Cytochrome P450 Oxidoreductase Influences CYP2B6 Activity in Cyclophosphamide Bioactivation

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Abstract

Introduction
Cyclophosphamide is commonly used as an important component in conditioning prior to hematopoietic stem cell transplantation, a curative treatment for several hematological diseases. Cyclophosphamide is a prodrug activated mainly by cytochrome P450 2B6 (CYP2B6) in the liver. A high degree of inter- and intra-individual variation in cyclophosphamide kinetics has been reported in several studies.

Materials and Methods
Hydroxylation of cyclophosphamide was investigated in vitro using three microsomal batches of CYP2B6*1 with different ratios of POR/CYP expression levels. Twenty patients undergoing hematopoietic stem cell transplantation were also included in the study. All patients received an i.v. infusion of cyclophosphamide (60 mg/kg/day, for two days) as a part of their conditioning. Blood samples were collected from each patient before cyclophosphamide infusion, 6 h after the first dose and before and 6 h after the second dose. POR gene expression was measured by mRNA analysis and the pharmacokinetics of cyclophosphamide and its active metabolite were determined.

Results
A strong correlation between the in vitro intrinsic clearance of cyclophosphamide and the POR/CYP ratio was found. The apparent Kₘ for CYP2B6.1 was almost constant (3-4 mM), while the CLₚ values were proportional to the POR/CYP ratio (3-34 μL/min/nmol CYP). In patients, the average expression of the POR gene in blood was significantly (P <0.001) up-regulated after cyclophosphamide infusion, with high inter-individual variations and
significant correlation with the concentration ratio of the active metabolite 4-hydroxy-cyclophosphamide/cyclophosphamide. Nine patients were carriers for POR*28; four patients had relatively high POR expression.

Conclusions
This investigation shows for the first time that POR besides CYP2B6 can influence cyclophosphamide metabolism. Our results indicate that not only CYPs are important, but also POR expression and/or activity may influence cyclophosphamide bioactivation, affecting therapeutic efficacy and treatment related toxicity and hence on clinical outcome. Thus, both POR and CYP genotype and expression levels may have to be taken into account when personalizing treatment schedules to achieve optimal therapeutic drug plasma concentrations of cyclophosphamide.

Introduction
Hematopoietic stem cell transplantation (HSCT) is a curative treatment strategy for malignancies, such as leukemia and lymphomas, and nonmalignant disorders such as metabolic disorders and aplastic anemia. Prior to HSCT, 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 [1]. Cyclophosphamide (Cy) in combination with busulphan or with total body irradiation (TBI) belong to the most common used conditioning regimens [2]. Cyclophosphamide is an alkylating agent that is widely used in the treatment of hematological malignancies as well as solid tumors. Cy is also a potent immuno-suppressive agent that affects both T- and B-lymphocytes, thus having effect on both humoral and cell-mediated immunity. Cy can also be used to treat rheumatoid arthritis, systemic lupus erythematosus, Sjögren’s syndrome, glomerulonephritis, multiple sclerosis, and in preconditioning of hosts to prevent transplant rejection [3]. Cy is a prodrug that is metabolized by several CYPs to become cytotoxic and effective in cancer chemotherapy [4]. The 4-hydroxylation product is the active metabolite of Cy that yields an active alkylating agent, phosphoramid mustard and a toxic by-product, acrolein. Acrolein is responsible for urotoxicity (hemorrhagic cystitis). Through another pathway, N-dechloroethylation, Cy is metabolized to an inactive metabolite (2-dechloroethyl-Cy) and the neurotoxic agent chloroacetaldehyde (CA) [5].

Cy is metabolized to the active metabolite, 4-hydroxy-cyclophosphamide (4-OH-Cy), mainly by CYP2B6 with contributions from some other CYPs. About 10% of the Cy dose is metabolized through the N-dechloroethylation pathway [5–7].

Several studies have shown high inter-individual variation in Cy kinetics including elimination half-life and clearance [5, 8]. Moreover, investigators have shown high inter-individual variation in expression and catalytic activity of CYP2B6 that is probably caused by the genetic polymorphism of this enzyme [9–11] or its regulation. In total, more than fifty different alleles containing point mutations have been identified to date (28-Nov-2013) (http://www.cypalleles.ki.se/cyp2b6.htm). Most of these mutations are silent but five of them result in amino acid substitutions in exons 1, 4, 5 and 9 [12].

