Optimization of busulfan dosing regimen in pediatric patients using population pharmacokinetic model incorporating GST polymorphisms

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Abstract

Aim: The aim of this study was to develop a population pharmacokinetic (PPK) model in Chinese children for intravenous busulfan, and to develop a novel busulfan dosing regimen to support better area under the concentration-time curve (AUC) targeting. Methods: We collected busulfan concentration-time samples from 69 children who received intravenous busulfan prior to allogeneic hematopoietic stem cell transplantation (allo-HSCT). A population pharmacokinetic model for busulfan was developed by nonlinear mixed effect modelling and was validated by an external dataset (n=14). A novel busulfan dosing regimen was developed through simulation on 1000 patients. Limited Sampling Strategy (LSS) was established by the Bayesian forecasting. Absolute Prediction Error (APE), Mean Absolute Prediction Error (MAPE) and relative Root Mean Squared Error (rRMSE) were calculated to evaluate predictive accuracy. Results: A one-compartment model with first-order elimination best described the data. GSTA1 genotypes, BSA and AST were found to be significant covariates of Bu clearance, and BSA had remarkable impact of the volume. Moreover, recommended dose regimens for children with different GSTA1 genotypes and BSA were developed with a perfect AUC targeting. A two-point LSS, two hours and four hours after dosing, behaved well with acceptable prediction precision. Conclusion: This study developed a PPK model for busulfan that firstly incorporated GSTA1 genotypes in an Asian pediatric population. We recommend a BSA-based dosing for personalizing busulfan therapy in pediatric population. Additionally, an optimal LSS (C2h and C4h) provides convenience for therapeutic drug monitoring (TDM) in the future.

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BSA and AST were found to be significant covariates of Bu clearance, and BSA had remarkable impact of the volume. Moreover, recommended dose regimens for children with different *GSTA1* genotypes and BSA were developed with a perfect AUC targeting. A two-point LSS, two hours and four hours after dosing, behaved well with acceptable prediction precision.

Conclusion : This study developed a PPK model for busulfan that firstly incorporated *GSTA1* genotypes in Chinese pediatric population. We recommend a BSA-based dosing for personalizing busulfan therapy in pediatric population. Additionally, an optimal LSS (C_{2h} and C_{4h}) provides convenience for therapeutic drug monitoring (TDM) in the future.

KEYWORDS : busulfan, HSCT, individualized therapy, population pharmacokinetics, pediatric patients

Introduction

Busulfan (Bu) is a common alkylating agent, which can bind to the guanine of intracellular DNA to damage its structure and function (1). Bu-based conditioning schemes are regarded as the cornerstone of allogeneic hematopoietic stem cell transplantation (allo-HSCT) because of their myeloablative activity (1, 2). However, IV Bu usually leads to large variability in pharmacokinetics and the treatment window is narrow. Higher exposure (expressed as area-under-the-curve of 0 - 6 h, $AUC_{0\text{-}6h}$) (>1350 μM·min) or low $AUC_{0\text{-}6h}$ (< 900 μM·min) of Bu may lead to a higher probability of sinusoidal obstructive syndrome (SOS) or acute graft-versus-host disease (aGVHD) (3-5). The exposure and clinical outcomes of Bu are largely affected by different conditioning regimen agents, which has been confirmed in children receiving Bu/cyclophosphamide (Bu/CTX), Bu/fludarabine (Bu/FLU) and Bu/ cyclophosphamide/etoposide (Bu/CTX/VP16) (6-8). Thus, therapeutic drug monitoring (TDM) for busulfan is the integral component of HSCT. Personalized Bu dosing via TDM based on the first-dose pharmacokinetics (PK) could make contribution to lower the occurrence of toxicity and to improve rate of engraftment (9, 10).

Bu is metabolized by the formation of a glutathione conjugate in the liver (11, 12). This reaction is primarily catalyzed by glutathione S-transferase (GST) enzymes, such as *GSTA1* ,*GSTM1* , *GSTT1* and *GSTP1* (13).*GSTA1* is the predominant GST enzyme involved in Bu metabolism, and the activity of *GSTM1* is close to the half of *GSTA1* . However, the activity of *GSTT1* and *GSTP1* is relatively weak (2, 14). Hence, polymorphisms in the *GSTA1* or *GSTM1* isoenzymes would more likely affect Bu metabolism.

