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ORIGINAL ARTICLE

# Cytochrome P450 2J2, a new key enzyme in cyclophosphamide bioactivation and a potential biomarker for hematological malignancies

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The role of cytochrome P450 2J2 (CYP2J2) in cyclophosphamide (Cy) bioactivation was investigated in patients, cells and microsomes. Gene expression analysis showed that *CYP2J2* mRNA expression was significantly ($P < 0.01$) higher in 20 patients with hematological malignancies compared with healthy controls. *CYP2J2* expression showed significant upregulation ($P < 0.05$) during Cy treatment before stem cell transplantation. Cy bioactivation was significantly correlated to *CYP2J2* expression. Studies in HL-60 cells expressing CYP2J2 showed reduced cell viability when incubated with Cy (half maximal inhibitory concentration = 3.6 mM). Inhibition of CYP2J2 using telmisartan reduced Cy bioactivation by 50% and improved cell survival. Cy incubated with recombinant CYP2J2 microsomes has resulted in apparent $K_m$ and $V_{max}$ values of 3.7–6.6 mM and 2.9–10.3 pmol/(min·pmol) CYP, respectively. This is the first study demonstrating that CYP2J2 is equally important to CYP2B6 in Cy metabolism. The heart, intestine and urinary bladder express high levels of CYP2J2; local Cy bioactivation may explain Cy-treatment-related toxicities in these organs.

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## INTRODUCTION

Cytochrome P450 2J2 (CYP2J2) is a recently discovered CYP involved in the metabolism of xenobiotics. It is encoded by the *CYP2J2* gene, which has been mapped to the short arm of chromosome 1 in humans and chromosome 4 in mice.[1]

CYP2J2 is mainly found in the intestine and cardiovascular system, and is involved in the metabolism of xenobiotics.[2–4] CYP2J2 has been reported to metabolize several drugs, particularly in extrahepatic tissues. High CYP2J2 activity in the intestine could explain the first-pass metabolism of several drugs.[5–7] Moreover, Matsumoto *et al.*[7] demonstrated that CYP2J2 is dominant in the small intestine and is involved in presystemic elimination of astemizole in human and rabbit.

In the human heart, CYP2J2 is responsible for the epoxidation of endogenous arachidonic acid to four regioisomeric epoxyeicosatrienoic acids released in response to ischemia.[8] CYP2J2 overexpression in transgenic mice was shown to be protective against global cerebral ischemia,[9] and these mice showed less extensive infarcts and complete recovery after ischemia.[10–12]

Chen *et al.*[13] reported that CYP2J2 is highly expressed in human and mouse hematological cell lines (K562, HL-60, Raji, MOLT-4, SP2/0, Jurkat and EL4 cells), as well as in peripheral blood and bone marrow cells of leukemia patients. High expression of CYP2J2 was associated with accelerated proliferation and attenuated apoptosis. CYP2J2 expression in HL-60 cells may explain the cytotoxic effect of Cy observed in these cells despite the lack of CYP2B6 (Xie *et al.*[14]). CYP2J2 is also overexpressed in ovarian and lung cancer,[15,16] and its inhibition was shown to suppress the proliferation of cancer cells *in vitro* and *in vivo*.[17]

Hematopoietic stem cell transplantation is a curative treatment approach for malignancies such as leukemia and lymphomas, as well as non-malignancies such as metabolic disorders and aplastic anemia. Before hematopoietic stem cell transplantation, chemotherapy with or without radiation is used as a conditioning regimen to eliminate malignant cells, provide space for donor cells and avoid graft rejection by suppressing the immune system.[18]

Cyclophosphamide is a prodrug that is metabolized mainly in the liver by cytochrome P450 to 4-hydroxy-cyclophosphamide (4-OH-Cy), which is the main active metabolite (90% of the total Cy).[19] Subsequently, 4-OH-Cy is degraded to phosphoramide mustard and acrolein. The latter is responsible for the urotoxicity. An alternative pathway is *N*-dechloroethylation in which Cy is metabolized to an inactive metabolite, *N*-dechloroethyl-Cy and the neurotoxic metabolite chloroacetaldehyde.[20–22] Phosphoramide alkylates the guanine base of DNA at the *N*7 position of the imidazole ring and hence triggers apoptosis due to the formation of guanine–adenine intrastrand cross-links.[23]

Cy is converted to 4-OH-Cy in the liver, predominantly by CYP2B6 (Ekhart *et al.*,[24] Raccor *et al.*[25] and Cho *et al.*[26]) and to a lesser extent by CYP3A4, CYP3A5, CYP2C9 and CYP2C19 (Afsar *et al.*[27] and Fernandes *et al.*[28]). High inter- and intra-individual variability in Cy kinetics has been reported, which affects treatment

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**Table 1.** Clinical characteristics among patients undergoing HSCT

| *Patient* | *Age (years)* | *Diagnosis* | *Conditioning regimen* | *CD 34 dose (kg)* | *Disease status at HSCT* | *Outcome* | *Cause of death* |
|---|---|---|---|---|---|---|---|
| P 1 | 51 | AML | Cy+TBI+ATG | $10.6\times10^6$ | Refractory | Alive 7.5 years | — |
| P 2 | 26 | Pre-B ALL | Cy+TBI+ATG | $13.5\times10^6$ | CR2 | [a]35 Months | Relapse |
| P 3 | 57 | B-CLL | Cy+fTBI (6 Gy)+ Alemtuzumab | $14.7\times10^6$ | Transformed | [a]10 Months | Relapse |
| P 4 | 31 | AML | Cy+TBI+ATG | $2\times10^6$ | CR2 | [a]6 Months | Pneumonia |
| P 5 | 41 | T-cell lymphoma | Cy+TBI+ATG | $9.3\times10^6$ | Relapse | [a]51 Days | Invasive fungal infection |
| P 6 | 25 | Pre-B ALL | Cy+TBI+ATG | $7.3\times10^6$ | CR2 | Alive 7.5 years | — |
| P 7 | 38 | T-cell lymphoma | Cy+TBI | $2.9\times10^8$ | PR | [a]19 Months | Relapse |
| P 8 | 10 | T-ALL | Cy+TBI+ATG | $6.48\times10^8$ | CR1 | [a]12 Months | Relapse |
| P 9 | 26 | T-ALL | Cy+TBI+ATG | $0.5\times10^5$ $0.2\times10^5$ | CR2 | [a]11 Months | Relapse |
| P 10 | 14 | T-ALL | Cy+TBI+ATG | $19.9\times10^6$ | CR2 | Alive 7.2 years | — |
| P 11 | 19 | ALL | Cy+TBI+ATG | $13.5\times10^6$ | CR3 | [a]9 Months | Relapse & pneumonia |
| P 12 | 49 | NHL | Flu+Cy+TBI | $4.0\times10^6$ | PR | [a]2 Months | Relapse |
| P 13 | 11 | Kidney Cancer | Flu+Cy+ATG | $12.9\times10^6$ | N/A | Alive 9 years | — |
| P 14 | 51 | CLL | Flu+Cy+TBI+Campath | $9.4\times10^6$ | PR | [a]2 Months | Septic shock |
| P 15 | 57 | NHL | Flu+Cy+TBI+ATG | $6.1\times10^6$ | CR4 | Alive 8.5 years | — |
| P 16 | 64 | NHL | Flu+Cy+TBI+Campath | $15.1\times10^6$ | CR2 | [a]6 Months | GI-bleeding |
| P 17 | 62 | NHL | Flu+Cy+TBI | $8.3\times10^6$ | CR2 | Alive 8 years | — |
| P 18 | 57 | ALL | Cy+TBI+ATG | $7.8\times10^6$ | CR1 | Alive 8 years | — |
| P 19 | 9 | CLL | Flu+Cy+Campath | $9.3\times10^6$ | Relapse | [a]6 Months | Relapse and pneumonia |
| P 20 | 9 | CLL | Flu+Cy+TBI | $11.0\times10^6$ | PR | [a]8 Months | Relapse, pneumonia and septicemia |