Some of the common non-synonymous polymorphisms are reported to affect Cy kinetics either by decreased liver protein expression [12, 13] or altered function [14, 15]. In addition,
several more rare non-synonymous SNPs have been found to result in the protein being absent or non-functional [16].

On the other hand, several other studies have reported that CYP2B6 genetic polymorphism doesn’t alter the Cy metabolism or the 4-OH-Cy formation in vivo or in vitro [17–19]. Moreover, Yao et al. suggested that clinical factors like patient age and cancer grade may contribute to the inter-individual variation in Cy kinetics [20].

Cytochrome P450 oxidoreductase (POR) is a membrane-bound enzyme in the endoplasmic reticulum that is involved in the electron transfer from the co-factor, NADPH, to CYP. POR influences the metabolic rate differently for different CYP alleles, as reported by Zhang et al [21]. POR is important in the metabolism of drugs and xenobiotics, and allelic variants and variability in expression may have clinical implications [22, 23].

POR deficiency may lead to serious complications such as disordered steroidogenesis, abnormal genitalia, bone abnormalities and William’s syndrome [24–26]. Recently, POR variants have been found to play an important role in CYP dependent metabolism of drugs and xenobiotics, at least in vitro. Some POR polymorphisms affect the amount of enzyme produced, which may affect the activities of cytochrome P450 enzymes such as CYP1A2, 2C9, 2C19, 2D6, 3A4 and 3A5, [27–30], especially for compounds which are rapidly metabolized and where the electron transfer from NADPH via POR may be rate-limiting.

POR variants have altered the CYP2B6 dependent activity in bupropion metabolism and S-Mephenytoin N-demethylation [31, 32]. POR*28 is the only polymorphism reported to increase CYP activity in vivo [33]. Many other polymorphisms are known to decrease its activity in vivo, for example POR*2, 3, 4 and 5 [http://www.cypalleles.ki.se/por.htm, 11-Oct-2011].

In the present study, we aimed to investigate the effect of POR levels on the cyclophosphamide 4-hydroxylation rate in vitro using microsomes containing CYP2B6.1. The effect of Cy treatment on POR gene expression in vivo in patients, prior to hematopoietic stem cell transplantation (HSCT), was also investigated.

**Materials and Methods**

**Chemicals**

Cyclophosphamide monohydrate and β-Nicotinamide adenine dinucleotide phosphate reduced form (β-NADPH) were purchased from Sigma Aldrich (Stockholm, Sweden). Maphosphamide was used to produce 4-OH-Cy by hydroxylation in water. Maphosphamide was kindly provided by Professor Ulf Niemeyer, Baxter Oncology GmbH (Frankfurt am Main, Germany). Maphosphamide (Asta-Z 7557; MW 500.4) is a stable form for 4-OH-Cy (4-OH-Cy connected to cyclohexylamine), that is suitable for ex vivo experiments. The compound hydrolyses spontaneously in aqueous solution producing 4-OH-Cy [34, 35].

Potassium phosphate buffer (50 mM, pH 7.4) was used for the microsomal incubations. Acetonitrile, phosphate salts, phosphoric acid, other chemicals and solvents used were of high performance liquid chromatography (HPLC) or analytical grade and were purchased from Merck, Germany.

QuickPrep Total RNA Extraction Kit (GE Life Sciences, Uppsala, Sweden), TaqMan Reverse Transcriptase-complementary DNA (RT-cDNA) Kit (Applied Biosystems, Roche, NJ, USA), and NimbleGen microarrays (Roche Diagnostics Scandinavia, Bromma, Sweden) were used for gene array experiments. TaqMan genotyping polymerase chain reaction (PCR) primer for POR*28 SNP (rs1057868C>T, catalogue #4362691) was purchased from Applied Biosystems (Stockholm, Sweden) while the genotyping master mix (catalogue # 208252) was from Qiagen (Stockholm Sweden).
Microsomes

Commercially available microsomes, containing human CYP2B6.1 and human POR coexpressed in *Escherichia coli* were purchased from Cypex Ltd. (Dundee, UK). Batches were available with up to approximately 18-fold differences in POR/CYP ratios (Table 1). Microsomes containing only human POR (without CYPs) from the same vendor have been used as controls (Table 1). The POR activities reported in the data sheets from the vendor were used.

