3	23.4	13.1	15.1
20	3.77	M	13.2	0.58	98		400	392	364	35.5	37.2	29.3	30.9
21	2.55	M	13.3	0.6	134		400	354	326	63.8	66.4	23.6	24.6
22	4.18	M	17.5	0.62	118		400	493	379	17.3	35.2	36.4	76.6

Table 5. Comparison to previously reported pharmacokinetic results

Author	Subjects	CY Dose (mg/m^2)	Timing	CY Clearance (L/h*m^2)	Subjects with detectable CEPM AUC	CEPM AUC (µM*h)
Tasso 1992[4]	9	125-1500	Variable	1.48 (0.6–5.2)	7	0.17[c] (ND–74.7)
Yule 1995[5]	36	368-2490	Variable	NA	28	105[c] (ND–1210)
Yule 1996[6]	38	370-2490	Dose 1	2.9 (1.2–106.1)	NA	NA
Yule 2001[7]	13	600-2000	Dose 1	3.1 (1.4–6.2)	11	99 (ND–274)
Yule 2004[8]	36	300-1000	Dose 1	3.6 (2.1–5.4)	8	ND (ND–206)
McCune 2008	22	400	Dose 1	3.58 (1.73–5.53)	22	16.5 (9.3-38)
			Dose 4	4.86 (1.93–7.37)	20	23.3 (12.7–76.6)

Figure 1. Metabolic schema for cyclophosphamide (CY), HCY and CEPM
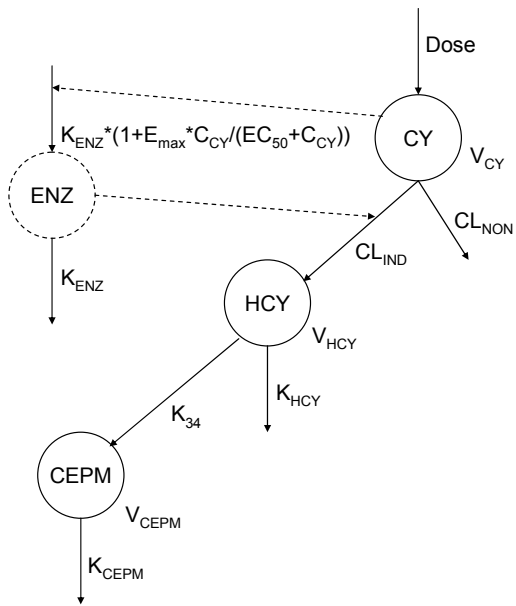
See Table 3 for explanation of the parameters (and final modeled values). Note, the inducible

enzyme formation rate depends on the modeled (time varying) concentration of

cyclophosphamide C_{CY}.