Abbreviations: ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; ATG, antithymocyte globuline; B, B lymphocyte; CLL, chronic lymphoblastic leukemia; CD 34, bone marrow-derived stem cells; CR, complete remission; Cy, cyclophosphamide; Flu, Fludarabine; HSCT, hematopoietic stem cell transplantation; NHL, non-Hodgkins lymphoma; P, patient; PR, partial remission; T, T lymphocyte; TBI, total body irradiation. [a]Survival time.

efficacy and toxicity.[26,29] This variability has been attributed predominantly to polymorphisms in the *CYP2B6* gene, such as CYP2B6*4, CYP2B6*6 and CYP2B6*9 (Ribaudo *et al.*,[30] Tsuchiya *et al.*[31] and Xie *et al.*.[32] However, recent studies have concluded that CYPs polymorphisms does not have a significant role in the response variation to Cy.[25,27,28]

In the present study, we investigated the expression of CYP2J2 as well as several other CYPs in 20 patients undergoing stem cell transplantation and treated with Cy compared with 12 healthy volunteers. We also investigated the role of CYP2J2 in Cy bioactivation *in vitro* using leukemic cell lines as well as complementary DNA-expressed CYP2J2 microsomes. These results were correlated to the kinetics of Cy and its active metabolite.

## METHODS

### Chemicals

QuickPrep Total RNA Extraction Kit (GE Life Sciences, Uppsala, Sweden), TaqMan Reverse Transcriptase complementary DNA Kit (Applied Biosystems, Roche, NJ, USA), and NimbleGen microarrays (Roche Diagnostics Scandinavia, Bromma, Sweden) were sourced for experiments. TaqMan-genotyping PCR primers for CYP2J2 single-nucleotide polymorphisms (rs72547599, rs1056595 and rs66515830; catalog number 4362691), genotyping master mix (catalog number 4371355), CYP2J2 (assay ID Hs00951113_m1) and the housekeeping gene *GAPDH* (assay ID; Hs02758991_g1) were purchased from Applied Biosystems (Stockholm, Sweden).

Cy, telmisartan, dansylhydrazine and dimethyl sulfoxide were purchased from Sigma-Aldrich (Stockholm, Sweden), while resazurin was sourced from R&D Systems. (Minneapolis, MN, USA). Acetonitrile (ACN) was purchased from Merck (Darmstadt, Germany). All other chemicals were purchased from Merck and were of analytical grade. Stock solutions were freshly prepared in $dH_2O$ for Cy and in dimethyl sulfoxide for telmisartan, and stored at − 20 °C.

### Equipment

Cells were incubated in 96-well BD BioCoat black culture plates (Stockholm, Sweden) and fluorescence signals were detected with a FLUOstar Optima system (BMG LABTECH, Ortenberg, Germany).

PAX and heparinized tubes were purchased from BD. PCR experiments were performed in 384-well plates, using a 7500 Fast Real-Time PCR System (Applied Biosystems Life Technologies, Stockholm, Sweden).

High-performance liquid chromatography (HPLC) consisted of an LKB-2150 pump, Gilson-234 auto-injector with a 100-µl sample loop and Extend-C18 column (150 × 4.6 mm (3.5 µm)) with a C18 guard column (Agilent, Santa Clara, CA, USA). For Cy measurements, the mobile phase consisted of ACN/0.05 M $KH_2PO_4$ buffer, pH 2.8 (24:76 v/v), and the flow rate was 1 ml $min^{-1}$. Cy was detected by a Milton Roy UV Spectro-Monitor 310 detector (Pont-Saint-Pierre, France).

For 4-OH-Cy measurements, the mobile phase consisted of ACN/phosphate buffer, pH 3.5 (1:2 v/v), and a flow rate of 1.6 ml $min^{-1}$. A Shimadzu RF-10XL fluorescence detector (Tokyo, Japan) was used to detect 4-OH-Cy. Integration was performed with a CSW32 (version 1.3) chromatographic station (DataApex, Prague, Czech Republic).

### Cells

HL-60 cells were obtained from Leibniz-Institute DSMZ–German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) 2007. The cell line has been tested and authenticated at least two times during this period. A confirmation was carried out using cytogenetic analysis at our cytogenetic laboratory. A very recent analysis through the provider (Leibniz-Institute) has confirmed the original character of the cell line. Cells were also tested negative for mycoplasma.

Cells were cultured in Roswell Park Memorial Institute 1640 medium, supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 U $ml^{-1}$) and streptomycin (100 µg $ml^{-1}$).

### Microsomes

Microsomes from *Escherichia coli* coexpressing human *CYP2J2* and CYP reductase were obtained from Cypex (Dundee, UK). The specific CYP

content was 127 pmol per mg protein and the reductase activity was 429 nmol/(min·mg) protein.

