Exposure-Toxicity Association of Cyclophosphamide and its Metabolites in Infants and Young Children with Primary Brain Tumors: Dosing Implications

SUPPLEMENTARY MATERIAL

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Throughout the data supplement, the following abbreviations will be used:

4OH-CTX, 4-hydroxy-cyclophosphamide, CEPM, carboxyethylphosphoramide mustard

SECTION 1. Bioanalytical Methods for Cyclophosphamide, CEPM, and Derivatized 4OH-CTX in Human Plasma Samples and Reproducibility of the Standardization Procedure

The LC-MS/MS methods for quantitation of cyclophosphamide and its metabolites in human plasma, previously developed by Kalhorn et al, were modified, validated, and applied in the current study (1,2). For quantitation of cyclophosphamide/CEPM in human plasma, 50 µL of patient plasma samples were used for solid-phase extraction. For dilution, 50 µL H_2O was added, followed by 4 µL of combined internal standard working solution at 50,000 ng/mL for both cyclophosphamide-d_4 and CEPM-d_8. The spiked plasma samples were loaded onto the Oasis HLB µElution plate, which was preconditioned with 100 µL of methanol and then washed with 200 µL of H_2O. The plate was washed with 150 µL of H_2O and then eluted with 100 µL of 50% methanol in H_2O. A 2- to 3-µL sample extract was injected for LC-MS/MS analysis.

For quantitation of 4OH-CTX in human plasma, 100 µL of derivatized patient plasma sample was transferred into 1.4-mL V-bottom pushcap tubes after an initial thawing of plasma samples at 25°C for 45 to 60 minutes. The calibrators and quality control samples were prepared in bulk by spiking an appropriate volume of 4OH-CTX and placed at 25°C for 45 to 60 minutes before being transferred to 1.4-mL V-bottom pushcap tubes in 100-µL aliquots. These calibrators and quality control aliquots were stored at –80°C until the day of analysis. Next, 10 µL of 5,000-ng/mL CEPM-d8 working solution was added to the derivatized calibrator, followed by the quality control and patient samples. This was followed by protein precipitation with 300 µL of methanol. The samples were vortexed for 30 seconds and centrifuged at 2,680x*g* for 5 minutes at 4°C. The supernatants (180-220 µL) were transferred to auto sampler vials, and 10 µL was injected for LC-MS/MS analysis.

LC/MS/MS analysis was performed with a Shimadzu HPLC coupled with an AB Sciex Qtrap 4000 mass spectrometer. Positive electrospray ionization was used. Chromatography separation was achieved by using a Gemini C18 column (3 µm, 100 mm x 4.6 mm) and gradient elution with 0.1% formic acid in water as mobile phase A and methanol as mobile phase B.

For cyclophosphamide and CEPM sample analyses, the gradient program was set as 0 to 2 minutes, 60% to 85% B; 2 to 2.5 minutes, 85% to 85% B; 2.5 to 2.51 minutes, 85% to 60% B; 2.51 to 6 minutes, 60% to 60% B. The flow rate was set as 0.7 mL/minute, and the column temperature was 30°C. Specific analytes and the corresponding internal standards were identified by retention time with the following precursor-to-product ion transitions, which were determined with analytical standards: m/z 261 → 140 (cyclophosphamide), m/z 293 → 221 (CEPM), m/z 265 → 235.2 (cyclophosphamide-d_4), and m/z 301 → 229 (CEPM-d_8). The calibration range for cyclophosphamide and CEPM in human plasma samples with IV administration was 0.18 to 161.29 μM and 0.2 to 40.96 μM, respectively.

For 4OH-CTX sample analyses, the gradient program was set as 0 to 4.0 minutes, 55% to 95% B; 4.0 to 4.50 minutes, 95% to 95% B; 4.51 to 5.50 minutes, 55% to 55% B. The flow rate was set at 1.0 mL/minute, and the column temperature was maintained at 30°C. The 4OH-CTX metabolite and internal standard (CEPM-d_8) were identified by retention time by using the following precursor-to-product ion transitions, which were determined with analytical standards: m/z 367 → 221 (4OH-CTX) and m/z 301 → 229 (CEPM-d_8). The calibration range for 4OH-CTX in human plasma was 14.18 to 6,805.70 ng/mL.