**In vitro cyclophosphamide metabolism assays**

The effect of different POR/CYP ratios on cyclophosphamide metabolism was studied.

Cy metabolism was studied with both time and CYP concentration, and the 4-OH-Cy formation was found to be linear up to 1 mM Cy and at CYP concentrations between 10 and 40 pmol/mL and incubation times up to 25 min. Enzyme kinetic parameters were determined under linear conditions.

Three batches of cDNA-expressed human CYP2B6.1 with similar specific CYP concentrations but different POR concentrations and thus POR/CYP ratios (Table 1) were incubated for 15 min in two independent experiments at 37°C, in a total volume of 100 μL, containing 50 mM potassium phosphate buffer, pH 7.4, and 1 mM NADPH. The reactions were stopped with 100 μL of ice-cold acetonitrile. The mixtures were vortexed and centrifuged at 3000 g for 5 min to remove precipitated protein. Negative control incubations were run in parallel without cyclophosphamide, without NADPH, or with control yeast microsomes not expressing the human enzymes. Microsomes containing POR only, were incubated as controls in parallel, under the same conditions, after adjusting the POR amount to be equal to that of the CYP2B6.1 batch with the highest POR content.

For the determination of apparent $K_m$ and $V_{max}$, nine substrate concentrations (0, 0.15, 0.25, 0.5, 1, 2, 4, 8 and 10 mM) were used. CYP content per incubation was kept the same (40 pmol/mL) for all batches.

Furthermore, two separate sets of experiments were performed with 0.75 mM Cy in each of the three batches. Incubations were run as described above (15 min). One set was run with constant CYP concentrations, the other with the POR concentration kept constant (356 nmol cyt c

| Batch No. | Batch 1 | Batch 2 | Batch 3 | POR |
|-----------|---------|---------|---------|-----|
| Catalogue No. | CYP/EZ016 | CYP/EZ041* | CYP020 | CYP004 |
| Lot. No | C2B6LR002/A | C2B6BR016A | C2B6R005 | RED004 |
| Vendor Company | Cypex | Cypex | Cypex | Cypex |
| P450 concentration** | 1 | 1 | 1 | N/A |
| Protein concentration*** | 10 | 10 | 10.6 | 12.7 |
| Specific P450 content # | 100 | 100 | 94 | N/A |
| Cytochrome c reductase activity## | 77 | 890 | 1302 | 2437 |

Batch 1–3 were microsomes (Bactosomes) from *E. Coli* containing co-expressed CYP2B6*1 and human POR. The POR batch was a control without CYP.

* Batch 2 also contained a supplement of purified human cytochrome b5

** P450 concentration (nmol/mL)

*** Protein concentration (mg/mL)

# Specific P450 content (pmol/mg protein)

## Cytochrome c reductase activity (nmol/min/mg protein) as marker for POR activity

N/A Not applicable

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reduced/min/mL, which is the same POR concentration as in batch 2 i.e. the batch with the median POR/CYP ratio), producing different concentrations of CYP and different POR/CYP ratios.

4-Hydroxy-cyclophosphamide preparation and detection

Maphosphamide was dissolved in phosphate buffer (50 mM, pH 7.4). The stock solution was added to plasma for the calibration curve for patient samples or further diluted with buffer to produce the standard curve for microsomal incubations. The produced 4-OH-Cy was stabilized and converted to a fluorescent dansylhydrazone derivative by adding dansylhydrazone (2 mg/mL) and one M hydrochloric acid. The mixture was vortexed and incubated at 50°C for 5 min. Thirty μL of the final sample was injected into the HPLC system which consisted of an LKB 2150 HPLC pump, a Gilson 234 auto-injector with a 100 μL sample loop and a Shimadzu RF-10XL Fluorescence detector. The detection was carried out with an excitation wavelength of 350 nm and an emission wavelength of 550 nm. The column was a Zorbax Extend C18, (150 x 4.6 mm, 5 μm microspheres) purchased from Agilent. The mobile phase was phosphate buffer (pH 3.5)/acetonitrile (2:1, v/v) and the flow rate was 1.4 mL/min. The retention time under these conditions was 13.4 min for 4 OH-Cy. The limit of quantitation was 100 pmol/mL and the calibration curve was linear between 60 and 2000 ng/mL [36, 37].

