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Characterization of the cytochrome P450 involved in side-chain oxidation of cyclophosphamide in humans

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Abstract *Objective*: Cyclophosphamide (CP) is an antineoplastic prodrug which requires bioactivation (4-hydroxylation) by the cytochrome P450 (CYP) enzymes in human liver. In parallel, P450-mediated side-chain oxidation (N-dealkylation) leads to the formation of the non-alkylating dechloroethylcyclophosphamide (DCl-CP) and chloroacetaldehyde, the latter being a potential neurotoxic agent. The enzyme responsible for side-chain oxidation has not been identified yet. We therefore used an in vitro approach to characterize the enzyme involved in N-dealkylation of CP.
Methods: CP was incubated with the microsomal fraction of human liver in the presence of specific inhibitors for some P450 enzymes and in the presence of stable expressed P450 enzymes. Dechloroethylcyclophosphamide was analysed using gas chromatography and nitrogen-phosphorus detection.
Results: Formation of DCl-CP increased linearly with substrate concentration over the entire concentration range (20 $\mu mol \cdot l^{-1}$ to 36 $mmol \cdot l^{-1}$). Saturation of the enzyme was not observed. Incubation with stable expressed P450 enzymes and inhibition experiments indicated that CYP 3A4 was the major enzyme involved in side-chain oxidation of CP.
Conclusion: Our in vitro data indicate that side-chain oxidation of CP occurs in dose-dependent fashion in men with no saturation of this pathway even following dose escalation. Thus enhanced neurotoxicity following CP administration may result in the setting of high-dose chemotherapy. Moreover, we conclude that CP has the potential to interact with other CYP 3A4 substrates.

Key words Cyclophosphamide · CYP 3A4; side-chain oxidation · dechloroethylcyclophosphamide

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Introduction

The alkylating antineoplastic drug cyclophosphamide is widely used in cancer chemotherapy of various malignancies. Since the parent drug shows no cytotoxicity it requires bioactivation via ring oxidation by the cytochrome P450 system in the human liver to finally yield the alkylating metabolite phosphoramide mustard and the urotoxic metabolite acrolein. Besides this metabolic activation there are some other pathways which lead to inactive metabolites. For example, formation of 4-ketocyclophosphamide and carboxy phosphamide by further oxidation of the unstable intermediates 4-hydroxycyclophosphamide and aldophosphamide is observed [1–3]. Another inactivation pathway of CP, the side chain oxidation (N-dealkylation), is also mediated by cytochrome P450 and leads to the formation of dechloroethylcyclophosphamide and chloroacetaldehyde [4]. The latter seems to be responsible for CNS toxicity of oxazaphosphorine therapy [5]. Figure 1 briefly summarizes these major pathways of CP metabolism.

Though CP metabolism has been extensively studied over the last 25 years, many questions remain unanswered. For example, little is known about the enzymes involved in the activation and deactivation of CP and the relative contribution of the different pathways. Only a few in vitro investigations were carried out to identify the P450 enzymes responsible for oxidation of CP. Chang et al. [6] showed that mainly CYP 2B6 catalyses the ring oxidation of CP. These findings appear to be questionable in view of the fact that 15% of Caucasian and 70% of Japanese patients do not express CYP 2B6 at all [7]. Moreover, Yule et al. [8] showed that CP activation was affected by substrates or inhibitors of CYP 3A. Activation by means of side-chain oxidation and bioactivation of ifosfamide, an isomer of CP, are catalysed by CYP 3A4 [6, 9]. Other P450 enzymes play only a minor role in these reactions. The enzyme(s) responsible for side-chain oxidation