### Patients

Twenty patients undergoing hematopoietic stem cell transplantation at the Center for Allogeneic Stem Cell Transplantation, Karolinska University Hospital-Huddinge, Sweden, were included in the study. The study was approved by the ethical committee of Karolinska Institutet (approval number 616/03). All patients were in molecular remission before hematopoietic stem cell transplantation and their characteristics are listed in Table 1. Twelve healthy volunteers were included as a control group. The control group aged between 18 and 55 years old, six males and six females, and the samples were obtained during the sampling period of the patients. Blood samples were taken from patients and healthy volunteers to obtain mononuclear cells.

## METHODS

### Patient treatment and blood sampling

Eleven patients were conditioned using Cy (60 mg $kg^{-1}$·per day) 1 h intravenous infusion for 2 days, followed by total body irradiation. Nine patients were conditioned using fludarabine (Flu) (30 mg $m^{-2}$·per day) by intravenous infusion for 5 days, followed by Cy (60 mg $kg^{-1}$·per day) 1 h infusion for 2 days and/or total body irradiation. Blood samples for RNA extraction were collected before Flu, before Cy, at 6, 24 and 30 h after termination of Cy infusion. For the determination of Cy and 4-OH-Cy kinetics, blood samples were collected at baseline and at 0.5, 1, 2, 4, 6 and 8 h after termination of the first infusion on day 1, and at baseline and 6 h after termination of the second infusion on day 2. Blood (2.5 ml) was collected in pre-chilled heparinized tubes and immediately centrifuged at 3000 *g* for 3 min at 4 °C. Plasma (0.5 ml) was transferred to new tubes containing 0.5 ml pre-chilled ACN, vortexed for 30 s and centrifuged at 3000 *g* for 3 min. The supernatant and remaining plasma were stored at − 80 °C until required for analysis.

### RNA extraction and complementary DNA preparation

RNA was extracted from mononuclear cells, according to the manufacturer's instructions, and complementary DNA was obtained by reverse transcription.

### Gene array and genotyping

Purified mRNA was analyzed using global gene expression (NimbleGen microarrays, Roche Diagnostics Scandinavia) and the data analyzed by Genespring GX (Agilent). Expression data of the probes and genes were normalized using quantile normalization and the Robust Multichip Average algorithm, respectively. Gene expression was determined by analysis of variance to be significantly differentially expressed if the selection threshold of a false discovery rate was $< 5\%$ and the fold change in SAM output result was $> 1.3$. The complete data set can be accessed in the Gene Expression Omnibus database with accession number GSE51907.[33]

### PCR analysis

Samples were scanned using 3 TaqMan genotyping PCR primers for *CYP2J2* single-nucleotide polymorphisms (rs72547599, rs1056595 and rs66515830). Samples (10 ng) were amplified, according to the manufacturer's instructions, in 384-well plates (10 µl total volume). Genotypes were assigned using the manual calling option in the allelic discrimination application, ABI PRISM 7500 SDS software version 1.3.1 (Applied Biosystems, Stockholm, Sweden).

Real-time PCR TaqMan gene expression assay was performed by means of the carboxyfluorescein dye-labeling system, according to the manufacturer's instructions. Assays were performed for *CYP2J2* against the housekeeping gene *GAPDH*. Controls were run using the healthy donors.

### Cell culture

HL-60 cells in the exponentially growing phase (passage 6) were incubated in a humidified incubator at 37 °C with 5% $CO_2$/95% $O_2$. Cells were incubated at a density of 10 000 cells per well with Cy at concentrations of 0.2–20 mM for 6–96 h or the CYP2J2 inhibitor[34] telmisartan at concentrations of 2.5–40 µM for 48 h.

To study the effect of telmisartan on Cy-induced toxicity, HL-60 cells were pre-incubated with telmisartan at 2.5, 5 or 10 µM concentrations for 2 h. Cy at a final concentration of 9 mM was then added and cells were incubated for 48 h. Positive controls for drug toxicity contained cells incubated with 9 mM Cy or 10 µM telmisartan, while negative controls were incubated in drug-free medium.

At appropriate time points, cell samples were removed to assess cell viability using resazurin assay or added to pre-cooled ACN for 4-OH-Cy analysis. Resazurin at a 10% final concentration was added to cell suspensions. The cells were then incubated for an additional 2 h at 37 °C and fluorescence signals were detected at 590 nm following excitation at a wavelength of 544 nm.[35] Experiments were performed in triplicate.

### Microsomal assay

Microsomes were incubated for 1 h at 37 °C with increasing concentrations of Cy (0-50 mM) in 100 µl of 50 mM $KH_2PO_4$ buffer, pH 7.4, containing 1 mM NADPH. The reaction was terminated by adding an equal volume of ice-cold ACN. The mixture was vortexed and centrifuged at 10 000 *g* for 10 min at 4 °C, to remove precipitated protein. 4-OH-Cy concentration was measured using HPLC equipped with a fluorescence detector.[36] Incubations were performed in triplicate and negative controls were run in parallel.

### Plasma Cy measurement

Plasma (1 ml) was extracted using diethyl acetate ester after the addition of ifosfamide as internal standard. The organic layer was evaporated to dryness. Next, 30 µl aliquots of residue dissolved in mobile phase were injected into the HPLC with the UV detector operated at a wavelength of 195 nm. The standard curve was linear within the range of 5–1000 µM.[37]

### 4-OH-Cy measurement

Dansylhydrazine and hydrochloric acid were added to samples, to enable the detection of 4-OH-Cy using HPLC equipped with a fluorescence detector.[36,38] The mixture was vortexed and incubated at 50 °C for 5 min. Phosphate buffer (pH 3.5) was added to 100 µl samples and 30 µl aliquots were injected into the HPLC. Detection was performed at excitation and emission wavelengths of 350 and 550 nm, respectively, and the retention time was 16.3 min. The standard curve was linear in the range of 50-600 µM.

### Data analysis

Kinetics estimations were performed using Microsoft Excel (Microsoft Sweden, Kista, Sweden) and WinNonLin software (Certara, Princeton, NJ, USA; standard edition, version 2.0). Graphs were plotted using GraphPad Prism (version 4.0, GraphPad Software, La Jolla, CA, USA) and SigmaPlot (version 12.5, Systat Software, Erkrath, Germany).