Figure 2. Observed vs. Population Predicted (PRED) and Individually Predicted (IPRED) concentration (µM) of cyclophosphamide, HCY and CEPM.*
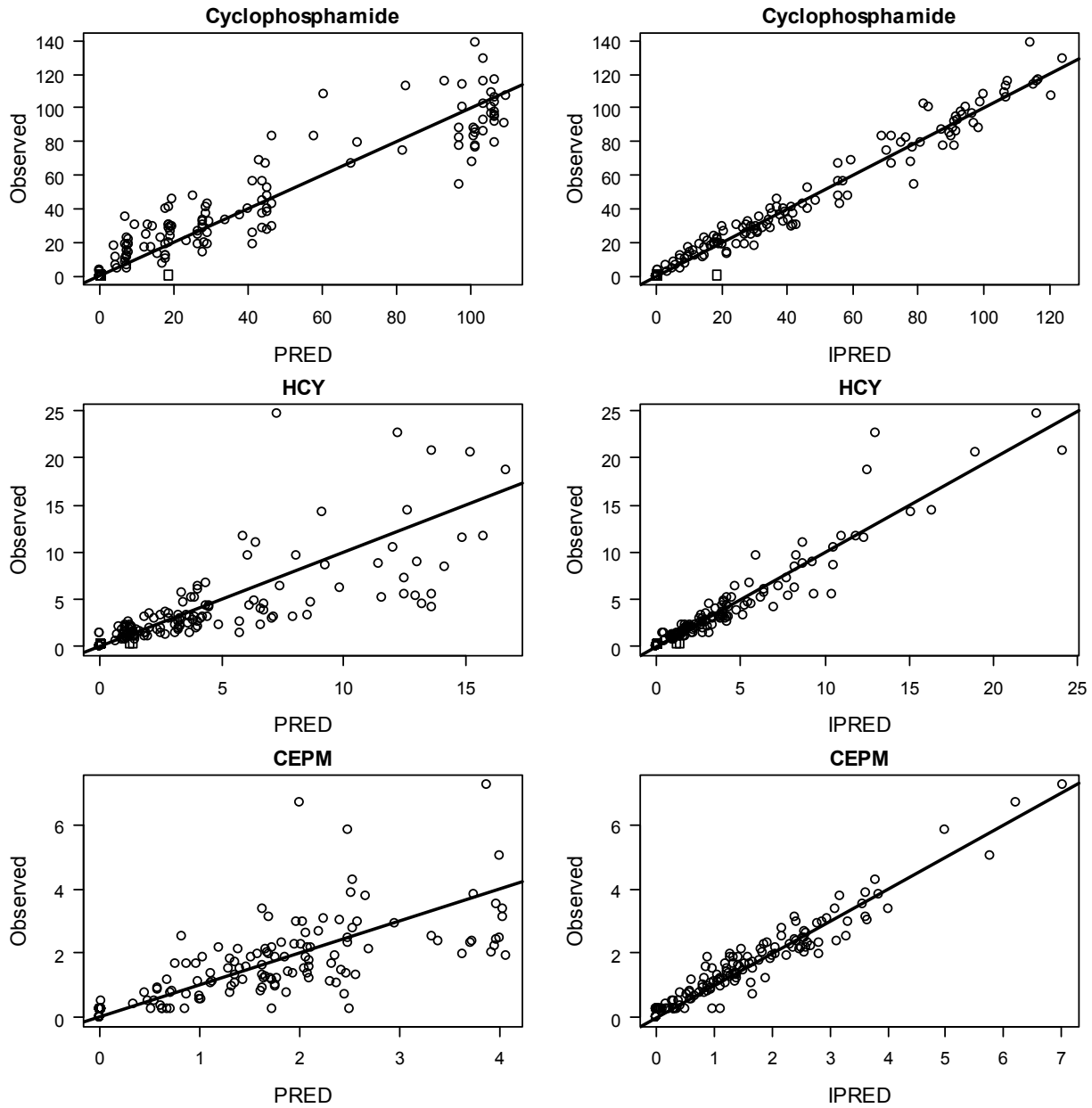
*Population Predicted (PRED) are predictions using population-level parameter values (Table 3). Individually Predicted (IPRED) are predictions using individual-level parameter values (not reported). Solid lines have slope=1. Data points denoted with a ▫ were BQL.