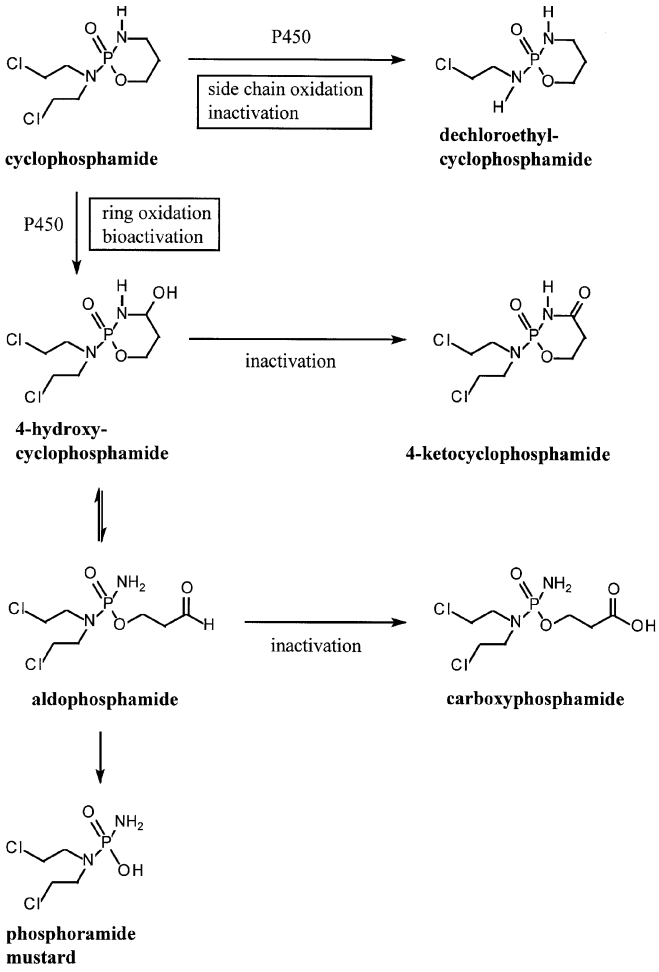

Fig. 1 Activation and inactivation pathways of cyclophosphamide metabolism

of CP has not been identified so far. Nevertheless this information is pivotal for optimization of CP therapy, as it contributes to the interaction potential and via formation of a neurotoxic compound to side effects.

We therefore characterized the enzyme catalysing the N-dealkylation of CP using in vitro techniques. In order to obtain enzyme kinetic data and to investigate interindividual variabilities in CP metabolism, formation of DCl-CP was analysed following incubation of CP with the microsomal fraction of different human livers. Identification of the enzyme was achieved by (a) correlating the rate of formation with the individual contents of various P450 enzymes, (b) incubation of CP with human liver microsomes in the presence of specific inhibitors for the individual P450 enzymes, and (c) incubation of CP with stable expressed P450 enzymes. For determination of DCl-CP we used a gas chromatographic method recently developed in our laboratory [10].

Materials and methods

Chemicals

All solvents used were of HPLC quality; chemicals were of analytical grade. 2-Dechloroethylifosfamide (2-DCl-IF), used as internal standard, and DCl-CP were a generous gift from Dr. J. Pohl (Asta Medica, Frankfurt). Cyclophosphamide monohydrate was obtained from ICN (Meckenheim, Germany). Trizma base (TRIS[hydroxymethyl]aminomethane) and Trizma -HCl (TRIS[hydroxymethyl]-aminomethane hydrochloride) were from Sigma (Deisen hofen, Germany).

Preparation of microsomes

Human and rat liver microsomes were prepared as described elsewere [11]. Individual protein and P450 contents were determined according to the method of Lowry et al. [12] and Omura and Sato [13], respectively.

Incubation conditions for side-chain oxidation of cyclophosphamide

Incubations were carried out at 37 °C for 30 min in the presence of 200 µg microsomal protein and magnesium chloride (final concentration 6 $mmol \cdot l^{-1}$) in a final volume of 250 µl. The system was buffered with 0.05 $mol \cdot l^{-1}$ TRIS buffer (pH 7.8). 2-DCl-IF was added as internal standard at a final concentration of 100 $ng \cdot ml^{-1}$. For kinetic studies increasing amounts of cyclophosphamide (20 $\mu mol \cdot l^{-1}$ to 36 $mmol \cdot l^{-1}$) were added to the incubation mixtures. The incubation was started by adding NADPH solution (final concentration 5 $mmol \cdot l^{-1}$) and stopped by heating at 90 °C for 10 min.