As part of our standard operating procedure, routine quality control studies were performed to assure the reproducibility of the standardization procedure. At the time new samples were processed, a calibration curve and six controls (2 at each level: low, medium, and high) were prepared, processed, and assayed after the curve and after the unknown samples. In order for the results of the analytical run to be acceptable, the values of 4 out of 6 of the quality controls must be within ±15% of their nominal value. In addition, at least one quality control at each level must pass to accept the results. For each run of new samples from a clinical study, we also included 6 incurred study samples from a previously accepted run. The results of 4 out of 6 of the incurred samples must be within ±20% of the mean result. These incurred samples were always chosen so that adequate

coverage of the expected study sample concentration range was represented. If incurred samples didn’t meet acceptance criteria, the run was rejected.

The samples were immediately processed at the bedside after collection and stored at -80°C until analysis, as described in the Methods section of the manuscript. A long-term stability study was performed and indicated that the samples needed to be assayed within at least 18 months from the time they were drawn.

TABLE S1. Characteristics of patients with 4OH-CTX measurements and patients without 4OH-CTX measurements

Patient Characteristics	Patients with 4OH-CTX measurements	Patients without 4OH-CTX measurements	[a]p-value
Total number of patients	131	40	
Sex			
Male, n (%)	59 (45.0)	17 (42.5)	0.87
Female, n (%)	72 (55.0)	23 (57.5)	
Age (months)	18.9 (0.85–58.5)	24.0 (12.4-37.4)	0.01
Total body weight (kg)	11.1 (3.6–20.1)	12.4 (7.8-17.3)	0.007
Body surface area (m^2)	0.52 (0.2–0.81)	0.56 (0.42-0.71)	0.002
Height (cm)	80.5 (48–113)	86.8 (72-99.5)	0.0006
Albumin (U/L)	3.8 (2.8–4.7)	3.75 (3.0-4.5)	0.58
Serum creatinine (mg/dL)	0.2 (0.1–0.4)	0.2 (0.2-0.3)	0.47
Phenobarbital use, n (%)	5 (3.8)	2 (5.0)	0.67
Dexamethasone use, n (%)	20 (15.3)	6 (15.0)	>0.99

Data represented as median (range) or frequency (%) for continuous or categorical characteristics.
[a]The p-values reported for continuous variables result from unpaired t-tests with Welch's correction. The p-values reported for categorical variables result from Chi-square and Fisher's exact test using contingency tables.

TABLE S2. Selected genotypic variants evaluated in the population-based pharmacokinetic analysis for cyclophosphamide and its metabolites

			WT Patients		HE Patients		HOM Patients	
SNP	**Gene**	**w/m allele**	**n**	**%**	**n**	**%**	**n**	**%**
rs7254579	CYP2B6	T/C	62	43.7	62	43.7	18	12.7
rs4802101	CYP2B6	C/T	65	45.8	60	42.3	17	12
rs8192709	CYP2B6	C/T	128	90.1	14	9.9	0	0
rs4803419	CYP2B6	C/T	70	49.3	55	38.7	17	12
rs3745274	CYP2B6	G/T	76	53.5	57	40.1	9	6.3
rs2279343	CYP2B6	A/G	76	53.5	58	40.8	8	5.6
rs3597956	CYP2B6	T/A	141	99.3	1	0.7	0	0
rs2279345	CYP2B6	C/T	64	45.1	62	43.7	16	11.3
rs4918758	CYP2C9	T/C	65	45.8	67	47.2	10	7
rs1057910	CYP2C9	A/C	122	85.9	20	14.1	0	0
rs1799853	CYP2C9	C/T	127	89.4	15	10.6	0	0
rs1224856	CYP2C19	C/T	96	67.6	39	27.5	7	4.9
rs378581	CYP2C19	G/A	122	85.9	20	14.1	0	0
rs4244285	CYP2C19	G/A	98	69.0	40	28.2	4	2.8
rs4986893	CYP2C19	G/A	142	100	0	0	0	0
rs776746	CYP3A5	C/T	92	64.8	37	26.1	13	9.2
rs4986910	CYP3A4	A/G	139	97.9	3	2.1	0	0
rs2740574	CYP3A4	T/C	111	78.2	25	17.6	6	4.2
rs2228100	ALDH3A1	G/C	76	53.5	52	36.6	14	9.9
rs3957356	GSTA1	C/T	55	38.7	65	45.8	22	15.5
rs1695	GSTP1	A/G	59	41.5	67	47.2	16	11.3
rs1138272	GSTP1	C/T	127	89.4	14	9.9	1	0.7
rs246221	ABCC1	T/C	58	40.8	64	45.1	20	14.1
rs4148350	ABCC1	G/T	116	81.7	24	16.9	2	1.4
rs9561778	ABCC4	G/T	91	64.1	39	27.5	12	8.5
rs717620	ABCC2	C/T	95	66.9	40	28.2	7	4.9
rs2273697	ABCC2	G/A	103	72.5	36	25.4	3	2.1
rs2032582	ABCB1	C/A	50	35.2	66	46.5	26	18.3
rs1045642	ABCB1	G/A	43	30.3	70	49.3	29	20.4