Figure 3. Model validation via a visual predictive check: observed data is compared with median prediction and 90% prediction intervals from 4400 subjects simulated from the final model.
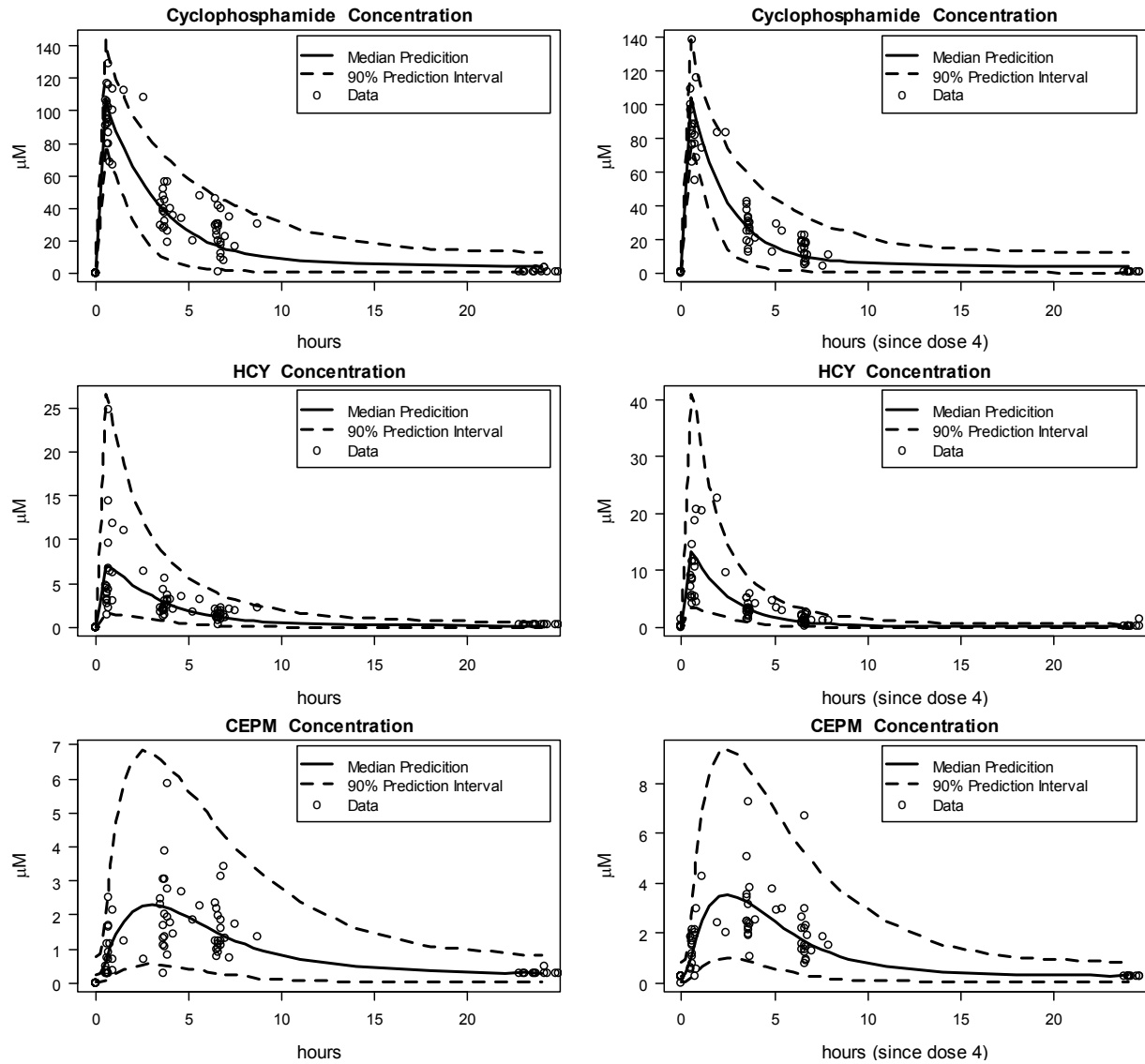
Figure 4. **Modeled cumulative and 24 hour interval AUC for cyclophosphamide, HCY and CEPM plotted for each individual. The curves show the mean AUC.**