## RESULTS

### CYP2J2 gene expression and genotype in patients

Mononuclear cells from peripheral blood of patients undergoing conditioning were investigated by gene array analysis. Patients were genotyped with regard to several CYPs, including CYP1A1, CYP1A2, CYP1B1, CYP2A6, CYP2A7, CYP2A13, CYP2B6, CYP2C8, CYP2C9, CYP2C18, CYP2C19, CYP2D6, CYP2E1, CYP2F1, CYP2J2, CYP3A4, CYP3A5 and CYP3A7. Significant upregulation of mRNA expression was only detected for *CYP2J2*. The heat map for CYP2J2 and other genes with similar expression pattern over 2 days of Cy treatment is presented in Figure 1.

The heat map shows gene expression before conditioning (S 1), 6 h after Cy administration (S2) on day 1, 24 h (S3; before conditioning on day 2) and at 30 h (S4; 6 h after the second Cy dose). The majority of genes in each cluster had the same pattern of up- and downregulation as observed for *CYP2J2*.

*CYP2J2* gene expression levels were confirmed with quantitative reverse-transcription PCR normalized to *GAPDH*. Quantitative reverse-transcription PCR experiments demonstrated that *CYP2J2* gene expression was elevated ($P < 0.01$, *t*-test) in pretreatment samples from patients with hematological malignancies compared with healthy controls (Figure 2).

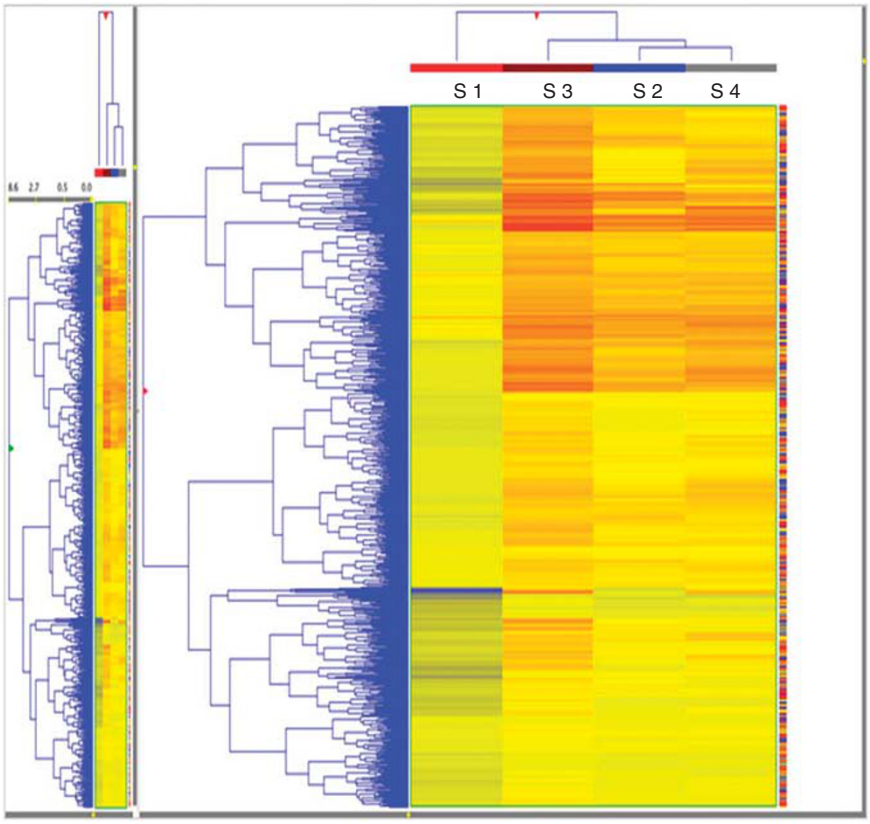


**Figure 1.** Heat map for gene expression including *CYP2J2* at baseline and during Cy treatment. S1, baseline; S2, 6 h after Cy administration on day 1; S3, 24 h; S4, 6 h after Cy administration on day 2. The majority of genes in each cluster had the same pattern of up- and downregulation as observed for *CYP2J2*.

Expression of the *CYP2J2* gene was significantly upregulated at 6 h after the first Cy infusion ($P < 0.05$, *t*-test). At S4, CYP2J2 expression was significantly reduced ($P < 0.05$, *t*-test) compared with measurements made at the S2 time point, but were elevated compared with pretreatment (S1) measurements (Figure 3). High inter-individual variation in gene expression after Cy infusion was observed.

Genotyping patients revealed that only one patient was carrier for CYP2J2 single-nucleotide polymorphism 'rs1056596' (A/T). This patient (P 11) had a lower expression level of *CYP2J2* compared with other patients (Figure 4).

Pharmacokinetic properties of Cy and 4-OH-Cy were calculated (Table 2). Cy and 4-OH-Cy kinetics showed a significantly higher ($P < 0.01$, *t*-test) 4-OH-Cy/Cy ratio at 6 h after the second infusion (S 4) compared with measurements recorded at 6 h after the first infusion (S 2) as can be seen in Figure 5. Furthermore, the concentration ratio 4-OH-Cy/Cy concentrations at 6 h after the first dose and at 0 and 6 h after the second dose was significantly ($P < 0.001$, $r^2 = 0.408$) correlated with *CYP2J2* expression as shown in Figure 6.

Clinical data, including diagnosis, type of donor, stem-cell dose, relapse, remission, mortality and complications were collected (Table 1). No correlation between clinical data and CYP2J2 results was observed.

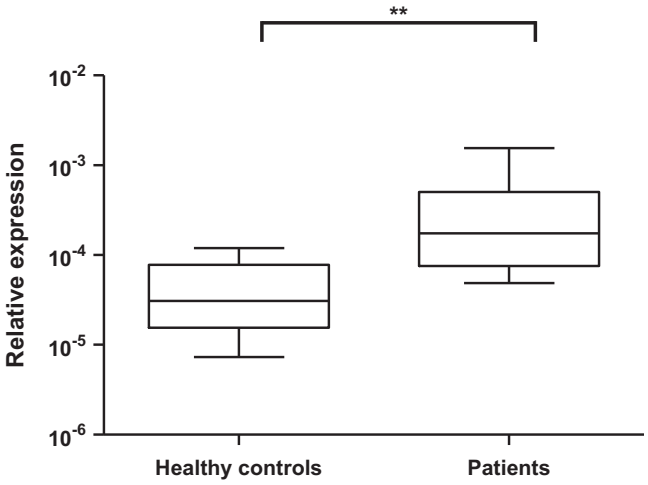


**Figure 2.** Expression of *CYP2J2* determined by quantitative reverse transcription PCR (qRT-PCR) in healthy controls and hematological cancer patients undergoing hematopoietic stem cell transplantation (HSCT). *CYP2J2* gene expression measured by qRT-PCR and normalized against *GAPDH* showed significantly ($P < 0.05$) higher levels in patients with hematological malignancies than in controls (healthy subjects) even before the start of conditioning.