Studies about the relationships between *GSTA1* polymorphisms and Bu PK have yielded inconsistent results. Although it has been reported that*GSTA1* haplotypes were not significant influence factors of intravenous (IV) Bu clearance (15), Yin *et al.* (16) stated adult patients with the *GSTA1 *A/*B* genotype had a significantly higher AUC, higher peak concentration (C_{max}) and lower clearance (CL). Pediatric patients have an increased Bu clearance when compared with adults (17), which is partially caused by higher expression or activity of *GST* enzymes in children (18, 19). Research on the relationship between *GSTA1* polymorphism and PK in children is also controversial. Ansari *et al.* (20) reported that*GSTA1 *A/*A* was associated with lower drug concentration and more extensive metabolism. Conversely, Zwaveling *et al.* (21) asserted that *GSTA1* polymorphisms had no influence on population PK parameters of IV Bu in children undergoing HSCT. In addition to age, the activity of *GST* enzymes is also affected by race. Minor allele frequencies (MAF) taken from HapMap were reported to be differing between Caucasian and Asian populations. Until now, the correlation between *GST* polymorphisms and PK in Chinese children has not been reported.

It has been suggested that, apart from *GSTs* polymorphism, the effect of age, body-weight (WT), primary disease, hepatic function, renal function and drug interactions (fludarabine and phenytoin) may partly explain inter-individual variability on Bu PK (22-25). Little is known about correlates of Bu PK in Chinese children, which may be potential factors to optimize dosage of Bu.

The aim of this study was thus to build a population pharmacokinetic (PPK) model with data from pediatric patients, so as to present the PK feature of Bu, to reveal the variability of PK parameters, and to identify the potential contribution of covariates on the disposition of Bu. Furthermore, Maximum A Posteriori (MAP) Bayesian forecasting made use of a PPK model and limited number of samples to forecast $AUC_{0\text{-}6h}$ and to

formulate an optimal LSS, which is an alternative monitoring strategy.

Methods

2.1 Patients and treatment regimens

From March 2019 to April 2020, we collected 76 patients received allo-HSCT. This study was approved by the Beijing's Children Hospital and all patients/parents provided informed consent.

In the pre-treatment regimen, intravenous infusion of busulfan (BUSULFEX, Otsuka Pharmaceutical Co. LTD, Zhejiang, China) typically started eight days prior to transplantation. The infusion frequency was once every six hours within 3-4 days and each infusion process last for two hours. The dosage of Bu was based on weight (1 mg/kg for children less than 9 kg, 1.2 mg/kg for children within a 9-16 kg range, 1.1 mg/kg for children between 16-23 kg, 0.95 mg/kg for children between 23-34 kg, and 0.8 mg/kg for children more than 34 kg) in accordance with the European Medicines Agency (EMA) (26). Different conditioning regimens were used dependent on the primary diseases and the types of donor. Briefly, Bu combined with cyclophosphamide (CTX) were the basic components of conditioning chemotherapy. For patients with malignant diseases, regimens containing cytarabine (Ara-C) were commonly applied, while fludarabine (FLU) was administrated for myeloablative treatment of patients with non-malignant tumors. Specific dosage and duration of different conditioning regimens have been exhibited in **Supporting Information Table 1** . Phenytoin (PHT) started 30 minutes before the initiation of Bu therapy in order to prevent central nervous system toxicity caused by Bu. Moreover, cyclosporine (CsA) with or without methotrexate (MTX) / mycophenolate mofetil (MMF) was given as GVHD prophylaxis.

2.2 Bu determination and genotyping

Blood samples for PK analysis and genotyping were withdrawn from central venous lines, in heparinized glass tubes, pre-infusion, 0.5, 1, 2, 2.5, 4 and 6 hours after the first infusion. Plasma concentrations of Bu were determined using a high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) (1). The lower limit of quantitation was 10 ng/mL and the range of quantitation was from 10 to 10000 ng/ml. Pre-transplant genomic DNA was isolated and extracted from whole blood or hemocyte prior to the first Bu infusion. GST genotypes of patients,*GSTA1* (rs3957356 and rs3957357, which defines haplotype **A*and **B*) and *GSTM1* (rs3754446), were detected with the ABI 3730XL DNA Analyzer (ABICo.; BioSune Biotechnology Co., Ltd, Shanghai, China). **Supporting Information Table 2** displays the primer sets and Tm used for the genotyping assays.

2.3 PPK analysis

BU plasma concentration-time data was analyzed using a Non-Linear Mixed-Effects (NLME) model implemented in Phoenix 8.0 (Certara USA Inc., Princeton, NJ, USA). Typical PPK parameters and their random inter-individual variability (IIV) were estimated using a First-order Conditional Estimation method with Extended Least Squares method (FOCE ELS). One- or two-compartment distribution with first-order elimination were tested as structural model. Exponential errors follows a log-normal distribution, assumed to describe the IIV in PK parameters by the equation $P_i = P \times e^{\eta i}$, where$P_i$ is the individual PK parameter of the$i$ th individual, P is the geometric average population value, and η_i is the subject-specific random effect value, normally distributed random variable with a mean of 0 and a variance of ω^2 (27). Additive, proportional, combined additive and proportional models were evaluated to account for the intra-individual (residual) variability. The selection of base model was based on changes in -2 log likelihood (-2LL) and on graphic analyses of the Goodness-of-fit (GOF).