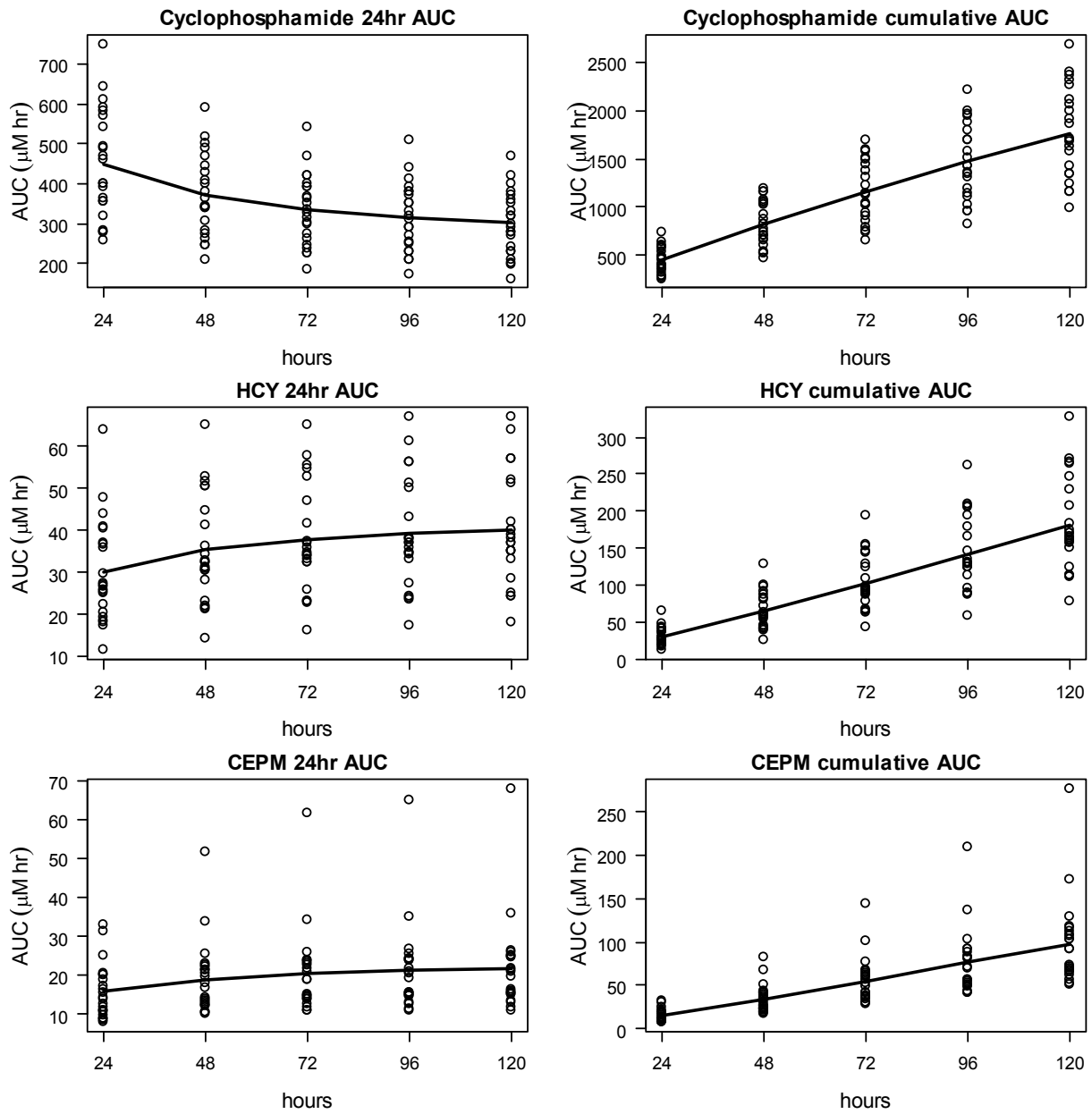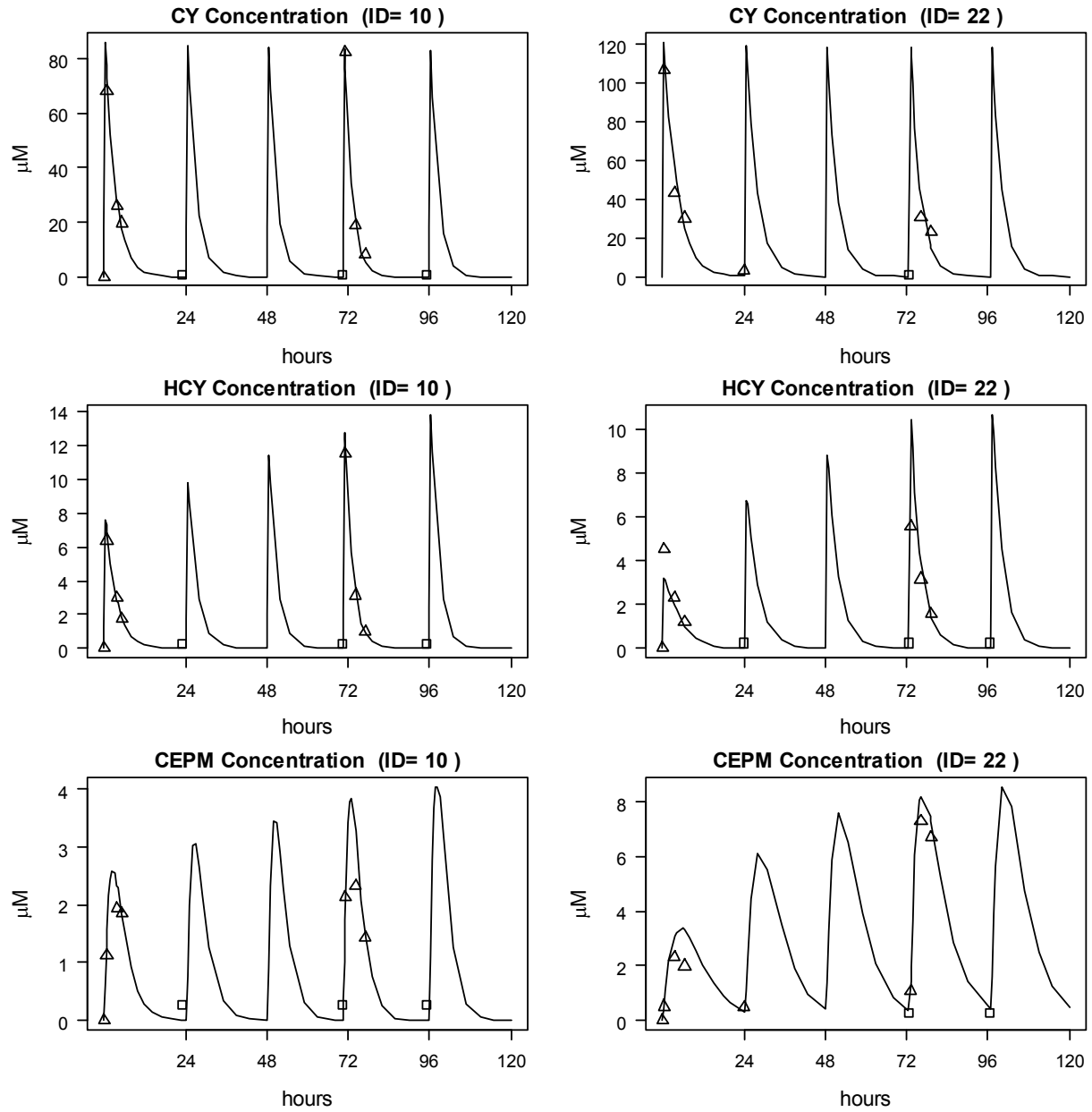
Figure 5. Concentration-Time profiles and simulations of cyclophosphamide (CY), HCY and CEPM for two study patients. Data points represented by a □ were BQL and have been set to QL/2.

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