### Role of CYP2J2 in Cy-induced HL-60 cytotoxicity

Cy reduced cell viability of HL-60 cells in a concentration- and time-dependent manner. The estimated half maximal inhibitory concentration was 3.6 mM (Figure 7) and a concentration of 9 mM was selected for further studies. Telmisartan at a concentration less than or equal to 10 μM did not affect viability of HL-60 cells. However, higher concentrations of the CYP2J2 inhibitor reduced cell viability in a concentration-dependent manner (Figure 8a). Telmisartan at a concentration of 10 μM reduced the formation of 4-OH-Cy by ~50%. Moreover, pre-incubation with telmisartan (10 μM) improved survival of cells treated with 9 mM Cy by 10% (Figure 8b). It is likely to be that the CYP2J2 inhibitor telmisartan

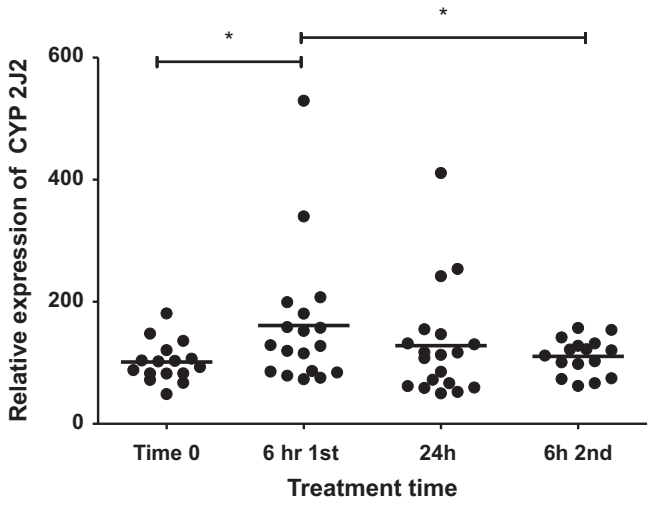


**Figure 3.** Expression of cytochrome P450 2J2 (CYP2J2) mRNA at baseline and during Cy conditioning. The gene expression of *CYP2J2* was significantly ($P < 0.05$, *t*-test) upregulated 6 h after the first Cy infusion. At the last sample (6 h after the second dose of Cy), CYP2J2 was significantly ($P < 0.05$) downregulated compared with the 24 h sample. The high inter-individual variation in gene expression during Cy treatment might be due to different inducibility of the polymorphic forms of *CYP2J2*.

reduces Cy bioactivation and hence increases survival of HL-60 cells.

Cy metabolism by recombinant CYP2J2

Incubation of Cy with microsomes containing recombinant human CYP2J2 showed CYP2J2 to be involved in Cy bioactivation. Fitting the data from two independent experiments performed on two different batches of microsomes to Michaelis-Menten kinetics (Figure 9a) gave an apparent $K_m$ within the range of 3.7–6.6 mM and an apparent $V_{max}$ 2.9–10.3 pmol/(min·pmol) CYP resulting in $V_{max}/K_m$ of 0.5-2.3 µl/(min·pmol) CYP. Comparing different enzyme kinetic models revealed that the curve of best fit was obtained with a substrate inhibition model (Figure 9b).

## DISCUSSION

The present investigation was designed to study CYP2J2 extrahepatic expression in patients with hematological malignancies and to examine its role in Cy metabolism. PCR analysis revealed that the *CYP2J2* gene is highly expressed in such patients before undergoing Cy conditioning as compared with healthy individuals. Similar to leukemic cell lines,[13] mononuclear cells from patients with hematological malignancies also express high levels of CYP2J2.

Gene array analysis demonstrated that the expression of *CYP2J2* was further upregulated in patients on treatment with Cy, indicating that CYP2J2 is induced by Cy. However, *CYP2J2* upregulation showed high inter-individual variation after the first Cy infusion. Genotyping patients for CYP2J2 single-nucleotide polymorphism 'rs1056596' (A/T) revealed lower *CYP2J2* expression detected in the patient carrier with this mutation. Interestingly, *CYP2J2* expression showed significant positive correlation with the concentration ratio 4-OH-Cy/Cy (as measure for the bioactivation of Cy), which strongly supports the fact that CYP2J2 is an important CYP in human Cy bioactivation.

Several studies have shown that CYP2J2 is involved in extrahepatic drug metabolism[4,5,7] and is highly expressed in hematological and tumor cells.[13,15–17,34,36,38] Incubation of HL-60 cells with the CYP2J2 inhibitor, telmisartan, reduced the viability in concentration- and time-dependent manners. CYP2J2 expression

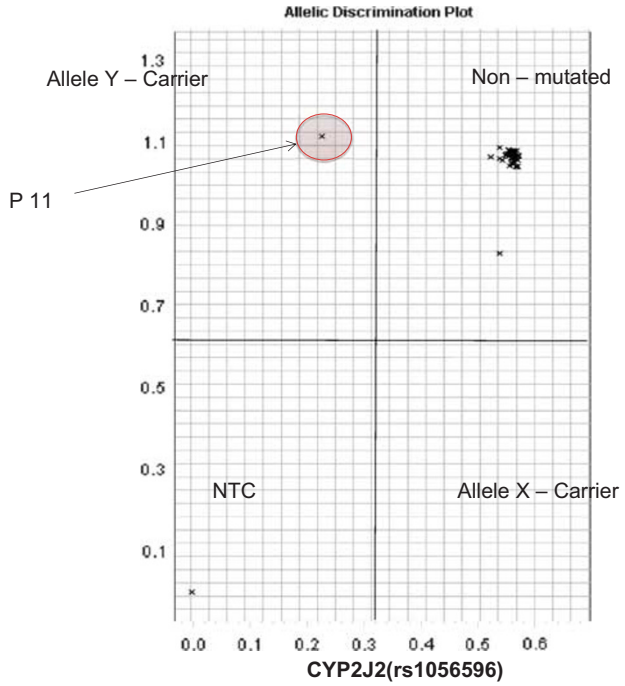


**Figure 4.** Genotyping for cytochrome P450 2J2 (CYP2J2) polymorphism (rs1056596). Genotyping of the patients showed that only one patient was carrier for CYP2J2 'rs1056596' (A/T) mutation. This patient (P 11) had a lower expression level of CYP2J2 compared with other patients. NTC, non-template control.