Calibration samples were prepared by adding increasing amounts of DCl-CP to mixtures which contained denaturized rat liver microsomes instead of human liver microsomes.

Isolation of 2-dechloroethylifosfamide and dechloroethylcyclophosphamide

For extraction of 2-DCl-IF and DCl-CP and separation of CP, each of the microsomal incubation mixtures was transferred to a C_8-cartridge (3 ml, 200 mg, Varian, Harbor City, USA) which was conditioned with 2 ml methanol and 2 ml water. The cartridges were then dried by vacuum. 2-DCl-IF and DCl-CP were eluted with 2.5 ml of a methanol-water mixture (10:90 v/v) and then extracted with 5 ml acetic acid ethylester by shaking for 15 min. After centrifugation the organic phase was separated and evaporated to dryness in a stream of nitrogen and the residue dissolved in 50 µl acetic acid ethylester. These extracts were analysed by gas chromatography (GC).

Gas chromatographic determination of dechloroethylcyclophosphamide

For gas chromatographic determination of 2-DCl-IF and DCl-CP, nitrogen-phosphorus detection was used. GC was performed in the splitless mode on a 15 m × 0.32-mm internal diameter DB-17 capillary column, film thickness 0.25 µm (J&W Scientific, Fisons, Mainz, Germany). Helium was used as carrier gas at an inlet pressure of 85 kPa according to a linear carrier gas velocity of 30 $cm \cdot s^{-1}$ (120 °C). The detector temperature was 250 °C, gas flows were 3 $ml \cdot min^{-1}$ (hydrogen), 100 $ml \cdot min^{-1}$ (air) and 25 $ml \cdot min^{-1}$ (Helium, make up gas), and the bead power was set at 30 pA. The

initial oven temperature of 120 °C was increased by 12 °C·min^{-1} to 200 °C; this temperature was held for 9 min. Retention times for 2-DCl-IF and DCl-CP were 6.5 and 7.3 min, respectively. Calibration curves were linear from 5 ng·ml^{-1} to 10 µg·ml^{-1}. The interassay variability averaged 4.8%.

Identification of dechloroethylcyclophosphamide formed during the incubation by gas chromatography/mass spectrometry (GC/MS)

An HP 5985 GC/MS system was used for proving the authenticity of the DCl-CP formed in the incubation experiments. The gas chromatographic conditions were the same as described above. Mass spectrometry was performed in the electron impact (EI) mode. MS conditions were: transfer-line temperature 250 °C, source temperature 200 °C, emission current 300 µA, electron energy 70 eV, multiplier voltage 2800 V. The spectrum obtained after incubation was compared to that of the authentic reference compound under the same conditions.

Incubation experiments using stable expressed cytochrome P450 isoenzymes

Incubation experiments using microsomes from human lymphoblast cells expressing CYP 1A2 and CYP 3A4 (Gentest Corporation, Woburn, USA) were carried out under the same conditions as the incubations with liver microsomes. Cyclophosphamide concentrations ranged from 500 µmol·l^{-1} to 14 mmol·l^{-1}.

Inhibition experiments

Ketoconazole, which at a concentration of 1 µmol·l^{-1} is a selective inhibitor of CYP 3A4 [14], was incubated simultaneously with four different concentrations of cyclophosphamide (500 µmol·l^{-1}, 2, 6 and 14 mmol·l^{-1}) under the same incubation conditions as described above. The inhibitor was added to the incubation mixtures in concentrations of 1, 10 and 50 µmol·l^{-1}. CYP 1A2 was inhibited by preincubation (10 min, 37 °C) of the microsomal mixture with furafylline (30 µmol·l^{-1}) [15, 16].

Statistical analysis

Statistical analysis was performed with the InStat software from GraphPad using Spearman's rank correlation.