w/m, wild-type/mutant allele; WT, wild-type; HE, heterozygous; HOM, homozygous mutant; ABCB1, multidrug resistance protein 1; ABCC (1-2-4), multidrug resistance-associated protein, ALDH (3A1), aldehyde deshydrogenase; CYP (2B6, 2C9, 2C19, 3A4, 3A5), cytochrome P450; GST (A1, P1), glutathione-S-transferase.

TABLE S3. Final pharmacokinetic parameter estimates

Parameter (Unit)	Symbol	Population Estimate (RSE%)	IIV (RSE%) [% of IIV Explained[a]]
Cyclophosphamide			
Total central clearance (L/h/m^2)	CL_{CTX}	2.1 (2.9)	0.208 (6.8)
Phenobarbital effect on CL_{CTX}	θ_P	0.87 (11)	[20]
CYP2B6 variant effect on CL_{CTX}	θ_{C1}	0.087 (43)	[4]
Central volume of distribution (L/m^2)	V_{CTX}	11.6 (1.6)	0.14 (9.4)
Age effect on V_{CTX}	θ_{A1}	0.11 (18)	[38]
Peripheral clearance (L/h/m^2)	Q_{CTX}	1.1 (14)	0.64 (64)
Peripheral volume (L/m^2)	Vp_{CTX}	2.8 (6.9)	0.20 (21)
Time-dependent coefficient for metabolic clearance (per hour)	β	0.029 (6.1)	0.28 (12)
CYP2B6 variant effect on β	θ_{C2}	0.23 (31)	[18]
Proportional error		0.11 (6.3)	-
4OH-CTX			
Central clearance (L/h/m^2)	CL_{4OH}	44.8 (3.2)	0.20 (7.8)
Age effect on CL_{4OH}	θ_{A2}	0.13 (20)	[11]
CYP2B6 variant effect on CL_{4OH}	θ_{C3}	–0.17 (23)	[6]
Metabolic clearance (L/h/m^2)	CLm_{4OH}	0.48 (2.8)	0.14 (16)
Time-dependent coefficient for metabolic clearance (per hour)	γ	0.034 (9.3)	0.16 (41)
Proportional error		0.21 (5.3)	-
CEPM			
Central clearance (L/h/m^2)	CL_{CEPM}	0.49 (3.5)	0.12 (18)
Age effect on CL_{CEPM}	θ_{A3}	0.18 (13)	[12]
Proportional error		0.13 (6.2)	-

RSE%, relative standard errors; IIV, interindividual variabilities reported as standard deviation.
The time-dependent metabolic clearances were expressed as followed:
Cyclophosphamide: $0.75 \cdot CL_{CTX} \cdot e^{\beta \cdot time}$ 4OH-CTX: $CLm_{4OH} \cdot e^{\gamma \cdot time}$
Final parameter–covariate associations for CL_{CTX}, β, V_{CTX}, CL_{4OH}, and CL_{CEPM} are as follows:

$$CL_{CTX} = CL_{CTX,pop} \cdot e^{\theta_P \cdot PHENO} \cdot e^{\theta_{C1} \cdot CYP2B6}$$
$$\beta = \beta_{pop} \cdot e^{\theta_{C2} \cdot CYP2B6}$$
$$V_{CTX} = V_{CTX,pop} \cdot \left(\frac{Age}{21.3}\right)^{\theta_{A1}}$$
$$CL_{4OH} = CL_{4OH,pop} \cdot e^{\theta_{C3} \cdot CYP2B6} \cdot \left(\frac{Age}{21.3}\right)^{\theta_{A2}}$$
$$CL_{CEPM} = CL_{CEPM,pop} \cdot \left(\frac{Age}{21.3}\right)^{\theta_{A3}}$$