is a protective mechanism for cancer cell survival and its inhibition has been shown to suppress the proliferation of cancer.[17]

We have previously reported that Cy induced concentration- and time-dependent cytotoxicity in HL-60 cells despite deficiency of CYP2B6 or other enzymes involved in Cy bioactivation.[14] HL-60 cells predominantly express CYP1A1 and CYP1B1;[14,39] however, neither is reported to be involved in Cy bioactivation. In the present study, reduction of 4-OH-Cy formation in HL-60 cells by the CYP2J2 inhibitor, telmisartan, and concomitant increase in cell viability, strongly support the role of CYP2J2 in Cy bioactivation.[13]

Expression levels of several CYPs were assessed over a 2-day Cy-conditioning period. No correlation was found between Cy pharmacokinetic parameters and other CYP expression, regardless of genotype. Several recent studies have reported that Cy clearance or disease-free survival is not affected by a patient's genotype; thus, genotype is not considered a predictor for dose adjustment.[28,40] Raccor *et al.*[25] reported that CYP2B6 polymorphism is not related to 4-OH-Cy formation and Cy kinetics *in vivo* and *in vitro*. In other studies, CYP2B6 polymorphism was reported to have a major role in the high variation of Cy kinetics *in vivo* and *in vitro*. Alleles such as CYP2B6*4 and CYP2B6*9 demonstrated lower activity compared with CYP2B6*1 (Xie *et al.*[29,32]).

As 4-OH-Cy was formed following incubation of Cy with recombinant CYP2J2, this finding provides confirmation that CYP2J2 is capable of bioactivating Cy. The apparent $K_m$ and $V_{max}$ were within the range 3.7–6.6 mM and 2.9–10.3 pmol/(min·pmol) CYP, respectively. This resulted in a $V_{max}/K_m$ of 0.5–2.3 µl/(min·pmol) CYP. A similar $K_m$ value was obtained from wild-type CYP2B6 (4.9 mM);[41] moreover, $V_{max}/K_m$ of CYP2J2 was even higher than some CYP2B6 polymorphisms,[41] suggesting that CYP2J2 may be a predominant enzyme responsible for Cy bioactivation in certain patients.

In the present study, we observed high inter-individual variability in kinetics and elevated levels of 4-OH-Cy/Cy 6 h after the second infusion compared with 6 h after the first infusion, thus

**Table 2.** Pharmacokinetics of Cy and 4-OH-Cy among patients undergoing HSCT

| *Patient* | *Cy* | | | *4-OH-Cy* | | | *4-OH-Cy/Cy AUC ratio* |
|---|---|---|---|---|---|---|---|
| | *AUC (μg.h ml$^{-1}$)* | *$C_{max}$ (μg ml$^{-1}$)* | *Half life (h)* | *AUC (ng.h ml$^{-1}$)* | *$C_{max}$ (ng ml$^{-1}$)* | *Half life (h)* | |
| **P 1** | 867.19 | 152.66 | 6.5 | 47501 | 1600.80 | 20.15 | 0.055 |
| **P 2** | 932.05 | 88.73 | 8.6 | 11531 | 1108.84 | 5.99 | 0.012 |
| **P 3** | 892.68 | 154.54 | 5.9 | 16675 | 1285.72 | 8.69 | 0.019 |
| **P 4** | 938.97 | 143.63 | 5.9 | 30432 | 1488.00 | 13.90 | 0.032 |
| **P 5** | 754.65 | 93.41 | 6.9 | 12165 | 977.39 | 7.39 | 0.016 |
| **P 6** | 1120.32 | 95.58 | 10.7 | 17870 | 1329.62 | 5.63 | 0.016 |
| **P 7** | 1310.99 | 118.39 | 8.7 | 13226 | 1216.67 | 4.24 | 0.010 |
| **P 8** | 808.97 | 113.08 | 5.5 | 8995 | 1330.53 | 4.69 | 0.011 |
| **P 9** | 654.13 | 76.81 | 6.5 | 6659 | 764.83 | 5.74 | 0.010 |
| **P 10** | 644.80 | 73.93 | 6.0 | 12870 | 1818.47 | 4.52 | 0.020 |
| **P 11** | 1020.95 | 105.76 | 8.6 | 17243 | 3252.19 | 3.68 | 0.017 |
| **P 12** | 712.278 | 40.87 | 11.7 | 22703 | 2052.46 | 2.76 | 0.032 |
| **P 13** | 736.275 | 75.73 | 6.3 | 11211 | 2201.24 | 3.29 | 0.015 |
| **P 14** | 416.505 | 53.39 | 5.0 | 24170 | 1161.98 | 14.06 | 0.058 |
| **P 15** | 402.327 | 42.3 | 6.3 | 23143 | 1854.52 | 7.65 | 0.058 |
| **P 16** | 467.306 | 36.35 | 8.5 | 5343 | 721.69 | 1.82 | 0.011 |
| **P 17** | 333.700 | 34.48 | 6.3 | 4658 | 714.74 | 4.23 | 0.014 |
| **P 18** | 603.092 | 60.47 | 6.6 | 14996 | 1325.54 | 7.52 | 0.025 |
| **P 19** | 640.961 | 90.73 | 4.5 | 11847 | 1764.20 | 4.13 | 0.018 |
| **P 20** | 554.941 | 78.65 | 8.45 | 14710 | 1138.02 | 7.83 | 0.027 |

Abbreviations: 4-OH-Cy, 4-hydroxycyclophosphamide; AUC, area under the curve; $C_{max}$, peak plasma concentration; Cy, cyclophosphamide; P, patient. Blood samples were withdrawn at baseline and at 0.5, 1, 2, 4, 6, 8 and 18 h after the first infusion of Cy. The results demonstrate high inter-individual variation in the kinetics of Cy and its metabolite.