Based on scatter-plots of individual parameters versus covariates and clinical plausibility, we selected candidate covariates for each PK parameters (28). The influence of potential covariates [sex, age, body weight, body surface area (BSA), alanine transaminase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), total bilirubin (TBIL), creatinine (CRE), creatinine clearance (CLcr), *GSTA1*genotypes (**A/*A, *A/*B, *B/*B*), *GSTM5* genotypes, primary disease (malignant disease, non-malignant disease) and drug interactions (FLU)] on clearance (CL) and volume (V) were further investigated using a stepwise

procedure. Categorical covariates were coded as number, and continuous covariates were centered on their median value (27). During forward selection, covariates were defined as significance if the -2LL decreased by at least 3.84 (p [?] 0.05) following their inclusion in the model. During backward elimination, one covariate could remain in the final model if the -2LL increased by at least 6.63 (p [?] 0.01) when removed at a time from the full model.

2.4 Model validation

Shrinkage in individual random effects were evaluated in order to assess whether the final model could be capable to estimate individual PK parameters by taking advantage of population typical values and sparse PK data. Shrinkage values of less than 20% indicate that the individual data are rich enough to compute the PK parameters, whereas larger shrinkage values generally mean that individual Bayesian estimates are biased towards the population mean values(29).

Graphical observation of the final model adopted GOF, including conditional weighted residuals (CWRES) over population predicted concentrations (PRED) or time after dose (TAD) and the relationship between observed (OBS) and PRED or individual predicted value (IPRED) (30). In addition, the final model was evaluated using visual predictive check (VPC) and bootstrap analysis. VPC was based on the final pharmacokinetic estimates and then calculated the 95% confidence interval (CI) for concentrations by simulating 1,000 individuals. Simulated concentrations were compared with the 5th, 50th, 95th percentiles of the observed concentrations. In the bootstrap analysis (31), the 95% CI of the parameter estimates were derived from 1000 datasets of 69 subjects generated by random sampling using the Phoenix NLME.

External validation of the model was performed using an external dataset to evaluate the predictive performance of the final PPK model. The external dataset consisted of 81 busulfan concentrations from 14 children undergoing allo-HSCT. Busulfan plasma concentrations were predicted by fixing the population PK parameters to the final estimates of the previously established model and setting maximum evaluations to 0. From this study, measured concentrations of individual patients assigned to busulfan were compared with calculated concentrations of these individual patients at the same time with our PPK model using their BSA, ALT and *GSTA1* genotypes of patients. Differences of $< 20\%$ between calculated and measured concentrations were allowed (32).

2.5 Dosing Simulations

A new dosage scheme using a simulated dataset of 1000 patients was designed to achieve a targeted AUC of 1125 μM·min. The BSA of simulated patients were distributed from 0.2 m^2 to 1.6 m^2. Meanwhile, the genotypes of *GSTA1* included **A*A and *A*B*and the range of AST was coincident with the clinical dataset. Then, 1000 $AUC_{simulated}$ values and their variabilities were generated in Phoenix-NLME. Model-based doses were recommended according to the following **Equation 1** :

$$Dose_{recommended} = 1125 \ / \ AUC_{simulated} \times Dose_{simulated} \quad (1)$$

Then, AUC values were calculated according to the new recommended doses. Variabilities of AUC were shown by the range between minimum and maximum values and the coefficient of variation (CV). The rate of achieving the targeted AUC window (900-1350 μM·min) was also listed.

2.6 Optimized Sampling Scheme

The final PPK model provides information on typical PK parameters and variabilities associated with these values as well as how specific covariates (e.g. BSA, AST, genotypes) influence the PK of BU. After evaluating final model predictive performance, filtrating optimal LSS was considered to optimize the sampling scheme for TDM. Candidate sampling points included: (1) 2, 2.25, 4 and 6 hours; (2) 2, 2.5, 4 and 6 hours; (3) 2.25, 2.5, 4 and 6 hours; (4) 2, 4 and 6 hours; (5) 2.25, 4 and 6 hours; (6) 2.5, 4 and 6 hours; (7) 2, 2.25 and 4 hours; (8) 2, 2.25 and 6 hours; (9) 2, 2.5 and 4 hours; (10) 2, 2.5 and 6 hours; (11) 2.25, 2.5 and 4 hours; (12) 2.25, 2.5 and 6 hours; (13) 2 and 4 hours; (14) 2 and 6 hours; (15) 2.25 and 4 hours; (16) 2.25 and