Data collection, chromatogram integration and determination of the 4-OH-Cy concentrations were carried out using a Clarity chromatographic station (version 2.8, DataApex Ltd., The Czech Republic) as integration software.

Patients

For the detection of POR expression during Cy treatment, twenty patients undergoing HSCT at the Center for Allogeneic Stem Cell Transplantation (CAST), Karolinska University Hospital, Huddinge, Sweden, were included in the study. The study was approved by the ethical committee of Karolinska Institutet (number 616/03 and 2014/150-32). The complete data (ID no. 20051907) is available via the GEO database with the accession number "GSE51907"[38]. Written consent was obtained for all patients (for pediatric patients, consent was obtained by a legal guardian), and the individual(s) in this manuscript have given informed consent to publish these case details.

All patient characteristics including diagnosis, disease status at the time of transplantation, conditioning, age, type of transplantation and cell dose are shown in Table 2. Prior to transplantation, eleven patients were conditioned using 1 h i.v. Cy infusions (60 mg/kg, once daily for two days) followed by fractionated total body irradiation (fTBI). Nine patients were conditioned using fludarabine (Flu) 1 h i.v. infusions (30mg/m2/day, for 5 days) followed by 1 h i.v. Cy infusions (60 mg/kg, once daily for two days) and/or TBI. Blood samples were collected from each patient before and after Flu treatment and at the start of Cy infusion, 6 h after the first dose, before and 6 h after the second dose of Cy. For the determination of drug kinetics, both Cy and 4-OH-Cy were measured from patient plasma samples at several time points as described previously [36, 37, 39]. Due to technical problems, some samples were missed as reported in the complete data (ID no. 20051907).

Patient mRNA was subjected to analysis of global gene expression by NimbleGen microarrays (Roche Diagnostics Scandinavia, Bromma, Sweden). Data were analyzed using GeneSpring GX (Agilent, CA, USA). The expression data were normalized using quantile normalization and the gene expression data were generated using the Robust Multichip Average algorithm. Significant differences in gene expression were determined by ANOVA. The selection threshold of a false discovery rate (FDR) was <5% and the fold change in the SAM
output result was $>2$. RNA extraction, cDNA preparation and gene array analysis were performed as described previously [38].

**PCR**

Samples were scanned using TaqMan genotyping PCR primer for the only polymorphism reported to increase CYP activity *in vivo*, POR*28 (catalogue #4362691, SNP rs1057868C>T, Applied Biosystems, Stockholm, Sweden). Samples were extracted (catalogue # 51304, Qiagen, Stockholm, Sweden) and amplified, according to the manufacturer’s instructions, in 72 rotor (20 μL total volume) containing 1x TaqMan SNP Genotyping master mix (catalogue # 208252, Qiagen, Stockholm, Sweden). Analysis was performed using Rotor Gene Real-Time PCR System (Qiagen, Stockholm, Sweden) by means of the VIC and FAM dye-labeling system according to the manufacturer’s instructions. Genotypes were assigned post-PCR end-point reading, using the manual calling option in the allelic discrimination application. Negative controls were run in parallel to the samples.