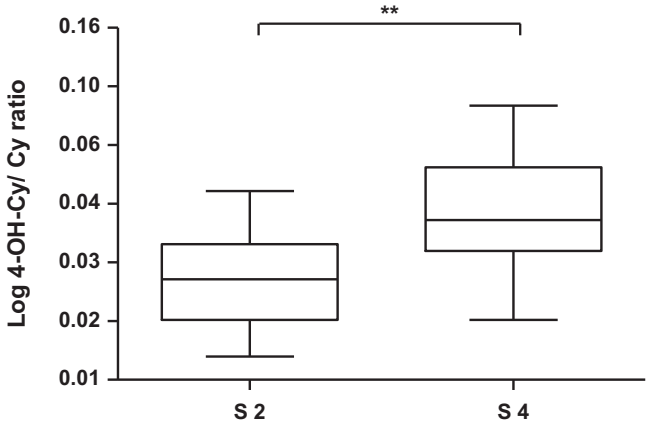


**Figure 5.** Log 4-hydroxycyclophosphamide (4-OH-Cy)/cyclophosphamide (Cy) concentration ratio at two time points. S2, 6 h after Cy administration on day 1; S4, 6 h after Cy administration on day 2. The figure shows significantly ($P < 0.05$) higher levels of 4-OH-Cy/Cy ratio (as measure for the bioactivation of Cy) after the second infusion indicating auto-induction of CYP2B6-dependent Cy metabolism.

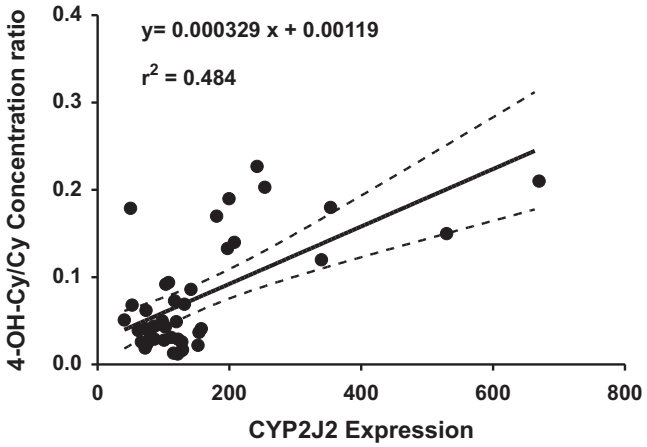


**Figure 6.** The correlation between *CYP2J2* expression and 4-hydroxycyclophosphamide (4-OH-Cy)/cyclophosphamide (Cy) concentration ratio. The concentration ratio 4-OH-Cy/Cy concentrations (as measure for the bioactivation of Cy) at 6 h after the first dose and at 0 and 6 h after the second dose was significantly ($P < 0.001$, $r^2 = 0.4084$) correlated with *CYP2J2* expression.

confirming auto-induction of CYP2B6-dependent Cy metabolism as previously reported.[42]

To the best of our knowledge, this is the first study that reports the involvement of CYP2J2 in Cy metabolism. Moreover, *CYP2J2* was upregulated during *in vivo* Cy treatment. CYP2J2 inhibition may be used as a marker for target treatment of hematological malignancies. Recently, several studies have reported that CYP2J2 is an important enzyme in the extrahepatic metabolism of drugs and is highly expressed in several tissues, including the urinary bladder, intestine and heart.[5,6] CYP2J2 has also been reported to have an important role in intestinal first-pass metabolism of antihistamines.[7] One study that investigated the effect of Cy on the intestinal barrier function reported modification of intestinal permeability and diarrhea during and after Cy conditioning,[43] which can be due to local Cy bioactivation in the intestine by CYP2J2. Involvement of CYP2J2 in Cy bioactivation may also explain its acute cardiotoxic effect reported by Gharib *et al.*,[44] as CYP2J2 is expressed in the human heart and is responsible for the epoxidation of endogenous arachidonic acid.

In conclusion, the present results demonstrate that *CYP2J2* gene is highly expressed in patients with hematological malignancies and its inhibition in HL-60 cells by the CYP2J2-selective inhibitor, telmisartan, has affected the cell viability. Moreover, Cy is metabolized in HL-60 cells despite lack of CYP2B6 expression. Cy

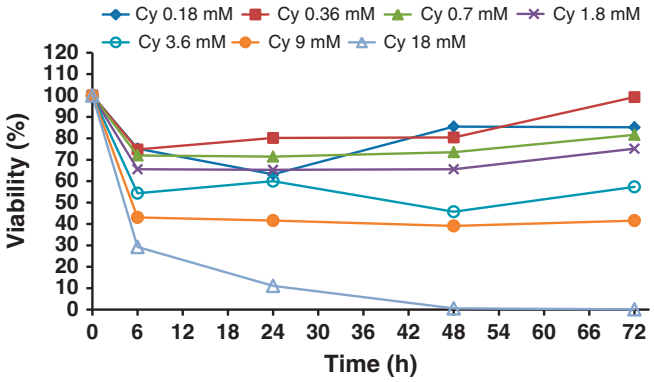


**Figure 7.** Cytotoxicity of cyclophosphamide (Cy) in HL-60 cells. Cy reduced cell viability of HL-60 cells assessed by resazurin assay in a concentration- and time-dependent manner. The estimated half maximal inhibitory concentration ($IC_{50}$) was 3.6 mM. A concentration of 9 mM was selected for further Cy bioactivation experiments.