6 hours; (17) 2.5 and 4 hours; (18) 2.5 and 6 hours after the beginning of first infusion. The blood drug concentration at 2.25 hours after administration was simulated by the final model. Each candidate LSS was tested by Bayesian method to obtain $AUC_{0\text{-}6h}$ of each patient. APE, MAPE and rRMSE were calculated by comparing the predicted $AUC_{0\text{-}6h}$derived from the LSS with the actual $AUC_{0\text{-}6h}$ obtained from each patient's full concentration-time samples, as according to**Equation 2, 3 and 4** to evaluate predictive accuracy:

$$\text{APE } (\%) = \frac{|predicted\ \text{AUC}_{0-6h} - \text{actual AUC}_{0-6h}|}{\text{actual AUC}_{0-6h}} \times 100\% \quad (2)$$

$$\text{MAPE } (\%) = \frac{1}{N}\sum\left(\frac{|predicted\ \text{AUC}_{0-6h} - \text{actual AUC}_{0-6h}\ |}{\text{actual AUC}_{0-6h}} \times 100\%\right) \quad (3)$$

$$\text{rRMSE } (\%) = \frac{1}{N}\sqrt{\sum\left(\frac{\text{predicted AUC}_{0-6h} - \text{actual AUC}_{0-6h}}{\text{actual AUC}_{0-6h}} \times 100\%\right)^2} \quad (4)$$

The lower the MAPE value, the better the LSS. A sampling strategy was considered to display a good predictive performance when the 95% confidence interval around the MAPE was less than 20% of the reference Bu $AUC_{0\text{-}6h}$ values (33). Bland-Altman plots were applied to assess the agreement between the predicted and the actual $AUC_{0\text{-}6h}$, and the difference was expressed as a mean ± 1.96 SD.

2.7 Definition of Clinical Outcomes

The definition of engraftment was absolute neutrophil count (ANC) [?] 0.5 x 10^9 per liter for the first three days in a row and platelet count (PLT) [?] 20 x 10^9 per liter for the first one week after HSCT (10, 16). Primary engraftment failure was defined as failing to reach an ANC of 0.5 x 10^9 /L within 30 days after HSCT (16). In brief, the diagnosis of SOS was according to the modified Seattle criteria (34). The Mount Sinai Acute GVHD International Consortium (MAGIC) was taken as the diagnostic criterion for aGVHD (35).

2.8 Statistical Analysis

Continuous data, like pharmacokinetics parameters, was compared using a two-tailed t-test, whereas time-to-event data was compared by the log-rank test. Univariate analysis was performed with GraphPad Prism (GraphPad Software, San Diego, CA). In this study, p values < 0.05 are considered as statistical significance, and$p$ values < 0.1 were deemed as trends.

Results

3.1 Population characteristics

From March 2019 to April 2020, 76 patients underwent allogeneic HSCT and received IV busulfan. The *GSTA1* genotypes for four patients and*GSTM1* genotypes for six patients were missing because of samples being unavailable or detection failure. *GSTA1-52C* and *T*defined haplotype **A* and **B* , respectively. In relation to*GSTA1-52T/C* and *-69A/G* SNPs, a higher percentage of*CC/GG* (78.08%) versus *TT/AA* (1.37%) and *CT/GA*(20.55%) was found in our model-building patient cohort. There was only one patient with *GSTA1 *B*B* . Thus, 69 patients were included in our final PPK analysis. **Table 1** summarizes characteristics of patients including demographics, primary diseases, donor types and conditioning regimens. **Supporting Information Table 3** further elaborates patients' diagnoses. The genotyping results are shown in**Table 2** .

3.2 PPK model

A total of 398 blood samples were collected for the PPK analysis.**Supporting Information Figure 1** plotted concentration–time profiles of Bu. A one-compartment model with first-order elimination was suitable to describe the profiles of busulfan pharmacokinetics, and the exponential model was feasible to estimate the inter-individual variability. Neither proportional error model nor additive error model could perform well, while a combined proportional and additive residual error model provided an adequate fit (Δ-2LL > 20, $p < 0.001$).

In the forward model building step, BSA, AST, types of primary diseases and *GSTA1* genotypes declined the value of -2LL by more than 3.84 ($p < 0.05$) at each addition. The *GSTA1* mutation, BSA and the level of AST significantly affected the clearance (CL) of busulfan. Meanwhile, the volume (V) of busulfan was influenced by BSA and types of primary diseases, while the *GSTM1* genotypes had no significant effect on PK parameters.

During backward elimination, the value of -2LL increased significantly (Δ-2LL >6.63, $p < 0.01$) when respectively removing the *GSTA1* mutation, BSA and the level of AST out of the model. Thus, the three covariates mentioned above could remain in the final model. Types of primary diseases were eliminated in the final model because it failed to significantly increase the -2LL value (Δ-2LL = 6.333, $p > 0.01$). **Table 3** presented the detailed PK parameters of the base and final model. The CL and V of busulfan were affected by the *GSTA1* genotypes, BSA and AST as illustrated by the following **Equation 5 and 6** :

$$\text{CL (L/h)} = \text{CL}_{\text{pop}} \cdot \left(\frac{\text{B}\Sigma\text{A}_i}{\text{B}\Sigma\text{A}_{\mu\varepsilon\delta\iota\alpha\nu}}\right)^{covBSA(CL)} \left(\frac{\text{A}\Sigma\text{T}_i}{\text{A}\Sigma\text{T}_{\mu\varepsilon\delta\iota\alpha\nu}}\right)^{covAST(CL)} e^{\varsigma o GSTA1(CL) GATA1} e^{\eta\hat{}\Lambda} \quad (5)$$

$$\text{V (L)} = \text{V}_{\text{pop}} \text{selectlanguagegreek } (\text{BSA}_i \overline{\text{BSA}_{\text{median}})^{\text{covBSA}(V)} e^{\text{V}}} \quad (6)$$

When patients carried the *GSTA1 *A/*B* mutation, *GSTA1* = 1, and when patients carried the **A/*A* genotype, *GSTA1* = 0.