**Table 2. Patient characteristics.**

| Patient | Age (y) | Diagnosis  | Conditioning regimen | CD 34 dose/kg | Disease status at HSCT | Outcome          | Cause of death |
|---------|---------|------------|----------------------|---------------|------------------------|------------------|----------------|
| P1  | 57 | B-CLL | Cy+TBI (6 Gy) + Alemtuzumab | 14.7x10⁶ | Transformed | † 10 months | Relapse |
| P2  | 38 | T-cell lymphoma | Cy+TBI | 2.9x10⁶ | PR | † 19 months | Relapse |
| P3  | 31 | AML | Cy+TBI+ATG | 2x10⁶ | CR2 | † 6 months | Pneumonia |
| P4  | 10 | T-ALL | Cy+TBI+ATG | 6.48x10⁶ | CR1 | † 12 months | Relapse |
| P5  | 26 | Pre-B ALL | Cy+TBI+ATG | 13.5x10⁶ | CR2 | † 35 months | Relapse |
| P6  | 19 | ALL | Cy+TBI+ATG | 13.5x10⁶ | CR3 | † 9 months | Relapse & pneumonia |
| P7  | 51 | AML | Cy+TBI+ATG | 10.6x10⁶ | Refractory | Alive 7.5 years | — |
| P8  | 25 | Pre-B ALL | Cy+TBI+ATG | 7.3x10⁶ | CR2 | Alive 7.5 years | — |
| P9  | 14 | T-ALL | Cy+TBI+ATG | 19.9x10⁶ | CR2 | Alive 7.2 years | — |
| P10 | 41 | T-cell lymphoma | Cy+TBI+ATG | 9.3x10⁶ | Relapse | † 51 days | Invasive fungal infection |
| P11 | 26 | T-ALL | Cy+TBI+ATG | 0.5x10⁶+2x10⁵ | CR2 | † 11 months | Relapse |
| P12 | 49 | NHL | Flu + Cy + TBI | 4.0 x 10⁶ | PR | † 2 months | Relapse |
| P13 | 11 | Kidney Cancer | Flu + Cy + ATG | 12.9 x 10⁶ | N/A | Alive 9 years | — |
| P14 | 51 | CLL | Flu + Cy + TBI + Campath | 9.4 x 10⁶ | PR | † 2 months | Septic shock |
| P15 | 57 | NHL | Flu + Cy + TBI + ATG | 6.1 x 10⁶ | CR4 | Alive 8.5 years | — |
| P16 | 64 | NHL | Flu + Cy + TBI + Campath | 15.1 x 10⁶ | CR2 | † 6 months | GI-bleeding |
| P17 | 62 | NHL | Flu + Cy + TBI | 8.3 x 10⁶ | CR2 | Alive 8 years | — |
| P18 | 57 | ALL | Cy + TBI + ATG | 7.8 x 10⁶ | CR1 | Alive 8 years | — |
| P19 | 9  | CLL | Flu + Cy + Campath | 9.3 x 10⁶ | Relapse | † 6 months | Relapse & pneumonia |
| P20 | 9  | CLL | Flu + Cy + TBI | 11.0 x 10⁶ | PR | † 8 months | Relapse, pneumonia & septicemia |

**Abbreviations:** ALL: acute lymphoblastic leukemia; AML: acute myeloid leukemia; ATG: antithymocyte globulin; 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-Hodgkin lymphoma; P: patient; PR: partial remission; T: T lymphocyte; TBI: Total body irradiation; †: survival time. fTBI: fractionated total body irradiation

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Data Analysis

Calculations, statistics and graphs were performed using GraphPad Prism (version 4.0, GraphPad Software, Inc.) and SigmaPlot (version 12.5, Systat software, Inc.). Prior to all analysis, all patient data were tested and approved to be normally distributed.

Results

In vitro cyclophosphamide metabolism

The linearity of Cy metabolism was studied with both time and CYP concentration, and the 4-OH-Cy formation was found to be linear up to 1 mM Cy and at CYP concentrations between 10 and 40 pmol/mL and incubation times up to 25 min.

4-OH-Cy produced from maphosphamide has a purity of 99.9% according to the manufacturer. Hydroxylation of maphosphamide in aqueous solution and/or plasma yielded 4-OH-Cy and cyclohexylamine. Cyclohexylamine did not interfere with the fluorescence detection of 4-OH-Cy.

The enzyme kinetics for 4-OH-Cy formation with microsomes containing human cDNA-expressed CYP2B6.1 and various ratios of POR/CYP was determined under linear conditions. Fitting the data from two independent experiments (each with the three different batches of microsomes at a constant concentration of 40 pmol CYP/mL and Cy concentration up to 10 mM) to Michaelis-Menten kinetics, the apparent $K_m$ for CYP2B6.1 was similar for the 3 batches (3–4 mM). However, the apparent maximum rate of Cy 4-hydroxylation ($V_{max}$) increased with the POR/CYP ratio, ranging from 12.6 to 99.0 nmol/min/nmol CYP (Fig 1). The CYP concentrations and the protein concentrations were almost identical in the different batches, indicating that the electron transfer by POR from NADPH to CYP may be rate limiting and that the POR/CYP ratio thus may be important for the Cy kinetics in vitro.