Results

The authenticity of DCl-CP formed during microsomal incubations was proven by GC/MS. Similar retention times and mass spectra were obtained for the analyte and an authentic reference compound. The electron impact mass spectral data (*m/z* and relative intensities) for DCl-CP were 149 (100), 56 (50), 120 (37), 94 (15), 57 (15), 92 (13), 121 (10), 198 (5).

Formation of DCl-CP following incubation of CP with human liver microsomes was linear in relation to substrate concentration over the entire concentration range (20 µmol·l^{-1}to 36 mmol·l^{-1}). Therefore the maximum rate of formation (V_{max}) and the Michaelis-Menten constant (k_M) could not be calculated. Higher CP concentrations could not be tested since CP was no longer soluble in the incubation buffer. As depicted in Fig. 2, for the concentration range up to 14 mmol·l^{-1} CP the formation of dechloroethylcyclophosphamide varied strongly in the different human livers investigated.

The rate of formation at the concentration of 6 mmol·l^{-1} CP was correlated with the contents of various P450 isoenzymes of six human liver preparations as shown in Table 1. Correlations were obtained with CYP 1A2 (r = 0.928) and CYP 3A (r = 0.714) contents whereas for the other isoenzymes no correlation was observed.

Dechloroethylcyclophosphamide formation was inhibited by ketoconazole up to 92% (6 and 14 mmol·l^{-1} CP) when the inhibitor was added at a concentration of 50 µmol·l^{-1}. At lower ketoconazole concentrations the inhibition was 75–82% (10 µmol·l^{-1}) and 61–66% (1 µmol·l^{-1}). Addition of furafylline did not affect the rate of formation of DCl-CP.

Incubation of cyclophosphamide with stable expressed CYP 1A2 and CYP 3A4 resulted in formation of dechloroethylcyclophosphamide with CYP 3A4 (V = 2.70 pmol·µg^{-1}·h, 6 mmol·l^{-1} CP; V = 8.08 pmol·µg^{-1}·h, 14 mmol·l^{-1} CP) and to a lower extent with CYP 1A2 (V = 1.01 pmol·µg^{-1}·h, 6 mmol·l^{-1} CP; and V = 3.22 pmol·µg^{-1}·h, 14 mmol·l^{-1} CP).

Discussion

Here we present data indicating that CYP 3A4 is the major enzyme responsible for side-chain oxidation of CP. Several lines of experimental evidence support this finding. We observed a significant correlation of CYP 1A2 and 3A content in different human livers with the

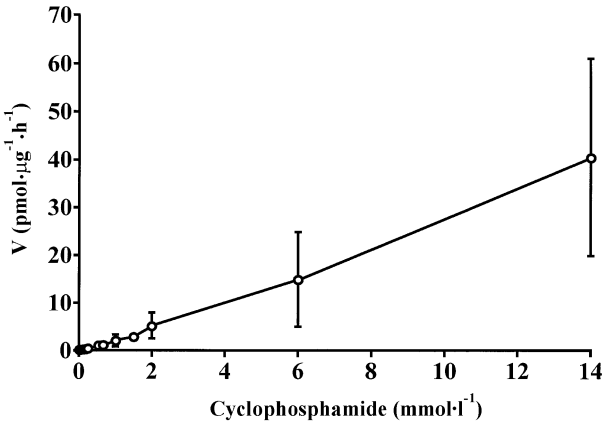

Fig. 2 Substrate dependency of the formation of dechloroethylcyclophosphamide following incubation of cyclophosphamide with human liver microsomes: means with standard deviation of six livers (200 µg microsomal protein incubated for 30 min at 37 °C)

Table 1 Correlation of various P450 enzyme contents versus rate of formation (V) of dechloroethylcyclophosphamide following incubation of cyclophosphamide (6 mmol·l^{-1}) with microsomes of six human livers