Population estimates for a patient with median BSA with *GSTA1 *A/*A* were CL 4.79 L/h and V 14.8 L. The values of inter-individual variability for CL and V were 18.65% and 23.63%, respectively. CL of patients carrying the *GSTA1 *A/*B* genotype was predicted to decline by 17.3% compared with those carrying *GSTA1 *A/*A* . As a result, CL/BSA between two genotypes had a significant difference ($p = 0.0103$), in spite of AUC/BSA without significant difference (**Figure 1**).

3.3 Model evaluation and validation

Shrinkage values of clearance and volume were respectively 0.207 and 0.130, which manifested that the model-building dataset was rich enough to compute the PK parameters of busulfan in children.

Compared with the base model, the -2LL value of final model declined by 175.26, which indicated that the model improved substantially after incorporating covariates, BSA, AST and *GSTA1* phenotype. As demonstrated in the scatter plots of OBS *vs.* PRED (**Figure 2A and 2B**), the PRED strongly deviated from the OBS in the base model, but PRED agreed with the OBS in the final model. In addition, the CWRES in the final model were more uniformly distributed within the accepted range (y = ± 2) than that in the base model (**Figure 2C and 2D**). As a whole, the final model was visually improved in terms of the GOF and had a more accurate predictive performance.

The estimated values generated by bootstrap analysis were close to the parameters in the final model, which also fell within 95% CIs (**Table 3**). Thus, it can be considered that the final model was accurate and robust. **Figure 3** clearly displayed that 5th, 50th and 95th percentiles of simulation values almost coincided with that of observed concentrations. The results of VPC combined with bootstrap analysis confirmed the exactitude of the parameter estimates and demonstrated the reliability of the final model. For external validation, a mean difference of 18.48% was observed in the simulated Bu concentrations generated using the established PPK model when compared with the observed Bu concentrations (**Figure 4**).

3.4 New dosing strategy and simulations

To achieve a target AUC of 1125 μM·min, a simple linear relationship between the total doses and BSA is illustrated in **Figure 5(A).** The linear **Equation 7 and 8** for *GSTA1 *A/*A* and **A*B* was respectively:

$$\text{Dose}_{\text{recommended}}\ (\text{mg}) = 34.14 \times \text{BSA}\ (\text{m}^2) + 3.75 \quad (7)$$

$$\text{Dose}_{\text{recommended}}\ (\text{mg}) = 30.99 \times \text{BSA}\ (\text{m}^2) + 3.21 \quad (8)$$

The dosages were normalized by BSA (mg/m^2) due to the fact that body size-based dosing is more familiar to pediatricians. As demonstrated in **Figure 5 (B)** , the doses normalized by BSA were shown to be a log-linear function of BSA. Dosages according to this log-linear curve would be inconvenient for application in clinical, so a nomogram of BSA categories was derived. Three dosages for each genotype were then defined according to selected BSA categories in order for convenient clinical application. Finally, a new dosing strategy based on BSA normalization was listed in **Table 4** .

BSA-based dosing recommended by the final PPK model was helpful for targeting the patient AUC. The rate of success in achieving the targeted AUC window (900-1350 μM·min) was 99.58% in simulated patients. The new dosage yielded homogeneous AUC values in different BSA categories, and the CV of 7.57% in AUC was low.

3.5 Limited sampling strategies

Eighteen models with different sampling time points are all listed in**Table 5** . The relationship between the predicted and actual $AUC_{0\text{-}6h}$ for these models is shown in **Supporting Information Figure 2** . From two to four-point models, 83.33% LSSs fitted well with the correlation (r^2) of more than 0.85. Prediction precision of LSSs expressed as rRMSE and MAPE is also given in **Table 5** . Model 2 (C_{2h}, $C_{2.5h}$, C_{4h} and C_{6h}) showed not only the best fit to the Bu $AUC_{0\text{-}6h}$, but also better prediction precision (rRMSE = 0.72% and MAPE = 4.55%) than other LSSs. Among the three-point models, Model 9 (C_{2h}, $C_{2.5h}$and C_{4h}) and Model 10 (C_{2h}, $C_{2.5h}$ and C_{6h}) both behaved well, and with similar prediction precision to Model 2. Within these models, no patients had an $AUC_{0\text{-}6h}$ lower than -15% or higher than +15% of the reference value. As for these two-sampling schemes, Model 13 (C_{2h} and C_{4h}), Model 14 (C_{2h} and C_{6h}), Model 17 ($C_{2.5h}$ and C_{4h}) and Model 18 ($C_{2.5h}$ and C_{6h}) had relatively low rRMSE and MAPE. The Bland–Altman plots have verified the excellent capacity of prediction of the seven models above **(Supporting Information Figure 3)** .