This also resulted in the intrinsic clearance, $CL_{int}$ (i.e. the ratio of $V_{max}/K_m$) being proportional to the POR/CYP ratio ranging between 3.1 and 33.7 μL/min/nmol CYP (Fig 2A and Table 3).
To further study the effect of different POR/CYP ratio in the three batches of CYP2B6.1, two separate sets of incubations were performed for 15 minutes with 0.75 mM Cy and each of the three batches. Experiments were performed with constant CYP amounts (40 pmol/mL) resulting in different POR concentrations (Fig 2B and 2C). Separate experiments were performed with constant POR activities (356 nmol cyt c reduced /min/mL) resulting in different CYP concentrations (Fig 2B and 2D). The CL\text{int} values were approximated from the \( \frac{v_0}{C_0} \) in experiments with either constant CYP (40 pmol/mL incubation) or constant POR (356 nmol/min/mL incubation) is plotted for the 3 batches. The CL\text{int} values are shown to increase proportionally with the POR/CYP ratio regardless of if POR or CYP is kept constant. With constant CYP, the CL\text{int} was found to be proportional to the POR concentration (Fig 2C) while it did not increase with CYP content in experiments with low, constant concentrations of POR (Fig 2D).

Fig 2. Cyclophosphamide intrinsic clearance for different CYP2B6.1 batches. Cy (0.75 mM) was incubated with different batches (Batch 1–3, Table 1) of microsomes containing CYP2B6.1. In Fig 2A, the intrinsic clearance, \( CL_{\text{int}} \), or ratio of \( V_{\text{max}}/K_m \) (3.1–33.7 \( \mu \)L/min/nmol CYP) determined from the Michaelis-Menten kinetics was found to be proportional to the POR/CYP ratio (0.77–13.8 nmol/min/pmol CYP). In Fig 2B, the CL\text{int} approximated from the \( \frac{v_0}{C_0} \) in experiments with either constant CYP (40 pmol/mL incubation) or constant POR (356 nmol/min/mL incubation) is plotted for the 3 batches. The CL\text{int} values are shown to increase proportionally with the POR/CYP ratio regardless of if POR or CYP is kept constant. With constant CYP, the CL\text{int} was found to be proportional to the POR concentration (Fig 2C) while it did not increase with CYP content in experiments with low, constant concentrations of POR (Fig 2D).

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To further study the effect of different POR/CYP ratio in the three batches of CYP2B6.1, two separate sets of incubations were performed for 15 minutes with 0.75 mM Cy and each of the three batches. Experiments were performed with constant CYP amounts (40 pmol/mL) resulting in different POR concentrations (Fig 2B and 2C). Separate experiments were performed with constant POR activities (356 nmol cyt c reduced /min/mL) resulting in different CYP concentrations (Fig 2B and 2D). The CL\text{int} values were approximated from the \( \frac{v_0}{C_0} \) and they correlated (\( R^2 = 0.99 \)) with the POR/CYP ratios (regardless of if POR or CYP concentrations were kept constant). The CL\text{int} values are shown to increase proportionally with the POR/CYP ratio regardless of if POR or CYP is kept constant. With constant CYP, the CL\text{int} was found to be proportional to the POR concentration (Fig 2C) while it did not increase with CYP content in experiments with low, constant concentrations of POR (Fig 2D) and an excess of CYP per POR. Thus, all experiments showed a strong correlation between the POR/CYP ratio and the Cy 4-hydroxylase activity, confirming that the POR/CYP ratio will affect the Cy kinetics, at least in vitro.
In microsomes containing POR only, no 4-OH-Cy formation could be detected, indicating that POR, by itself, could not bioactivate Cy.

**Patients**

The clinical outcome and survival time for the patients that participated in the study is listed in **Table 2**. As shown, the majority of patients died due to relapse or infection.

The average expression level of the POR gene was significantly (p < 0.001, t-test) up-regulated 24 h and 30 h after the first Cy infusion (i.e. before and 6 h after the second infusion), compared to the levels before conditioning. However, a high inter-individual variation in POR gene expression after Cy infusion was observed, with a difference in individual relative POR expression of 4.3-fold before Cy conditioning increasing to 9.9-fold after 24 h and 16.5-fold by the end of the treatment, i.e. the expression levels increased in some patients more than others. The average expression levels after Cy treatment increased by 2.2 fold compared to the average before the start of the treatment (Fig 3).