Liver number	P450 content (units·mg^{-1} protein)					V at 6 mmol·l^{-1}CP (pmol·μg^{-1}·h^{-1})
	CYP 1A2	CYP 2C	CYP 2D6	CYP 2E1	CYP 3A	
8	0.2	0.8	1.3	0.3	0.3	9.94
12	0.1	0.9	0.5	0.4	0.9	11.4
13	0.7	1.2	0.8	0.3	0.7	17.5
14	0.0	0.5	1.2	0.3	0.5	5.81
16	0.0	0.3	1.7	0.2	0.4	6.60
19	2.8	0.1	1.1	0.2	1.2	34.5
Correlation	r = 0.928	r = 0.086	r = −0.600	r = −0.093	r = 0.714	

rate of formation of DCl-CP. These data indicate involvement of both enzymes in CP metabolism. Therefore both enzymes were further tested for their involvement in N-dealkylation of CP. Formation of DCl-CP was inhibited to more than 90% by ketoconazole. Recent data indicated that ketoconazole at 1 μmol·l^{-1} is a selective inhibitor of CYP 3A4 [14]. We observed 61–66% of DCl-CP formation to be inhibited by 1 μmol·l^{-1} ketoconazole in the concentration range of 2–14 mmol·l^{-1} CP. In contrast, no inhibition of side-chain oxidation was observed after preincubation of the microsomal fraction with the CYP 1A2 inhibitor furafylline [15, 16]. These data clearly indicate that identification of enzymes involved in drug metabolism should be carried out using different techniques in a complementary manner. Thus the inhibition experiments clearly indicate that CYP 3A4 is the major enzyme involved in side-chain oxidation of CP. Data obtained from incubations with stable expressed cytochrome P450 enzymes are compatible with this hypothesis. We observed formation of DCI-CP with stable expressed CYP 3A4 and CYP 1A2. The rate of formation, however, was considerably higher for CYP 3A4 (2.70:1.01 pmol·μg^{-1}·h at 6 mmol·l^{-1}; 8.08:3.22 pmol·μg^{-1}·h at 14 mmol·l^{-1} CP for CYP 3A4:CYP 1A2). Moreover, the natural abundance of CYP 1A2 in human liver is at most one-third of that of CYP 3A4 [7]. Taking these data into account further supports the hypothesis that CYP 1A2 contributes only to a minor extent to side-chain oxidation of CP.

CYP 3A is a major enzyme in the metabolism of a variety of drugs including other cytostatics such as etoposide [17]. The latter drug is often used in combination with CP, giving rise to the potential of a drug/drug interaction. Since the side-chain oxidation contributes only to a minor extent to overall clearance of CP [18], such interactions are unlikely to change the disposition of CP itself but rather that of the other compound involved. It is of interest in this context that Murray et al. [19] described the CYP 3A4 dependent 6β-hydroxylation of testosterone to be inhibited by CP.

Aside from the qualitative aspect of enzyme identification, important inferences can be made from enzyme kinetics of CP side-chain oxidation. Over a wide range of substrate concentrations a saturation of the enzyme was not reached. In other words, product formation is linearly related to the substrate concentrations. The resulting high k_M leads to a low intrinsic clearance of this pathway, which is the confounding factor for the in vivo observation of a minor contribution to overall CP clearance. The linearity of the substrate product relationship, however, has potential clinical implications in particular in the setting of high-dose therapy with CP. With high-dose CP therapy doses of up to 6000 mg·m^{-2} are administered [20–22]. If the enzyme for side-chain oxidation is not saturable, formation of the neurotoxic chloroacetaldehyde will increase in a dose-proportional fashion. Since the bioactivation pathway of CP is saturable [6], an increase in neurotoxicity would not be paralleled by an increase in therapeutic efficacy once a threshhold dose is exceeded. These assumptions are intertwined with the observation in the present study that CP side-chain oxidation shows a considerable interindividual variability (see Fig. 2). Therefore, only a certain subset of patients with high CYP 3A4 activity are likely to develop neurotoxicity. Our in vitro data concerning variability of side-chain oxidation of CP are in good agreement with in vivo results [18].

In summary we conclude that CYP 3A4 is the major enzyme responsible for side-chain oxidation of CP. The enzyme is not saturable over the concentration range from 20 μmol·l^{-1} to 36 mmol·l^{-1} CP. These findings have potential implications for both interaction potential and side effects of CP.

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