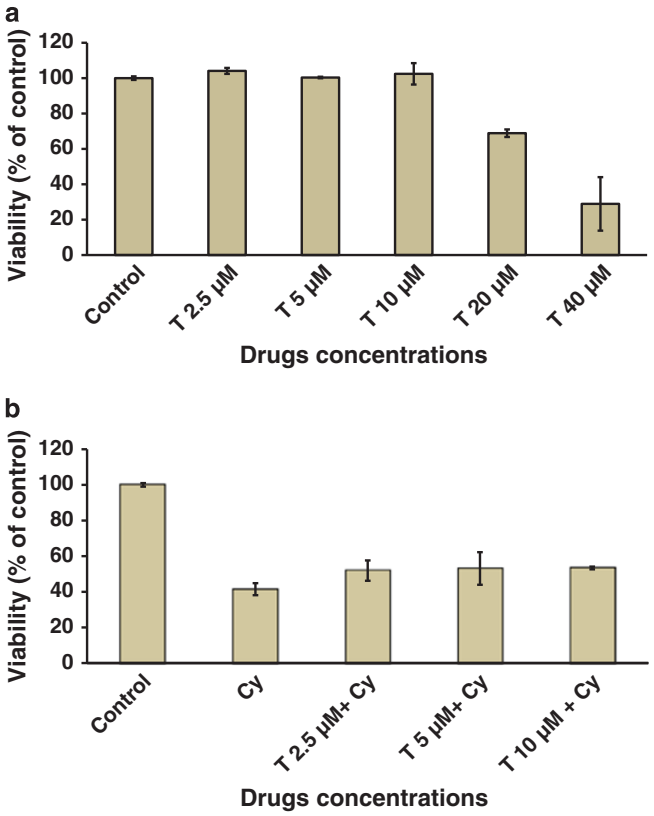


**Figure 8.** Effect of telmisartan (T) pre-incubation on cyclophosphamide (Cy)-induced cytotoxicity. HL-60 cells were incubated with Cy (0.2–20 mM) for 6–96 h. Cy reduced HL-60 cell viability in the incubations in a both concentration- and time-dependent manner. The estimated half maximal inhibitory concentration ($IC_{50}$) was 3.6 mM. HL-60 cells were incubated with telmisartan (CYP2J2 inhibitor) at concentrations of 2.5–40 µM for 48 h. Telmisartan has reduced HL60 cell viability in a concentration-dependent manner (**a**). Telmisartan at a concentration of 10 µM or lower did not affect HL60 cell viability but reduced the formation of 4-hydroxycyclophosphamide (4-OH-Cy) by ~50%. HL 60 cells were either pre-incubated with telmisartan at concentrations of 2.5, 5 or 10 µM for 2 h, before adding Cy (9 mM). Cells were incubated for an additional 48 h. HL-60 cells incubated with 9 mM Cy or 10 µM telmisartan alone served as controls for drug toxicity. Negative controls have been incubated in parallel. Preincubation with telmisartan showed 10% improvement in the survival of cells treated with Cy compared with cells treated with Cy alone (**b**). Cell viability was assessed using resazurin assay.

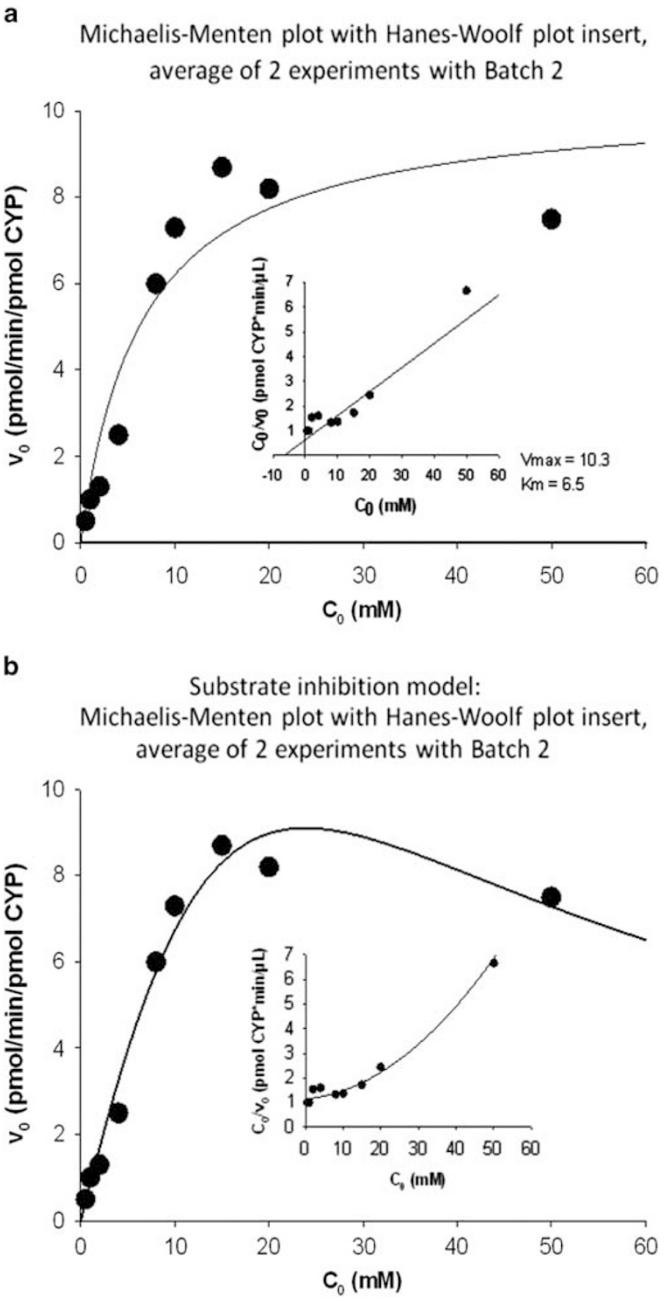


**Figure 9.** Michaelis–Mentin plot with Hanes–Woolf plot (inset) of 4-hydroxycyclophosphamide (4-OH-Cy) kinetics after incubation with recombinant cytochrome P450 2J2 (CYP2J2). $C_0$, concentration; Cy, cyclophosphamide; CYP, cytochrome P450; $V_0$, initial rate. Cyclophosphamide (Cy) at different concentrations was incubated in two independent experiments with two different batches of microsomes containing recombinant human CYP2J2. The 4-OH-Cy formation was measured using high-performance liquid chromatography (HPLC). The results shown are the average obtained with batch 2. Fitting the data to Michaelis-Menten kinetics (**a**) gave an apparent $K_m$ of 6.5 mM and an apparent $V_{max}$ of 10.3 pmol/(min·pmol) CYP resulting in $V_{max}/K_m$ of 1.6 µl/(min·pmol) CYP. Comparing different enzyme kinetic models revealed that the best curve fit was obtained with a substrate inhibition model (**b**). These results showed CYP2J2 to be involved in Cy bioactivation.

metabolism was reduced by CYP2J2 inhibition implying that Cy is metabolized predominantly by CYP2J2. The role of CYP2J2 in Cy metabolism was further confirmed following incubation with recombinant CYP2J2. Enzyme kinetics revealed that CYP2J2 is

as important as CYP2B6 in metabolizing Cy, and relative expression levels of CYP2B6 and CYP2J2 will help determine *in vivo* kinetics.

The findings are of high clinical relevance in patients with certain CYP2B6 polymorphisms and may assist in developing strategies for personalized medicine. *CYP2J2* upregulation *in vivo* during Cy treatment may in part explain cardiotoxicity, urotoxicity and gut toxicity observed during high-dose treatment with Cy in transplanted patients. This toxicity in combination with the alloreactivity may intensify graft versus host disease observed in transplanted patients.

The present results increase our understanding of Cy metabolism and may help in optimizing treatment strategies and improve treatment outcome.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## ACKNOWLEDGMENTS

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