For the patients treated with fludarabine before Cy, the increase in POR expression after treatment with Cy was similar to that found in patients treated with Cy. Moreover, no significant change was found in POR expression after fludarabine treatment and prior to Cy (Fig 4).

The concentration ratio (4-OH-Cy/Cy) at 6 h after first dose and at 6 h after the second dose was significantly (P < 0.001, r² = 0.308, linear regression) correlated with POR expression as shown in **Fig 5**.

**Discussion**

Genetic polymorphism has been associated with variable levels of gene expression in the liver, and CYP2B6 appears to be one of the most polymorphic human P450s.

Human CYP2B6 expression levels for the C1459T mutation (alleles *5 and *7) have been reported to be significantly lower than for CYP2B6*1 [12], while this mutation leads to a higher
Cy intrinsic clearance both \textit{in vitro} and \textit{in vivo} \cite{8, 15}. In Caucasians, the SNP frequency has been reported to be 33\% and 29\% for A785G and G516T, respectively. \cite{12}. These SNPs are present in several \textit{CYP2B6} allelic variants such as (2B6*/C3*4, 2B6*/C3*6, 2B6*/C3*7 and 2B6*/9).

Ariyoshi \textit{et al.} have reported higher activity of \textit{CYP2B6*6} in 4-hydroxylation of Cy and decreased activity of 8-hydroxylation of efavirenz metabolism. The authors used microsomes containing CYP2B6.1, CYP2B6.4 or CYP2B6.6, but with the same POR activity and POR/CYP ratio in each batch for their \textit{in vitro} experiments \cite{40}.

On the other hand, Raccor \textit{et al.} have reported that \textit{CYP2B6} genotype is not related to 4-OH-Cy formation either \textit{in vitro} or \textit{in vivo} \cite{18}. Moreover, other studies have shown that the genotype of \textit{CYP2B6}, or other CYPs involved in Cy metabolism, do not affect Cy kinetics and suggest that clinical factors such as patient age and cancer grade may be more significant \cite{17, 19, 20}.

\textbf{Fig 3. Up-regulation of POR mRNA during Cy conditioning.} The gene expression of \textit{POR} was significantly ($p < 0.001$, t-test) up-regulated 24 hours after the first Cy infusion and 6 hours after the second dose of Cy, compared to pre-Cy conditioning. The inter-individual variation in relative POR expression increased from 4.3-fold before Cy conditioning to 9.9-fold before the 2nd dose of Cy and to 16.5-fold by the end of the treatment. The high variation in up-regulation of \textit{POR} gene expression during Cy treatment may contribute to the high inter-individual variations in Cy kinetics reported in patients, possibly in part due to different inducibility of the polymorphic forms of \textit{POR}. Circles in the figure are for four patients with \textit{POR*28} polymorphism.
POR is the main electron donor for all microsomal CYP monooxygenases and its polymorphisms have been shown to affect CYP-mediated drug metabolism as well as direct bioactivation of prodrugs [41]. Chen et al. studied the effect of POR genotype on CYP2B6-dependent bupropion metabolism. Their results showed a 70–74% reduction in CYP2B6 activity with some POR polymorphisms in vitro [31]. That was in agreement with another study, which also reported that S-Mephenytoin N-demethylation by CYP2B6 varied with the POR polymorphism in the human liver [32]. CYP-dependent drug metabolism, like Cy 4-hydroxylation, depends on POR for the electron transfer from NADPH to CYP. POR expression levels and its electron transfer efficiency may be rate limiting for fast reactions and could affect drug kinetics, overall exposure and metabolic pattern and hence affect efficacy and side effects.

The formation of 4-OH-Cy is a fast reaction, which means that the rate limiting step may be the electron transfer from the POR to CYP [41]. In an experiment with different batches of recombinant human CYP2B6.1, the intrinsic clearance of Cy was clearly proportional to the POR/CYP ratio ranging between 3.1 and 33.7 μL/min/nmol CYP despite the fact that $K_m$ was almost constant in all batches. This experiment was performed once with constant CYP in all incubations and then repeated with constant POR in all incubations with the same batches. The results showed good linear correlation between 4-OH Cy formation and POR/CYP ratio.

POR variability also affects CYPs other than CYP2B6, such as CYP2C9 activities, when incubated with flurbiprofen, diclofenac, and tolbutamide. These drugs, like Cy, are metabolized rapidly [29]. The effect of POR variants and expression levels varies with the substrate and the CYP

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**Fig 4. Effect of fludarabine on POR gene expression.** The gene expression of POR was not significantly changed after treatment with fludarabine.