3.6 Clinical outcomes

Among the clinical outcomes analyzed, five patients died after HSCT, one of whom died of SOS and the other four died of severe infection. Graft failure occurred in two patients with $AUC_{0\text{-}6h}$ of 659.9 and 482.7 μM·min. Engraftment was achieved for 97.10% of patients (median time 12 days, range 10-19 days) for neutrophils within 30 days after transplantation and 59 patients achieved engraftment for platelets within a median of 15 days (range 7-30). As shown in **Figure 6** , there were significant differences in ANC recovery and survival rate between patients with two *GSTA1*genotypes ($p < 0.05$). Seven patients (10.14%) developed SOS and aGVHD I–IV was documented in 57 patients (82.61%), of whom seventeen had grade III–IV disease (24.64%). The clinical outcomes of patients with two*GSTA1* genotypes were compared in **Supporting Information Table 4** .

In a univariate model, type of donor (HLA matched *vs.* mismatched) was the single variable that had significant association with the incidence of aGVHD ($p = 0.022$) (**Figure 7**). The logistic regression model was again put into use to investigate the relationship between the incidence of aGVHD I–IV, aGVHD III/IV, SOS and Bu $AUC_{0\text{-}6h}$, while **Supporting Information Figure 4**revealed that no correlation between Bu $AUC_{0\text{-}6h}$ and regimen-related toxicity or mortality was observed.

Discussion

This study developed the first PPK model for busulfan that successfully incorporated *GSTA1* genotypes in Chinese pediatric population and partly explained the source of large variability of busulfan exposure, suggesting that GST genotyping would be necessary for optimization of pediatric Bu treatment.

The literature until now has been controversial regarding the correlation between Bu clearance with genetic polymorphisms (36). There have been several researches supporting the positive association between *GSTs* polymorphisms and Bu clearance since 2016. In a pediatric multicenter study, Ansari *et al.* (37) reported that the activity of *GSTA1* promoter was significantly descended in the case of **B* haplotype compared with **A* haplotype, thus the correlation between *GSTA1* genotypes and clearance was distinguished. In addition, *GSTA1* diplotypes with slow metabolizing capacity were associated with higher incidence of SOS, aGVHD and combined treatment-related toxicity. Another study succeeded in incorporating the *GST* genetic variants (*GSTA1*) into a PPK model for Bu in a Caucasian pediatric population, and then tailored the dose according to the individual metabolic capacity (38). For Chinese adults, Yin *et al.* (16) concluded that patients with *GSTA1 *A/*B* genotype possessed a higher C_{max}, higher AUC and lower clearance than the group with *GSTA1 *A/*A* genotype. Furthermore, *GSTA1* expression in young children has been reported to be higher than adults (19). In a recent study of Japanese pediatric population (n=20), Nishikawa *et al.* (39) stated the correlation between *GST* polymorphisms and clearance was distinguished.

In our two cohorts (n=84), the frequency of *GSTA1 *B*haplotype (*GSTA1-52T/-69A*) was 11.9% (MAF = 0.119). In other words, a minority of patients had the **B* haplotype, of which 1.19% were homozygous (*GSTA1 *B/*B*). Therefore, the MAF of Chinese patients was lower than that of global population (MAF = 0.306) taken from 1000 Genomes. Due to the significant discrepancy of the frequency of haplotype **B* between different populations, studies in Caucasians may not be able to represent that in the Asian population. Besides, in a study conducted in patients with acute myeloid leukemia, Yee *et al.* (40) found that C allele in the *GSTM1* locus (rs3754446) was associated with decreased Bu AUC of first dose and lower disease-free survival. Thus,*GSTA1* and *GSTM1* genotypes were taken into consideration in the present study when exploring the influence of *GST*polymorphisms on Bu PK in Chinese children.

A population pharmacokinetic model of Bu was developed to test the influence of gene mutation on the pharmacokinetic characteristics. In the final model, the estimate value of CL was 4.79 L/h and of V was 14.8 L, consistent with those results in previous studies (38). The PPK model demonstrated that the *GSTA1* polymorphisms were associated with Bu clearance. Patients carrying the *GSTA1 *A/*B* genotype had a 17.3% lower clearance than those carrying *GSTA1 *A/*A* , which is consistent with previous studies reporting that the presence of mutation allele probably resulted in the decreased activity of GST enzyme (11, 37). However, genetic variation in *GSTM1* showed no significant impact on Bu CL, likely because the function of the *GSTM1*enzyme involved in Bu metabolism was less than the *GSTA1* enzyme.