PHENO was assigned a value of 0 if no phenobarbital or 1 if phenobarbital cotreatment was used.
CYP2B6 was assigned a value of 0 or 1 for CYP2B6 rs4802101 wild-type genotype or heterozygous/homozygous mutant genotype, respectively.
Parameter shrinkage values were below 40% to 50%, except for cyclophosphamide peripheral clearance Q_{CTX} and volume Vp_{CTX} and time-dependent coefficient γ.
The time-dependent coefficients β and γ exhibited moderate correlation (r = 0.204).
[a]The percent of initial IIV explained by the inclusion of the covariate effect on the parameter is reported. Initial IIV represents IIV values obtained in the model before the inclusion of the covariates. The IIV values reported in this table are the final IIV values, i.e., after the inclusion of the covariates on the parameters.

TABLE S4. Characteristics of the 20 validation sets used in the Bayesian analysis evaluating the prediction of 4OH-CTX exposure based on cyclophosphamide and CEPM data only

Set	Age (month)	BSA (m^2)	WT (kg)	Height (cm)	Albumin (U/L)	SCR (mg/dL)	Sex*	Phen†	Dex‡
S1	18.9 (2.1–58.5)	0.5 (0.3–0.8)	10.9 (3.8–18.3)	81 (56–108)	3.9 (3.1–4.4)	0.2 (0.1–0.4)	21/19	1/39	8/32
S2	21.3 (2.1–47.8)	0.6 (0.3–0.7)	11.8 (3.8–18.1)	84 (59–102)	3.9 (3.1–4.4)	0.2 (0.2–0.4)	16/24	1/39	4/36
S3	16.4 (1.6–55.1)	0.5 (0.2–0.8)	10.6 (3.8–20.1)	79 (48–113)	3.8 (2.8–4.5)	0.2 (0.1–0.4)	22/18	1/39	5/35
S4	22.0 (1.3–50.3)	0.5 (0.3–0.7)	10.9 (3.7–19.1)	83 (53–100)	3.8 (3.1–4.5)	0.2 (0.1–0.3)	20/20	1/39	7/33
S5	25.2 (4.2–58.5)	0.5 (0.3–0.8)	11.2 (5.4–18.3)	81 (60–113)	3.9 (2.8–4.7)	0.2 (0.1–0.4)	19/21	1/39	5/35
S6	13.7 (1.3–50.3)	0.5 (0.2–0.7)	10.4 (3.7–17.8)	77 (48–102)	3.8 (2.8–4.7)	0.2 (0.1–0.4)	19/21	1/39	7/33
S7	17.5 (1.6–36.5)	0.5 (0.2–0.7)	10.9 (3.6–19.1)	80 (48–98)	3.8 (2.8–4.5)	0.2 (0.1–0.4)	14/26	2/38	8/32
S8	18.3 (1.6–50.3)	0.5 (0.2–0.7)	11 (3.8–18.1)	80 (48–100)	3.7 (2.8–4.4)	0.2 (0.1–0.4)	20/20	2/38	7/33
S9	21.8 (0.9–55.1)	0.5 (0.2–0.8)	12 (3.6–20.1)	84 (51–113)	3.8 (2.8–4.5)	0.2 (0.1–0.4)	21/19	3/37	7/33
S10	18.5 (2.0–39.2)	0.5 (0.2–0.7)	11.4 (3.8–19.1)	84 (49–98)	3.7 (2.9–4.7)	0.2 (0.1–0.4)	17/23	0/40	4/36
S11	18.8 (1.3–39.2)	0.5 (0.2–0.7)	10.9 (3.7–17.8)	82 (48–97)	3.8 (2.8–4.4)	0.2 (0.1–0.4)	17/23	1/39	6/34
S12	18.2 (1.6–55.1)	0.5 (0.2–0.8)	11.1 (3.6–19.1)	80 (48–113)	3.9 (2.8–4.5)	0.2 (0.1–0.4)	19/21	1/39	9/31
S13	19.0 (1.3–58.5)	0.5 (0.3–0.8)	11.2 (3.7–20.1)	81 (53–111)	3.8 (2.8–4.7)	0.2 (0.1–0.4)	17/23	1/39	3/37
S14	15.6 (1.7–58.5)	0.5 (0.3–0.8)	10.7 (3.6–18.3)	79 (55–108)	3.8 (2.9–4.4)	0.2 (0.1–0.4)	21/19	1/39	8/32
S15	18.6 (1.6–36.5)	0.5 (0.2–0.7)	11.0 (3.8–16.8)	80 (48–97)	3.8 (2.8–4.4)	0.2 (0.1–0.4)	18/22	2/38	7/33
S16	18.0 (1.7–58.5)	0.5 (0.3–0.8)	10.8 (3.6–18.3)	81 (55–108)	3.8 (2.8–4.7)	0.2 (0.1–0.4)	19/21	2/38	6/34
S17	17.6 (1.6–58.5)	0.5 (0.2–0.8)	11 (3.8–19.1)	81 (48–108)	3.8 (2.8–4.7)	0.2 (0.1–0.4)	18/22	1/39	5/35
S18	19.8 (2.0–55.1)	0.6 (0.2–0.8)	12.4 (3.8–20.1)	84 (49–113)	4.0 (3.1–4.5)	0.2 (0.2–0.4)	16/24	0/40	5/35
S19	16.8 (1.3–58.5)	0.5 (0.3–0.8)	10.9 (3.7–18.3)	79 (53–108)	3.8 (3.1–4.4)	0.2 (0.1–0.4)	22/18	2/38	3/37
S20	20.9 (2.0–37.8)	0.5 (0.2–0.7)	11.0 (3.8–19.1)	82 (49–98)	3.9 (2.9–4.7)	0.2 (0.1–0.3)	15/25	1/39	4/36