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enzyme variant; for example, POR polymorphic variants with A287P and/or R457H are associated with no detectable CYP2D6 metabolism of 7-ethoxymethoxy-3-Cyanocoumarin (EOMCC), while Q153R polymorphism increased CYP2D6 activity with EOMCC in vitro [28].

Steroidogenic activity is dependent mainly on CYP1A2 and CYP2C19. POR variants affected the activities of these enzymes to different extents. POR polymorphisms A287P and/or R457H have reduced CYP1A2 and CYP2C19 catalytic activities to EOMCC. The A503V polymorphism gave 85% of wild-type activity with CYP1A2 and 113% of wild-type activity with CYP2C19, while Q153R polymorphism increased both CYP1A2 and CYP2C19 activities [27].

CYP3A4 completely lost its capacity for 6-beta-hydroxylation of testosterone in vitro by two of the POR polymorphisms, Y181D and A287P. Other POR polymorphisms, such as K49N, A115V and G413S, resulted in increased CYP3A4 activity for testosterone with up to 65% [31]. Tacrolimus is metabolized by CYP3A5 and the POR*28 polymorphism was shown to result in a significant decrease in tacrolimus exposure. POR*28 along with the CYP3A5 polymorphism was responsible for the inter-individual variability of tacrolimus [30].

A study on human liver samples showed that four POR polymorphisms (K49N, L420M, A503V, and L577P) have both reduced POR expression levels and drug-metabolizing CYP activity. The same study also showed that intron polymorphisms altered POR activity [41].

Fig 5. The correlation between POR expression and 4-OH-Cy/Cy concentration ratio. The concentration ratio for 4-OH-Cy/Cy (as measure for the bioactivation of Cy) in plasma at 6 and 24 h (0 time before next dose) after first dose and at 6 h after the second dose was significantly (P < 0.001, r² = 0.308, linear regression) correlated with POR expression in patients (in vivo).

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CYP2B6 polymorphism can affect the drug kinetics. In the present study, however, the influence of POR was studied for only one CYP2B6 variant (CYP2B6.1). The relative amount of POR significantly influenced the drug metabolic activity.

Results obtained from POR gene expression measurements in samples from 20 patients conditioned with Cy showed significant ($p<0.001$) POR up-regulation at 24 h and 30 h after the first Cy infusion (i.e. before and 6 h after the second Cy infusion). However, high inter-individual variations in gene expression before and after Cy infusion was observed. POR expression showed significant positive correlation with the concentration ratio 4-OH-Cy/Cy (as measure for the bioactivation of Cy), which may explain part of the inter-individual variation in Cy kinetics.

Although half of the patients had been treated with Flu before Cy, Flu didn’t show any effect on the POR up-regulation. Flu is a prodrug which is converted to its active metabolite through a process of dephosphorylation and phosphorylation, neither of which need any of the CYPs or POR [42].

PCR results for POR*28 genotype showed that almost half of the patients were carriers for POR*28. Five of them had significantly higher POR expression after Cy conditioning. However, four other patients also had high POR expression, possibly due to other POR polymorphisms not yet described, or to effects on nuclear receptors or other factors involved in POR regulation, also resulting in higher inducibility.

Determination of patient genotype and expression levels for POR [43] as well as CYP2B6 before Cy therapy, may be utilized for personalizing treatment. Moreover, it might help make the clinical decision for concomitant treatment with other drugs that are metabolized by these enzymes. This may improve Cy treatment, decrease its adverse effect and hence improve the clinical outcome. More studies are warranted to clarify the role of POR in Cy metabolism.

Conclusions

The present results indicate that not only CYPs are important, but also POR expression and/or activity may influence Cy bioactivation affecting treatment efficacy. Thus, both POR and CYP genotype and expression levels may have to be taken into account when personalizing treatment schedules to achieve optimal therapeutic drug plasma concentrations.

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Author Contributions

Conceived and designed the experiments: MH YT IES. Performed the experiments: IES PA AM. Analyzed the data: IES PA AM YT MH. Contributed reagents/materials/analysis tools: AM MH. Wrote the paper: IES PA AM YT MH.

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