As shown in **Figure 1** , there was a significant difference in clearance per body surface area between the two *GSTA1* genotypes, but there was no significant difference in AUC per body surface area. These results were indeed confusing. As is well-known, AUC of drug administered intravenously is affected by the distribution, metabolism and excretion of drug, while CL is influenced only by metabolism and excretion. There are many complex physiological factors that affect drug distribution, especially in children. Thus, AUC may be not completely inversely proportional to CL. As shown in **Supporting Information Figure 1** , the distribution phase (0-2 hours after dosing) shown large heterogeneity between two genotypes, which may slightly interfere the statistical analysis of AUC. Nevertheless, in the eliminate phase, it was obvious that patients with *GSTA1 *A*A* appeared faster clearance than those with *GSTA1 *A*B* . Generally, the non-obvious significance of $AUC_{0\text{-}6h}$ seemed reasonable. Moreover, the number of patients treated with busulfan with an AUC value of more than 900 μM·min was eight. In other words, 88.4% of children with HSCT could not reach the range of 900-1350 μM·min recommended by FDA. The main factor resulting in the low $AUC_{0\text{-}6h}$ may be the fact that, for Chinese patients, the frequency of *GSTA1 *A* with high metabolizing capacity was much higher when compared to *GSTA1 *B* with slow metabolizing capacity. Thus, the relatively low level of $AUC_{0\text{-}6h}$ in this study population was rational.

In the final PPK model constructed in this study, AST levels were negatively correlated with CL of Bu in pediatrics and CL declined 38.34% when AST increased from 12.7 to 127.4 U/L. This is unsurprising because busulfan is mainly eliminated by the liver as previously described. This study divided the patients into two subpopulations (malignant and non-malignant diseases), which had no significant effect on the

pharmacokinetic parameters of Bu. In fact, malignant diseases could be subdivided into seven types according to pathology, and non-malignant diseases included six types. Nevertheless, some of the pathologies, such as osteopetrosis, were rare and present in only a small number of patients, resulting in an impossible comprehensive evaluation of variability of Bu PK. In oral busulfan-based pediatric research, the influence of underlying diseases on busulfan disposition was significant and CL/F was significantly lower in group with immune deficiencies than other groups (metabolic diseases, hemoglobinopathies and hematological malignancies) (25). Hence, the number of different types of cases needs to be expanded in order to analyze the specific impact of every primary disease on Bu PK.

There are theoretical and documented medication interaction with fludarabine and busulfan (41). Clearance of IV Bu decreased significantly in patients receiving concomitant fludarabine administration ($p = 0.0016$) and the average of reduction was 9.7% (42). However, there was no other studies drawing similar conclusions (43, 44). Not surprisingly, in this PPK model, the inclusion of fludarabine as a covariate failed to significantly decline the value of -2LL during the forward selection. In fact, patients in our cohort received one of the two regimens, Bu/CTX/FLU and Bu/CTX/Ara-C, as basic conditioning therapies. Due to the use of Bu/CTX in all patients without exception, the influence of CTX has already existed in the model. Although we only regarded FLU as a candidate covariate, in fact the influence of Ara-C has been also reflected in the model. When FLU=1, the impact of FLU was added to the model, and when FLU=0, the effect of Ara-C was included.

BSA was the most predictive covariate for CL and V, explaining 25.50% and 24.17% of the observed IIV, respectively. Weight-based dosing schedules were calculated with five fixed doses, however the model established by this study showed that body weight is not a significant predictor of Bu PK in children. Additionally, in a retrospective study, SOS and early infectious complications occurred more frequently in the weight-based dosing group (45). Besides, a PPK model, developed among patients of all ages, revealed that the maturation of Bu clearance reaches half of adult values at 6 weeks after birth (46). Also, in children, Bu concentration did not show an obvious trend of change with postnatal age, which strongly supported the conclusion drawn from our cohort that age was not a significant factor affecting Bu clearance. According to the PPK model established with Chinese pediatric patients, BSA-based dosing scheme was recommended. When compared with weight-based dosage, the new dosing scheme not only took the influence of*GSTA1* polymorphism into consideration, but could also be applied to patients with different liver function. More importantly, the simulation analysis demonstrated that the three fixed doses given on an mg/m^2 basis enabled almost all of the young patients ($0.2^{\sim}1.6$ m^2) to achieve the target $AUC_{0\text{-}6h}$. Since this new dosing regimen was based on a retrospective analysis, a prospective study is necessary to confirm the benefits in terms of efficacy and safety.