Data represented as median (range) for continuous characteristics and frequency for categorical characteristics.

Abbreviations: BSA, body surface area; WT, bodyweight; SCR, serum creatinine; Phen, phenobarbital cotreatment; Dex, dexamethasone cotreatment.

*Data represents number of male/number of female patients.

†Data represents number of patients with phenobarbital cotreatment/without cotreatment.

‡Data represents number of patients with dexamethasone cotreatment/without cotreatment.

TABLE S5. Precision (MAPE%) and bias (MPE%) of Bayesian estimates for 4OH-CTX plasma exposures in each of the 20 tested validation sets

Set	No Covariate Included		Age Effect Included	
	Precision MAPE%	Bias MPE%	Precision MAPE%	Bias MPE%
S1	10.8	3.0	10.0	2.7
S2	11.3	6.1	10.7	5.8
S3	11.3	0.1	11.2	3.1
S4	11.7	2.1	11.1	2.9
S5	16.4	7.9	15.2	5.3
S6	10.8	4.7	8.3	2.5
S7	11.2	–2.9	12.6	3.8
S8	12.0	4.4	11.3	5.6
S9	14.0	7.9	13.7	8.7
S10	14.3	7.2	12.3	5.9
S11	11.3	–3.5	11.7	–1.5
S12	12.1	2.6	12.4	2.2
S13	15.0	–1.9	15.2	–4.0
S14	14.3	7.6	14.7	7.9
S15	12.4	–3.3	11.6	–2.0
S16	11.9	2.0	12.4	3.0
S17	12.1	0.6	12.6	2.6
S18	14.4	9.1	11.7	4.8
S19	11.4	0.5	12.2	0.4
S20	10.9	2.3	10.3	2.0
Average ± SD	12.5 ± 1.6	2.8 ± 3.9	12.1 ± 1.7	3.1 ± 3.1

MAPE% was calculated as follows: $MAPE(\%) = \frac{1}{N} \cdot \sum_{i=1}^{N} \left(\left| \frac{AUC_{pred} - AUC_{ref}}{AUC_{ref}} \right| \cdot 100 \right)$

MPE% was calculated as follows: $MPE(\%) = \frac{1}{N} \cdot \sum_{i=1}^{N} \left(\frac{AUC_{pred} - AUC_{ref}}{AUC_{ref}} \cdot 100 \right)$

AUC_{pred} represents 4OH-CTX exposure predicted by the Bayesian model on the basis of cyclophosphamide and CEPM data only. AUC_{ref} represents 4OH-CTX exposure estimated by the full model with all data. MAPE% and MPE% < 15% were considered as clinically acceptable.(3)

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