Based on the final PPK model, Bu Bayesian estimation of individual $AUC_{0\text{-}6h}$ values were performed by using various combinations of 2-4 sampling times within 6 hours following busulfan administration. Until this point, several LSS strategies have been established to predict BU exposure. Most LSS strategies published were estimated by using the trapezoidal rule (TR) or multiple linear regression (MLR), which usually reduced the accuracy of estimation and lacked professionalism. For example, Vaughan *et al.* (47) concluded that 4-5 sampling points (3, 4, 5 and 6 hours or 2, 3, 4, 5 and 6 hours after dosing) could predict well in adult patients receiving IV Bu four times daily. Teitelbaum *et al.* (48) developed an LSS with four sampling points (0, 2, 3 and 4 hours after the start of the second infusion). To achieve a more accurate estimate of AUC of every LSS, PPK models, considered to be the gold standard, should be applied. In this study population, C_{max} generally appeared in 2-2.5 hours after dosing according to the concentration-time curve. Therefore, peak concentration was picked among C_{2h}, $C_{2.25h}$ or $C_{2.5h}$, and $C_{4h}$and C_{6h} were regarded as time points of elimination phase. Since the metabolism of busulfan conformed to the one-compartment model, selecting C_{4h} or C_{6h} on behalf of elimination phase had a similar predictive function. Model 9 (C_{2h}, $C_{2.5h}$ and C_{4h}) and Model 10 (C_{2h}, $C_{2.5h}$ and C_{6h}) elevated the accuracy and precision of prediction while increasing medical costs and pain for children. Model 13 (C_{2h} and C_{4h}) not only had a better predictive performance by rRMSE, MAPE and Bland-Altman analysis, but was also more in line with the clinical requirement of reducing sampling points for TDM. Finally, considering the accuracy of prediction and the feasibility of pediatric clinical practice

synthetically, Model 13 was selected as the optimal LSS.

The relationship between pharmacokinetics and outcome or toxicity of busulfan reported previously are summarized in **Supporting Information Table 5** . A multicenter retrospective research stated that graft-failure occurred more frequently in the low AUC group, meanwhile, acute toxicity and TRM were significantly higher in the high AUC group (5). Additionally, Andersson *et al.* (49) found that the probability of developing aGVHD increased with increasing AUC. Similar correlations between toxicities and AUC have been verified in both Korean children and adult groups (14, 50). However, no relationship between busulfan PK and toxicity was observed. Based on the data of 27 children with sickle cell disease undergoing HSCT, hepatic toxicity (SOS) was not associated with busulfan AUC (51). Moreover, Jessica*et al* . (52) concluded that there was no significant association between AUC $_{\text{dose1}}$ and death, relapse, or a composite of the two.

In terms of ANC recovery and survival rate, there were significant differences between patients with two GSTA1 genotypes ($p< 0.05$) in our study. Till now, no research has been reported concerning the relationship between *GSTs* polymorphisms and engraftment or mortality. However, Ansari *et al.* reported that GSTA1 *B haploid was associated with higher incidence of treatment-related toxicity (37). Hence, we deduced that the high incidence of toxicity was likely connected to the postponement and the low rate of ANC recovery, which further led to higher chance of infection, the main cause of death in this study. In the next stage, we will include more abundant samples to verify the above conjecture.

In this study population, type of donor was the major predictor of the occurrence of aGVHD, whereas neither *GSTM1* genotypes nor pharmacokinetic parameters were found to be significantly correlative to SOS. These results are difficult to interpret because multiple pre-transplantation and transplantation-related factors have been implicated in the rate of engraftment and the incidence of toxic effect. So far, no single factor has been reported to result in aGVHD or SOS independently. On one hand, it is worth noting that the homogeneity of patients' disease would be beneficial to the correlation analysis. For example, Srivastava at al. (53) concluded that GSTM1-null genotype was relevant to the incidence of SOS in 114 patients undergoing HSCT with uniform disease, β-thalassemia. On the other hand, the influence of complex combined medicines could not be ignored. Sixty-eight out of 69 patients adopted a combination of three or more myeloablative drugs and 57.97% of pediatric patients used four drugs with myeloablative activity in succession, which led that the AUC of a single drug, Bu, may be not the only influencing factor in curative effect or toxic reaction. It should be also recognized that when Bu was combined with different agents, relationships between Bu PK and outcome or toxicity would be varied (54), as has been confirmed in BU/CY/TBI regimens (55). Consequently, in order to clarify the correlation, analysis should be conducted according to disease types and conditioning regimens in the further.

CONCLUSION

In summary, this study was the first PPK model for busulfan that successfully incorporated *GSTA1* genotypes in a Chinese pediatric population and partly explained the source of large variability of busulfan exposure, suggesting that TDM would be necessary for optimization of pediatric Bu treatment. Moreover, a BSA-based dosing regimen is recommended for individual busulfan therapy in order to weaken variability in busulfan exposure and to enhance the safety and efficacy of Bu treatment. Finally, an optimal LSS (C_{2h} and C_{4h}) would be convenient for TDM in the future.

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