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European Journal of Pharmacology 449 (2002) 197–205

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Effect of cyclophosphamide on gene expression of cytochromes *P*450 and β-actin in the **HL-60 cell line**

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Received 5 April 2002; received in revised form 18 June 2002; accepted 21 June 2002

Abstract

Many studies have demonstrated that cyclophosphamide (CPA) can affect hepatic cytochrome *P*450 (CYP) isoenzyme activity in animals. We have investigated the effect of CPA on gene expression of various CYP enzymes as well as β-actin in the human acute promyelocytic leukemia cell line (HL-60S) and its multidrug-resistant (MDR) phenotype HL-60R. Cells were incubated at different concentrations of CPA ranging between 50 μg/ml and 5 mg/ml. In determination of cytotoxicity and resistance factor (RF: IC_{50} HL-60R/IC_{50} HL-60S), concentrations of 100 and 500 μg/ml CPA were selected to treat HL-60S and HL-60R up to 72 h. CYP gene expression in the cells prior to and after treatment with CPA was determined using semiquantitative reverse transcriptase–polymerase chain reaction (RT-PCR) and real-time PCR. Unexposed cell lines did not contain measurable levels of mRNA for CYP2B6, CYP3A4, CYP2C9 and CYP2C19 and no induction was observed after exposure. However, CYP1B1-specific mRNA, which is predominantly expressed in HL-60 cell line, was suppressed after exposure to CPA in a concentration-dependent manner. β-Actin gene expression was also decreased. The HL-60 RF to CPA was calculated to 0.71, indicating that the multidrug-resistant (MDR) phenotype is not involved in the mechanism of resistance to CPA. No CYPs were induced by CPA in vitro, which probably indicates that the CYP inducibility in blood cells is poor. Our study suggests that suppression of β-actin gene expression contributes or is involved in the CPA cytotoxicity.

Keywords: Cyclophosphamide; HL-60; CYP1B1; RT-PCR (reverse transcriptase–polymerase chain reaction); PCR (polymerase chain reaction), real-time; β-Actin; Cytotoxicity; Multidrug resistance

1. Introduction

Cyclophosphamide (CPA) is one of the most widely used anticancer drugs that requires bioactivation by hepatic cytochromes *P*450 (CYP) to exhibit its cytotoxic activity (Moore, 1991). Although the parent drug is relatively stable in aqueous solution and displays only weak activity, it is generally considered that its active metabolite determines the therapeutic effect (Fleming, 1997; Moore, 1991).

The CYP-catalyzed metabolism of CPA involves at least two pathways (Fleming, 1997; Yu et al., 1999) (Fig. 1). One is CPA 4-hydroxylation yielding 4-hydroxy-CPA (4-OH-CPA), which is in equilibrium with the ring-opened tautomer aldophosphamide. The latter metabolite undergoes chemical decomposition to generate the cytotoxic phosphamide mustard with acrolein as byproduct. When transported into tumor cells, phosphamide mustard acts as the bifunctional alkylating agent following cross-linking of DNA. By processing protein alkylation, acrolein is responsible for the urotoxicity (Brock et al., 1979). Alternatively, the primary 4-hydroxy metabolite may be detoxified by aldehyde dehydrogenase to yield the inactive carboxyphosphamide (Sladek, 1999). The other pathway involves an *N*-dechloroethylation that yields equal molar amounts of dechloroethyl-CPA and chloroacetaldehyde (Huang et al., 2000). Both products have no anti-tumor activity; however, chloroacetaldehyde is responsible for various toxic effects, including neurotoxicity and urinary tract toxicity (Springate et al., 1997).

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PII: S0014-2999(02)01995-7

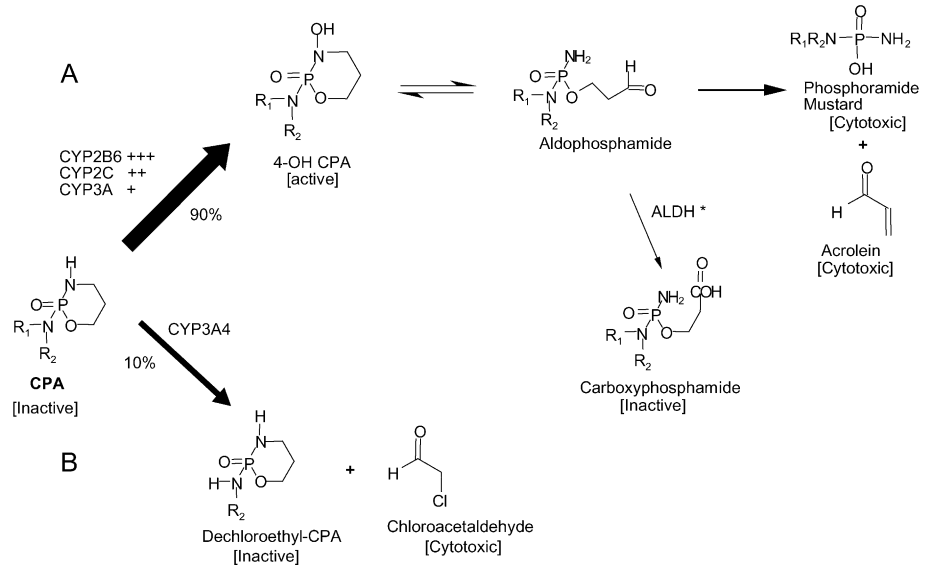

Fig. 1. Two CYP450-catalyzed pathways of cyclophosphamide (CPA). (A) Active pathway, (B) Inactive pathway. * ALDH: Aldehyde dehydrogenase.

The most important pharmacological cytotoxic action of CPA is that it disturbs the fundamental mechanism concerned with cell proliferation, in particular, DNA synthesis and cell division (Hengstler et al., 1997). Although CPA is a non-cell cycle-specific chemotherapeutic drug, it exerts most cytotoxicity in rapidly proliferating tissues. The mechanism of DNA-link formation and cell death, as well as the exact CPA pharmacological route remains unclear (Boddy and Yule, 2000).

The CYP enzymes responsible for CPA activation have been identified in both humans and rats. In humans, CYP2B6 (Code et al., 1997; Gervot et al., 1999; Roy et al., 1999), which is strongly inducible by specific drugs (e.g. phenobarbital, phenytoin, rifampin) (Chang et al., 1997b; Ducharme et al., 1997), plays a major role in the 4-hydroxylation of CPA (Chang et al., 1993). CYPs, 3A4, 3A5, 2A6 and three 2C enzymes exhibited significant oxazaphosphorine 4-hydroxylase activity, whereas CYPs 1A1, 1A2, 1B1, 2C8, 2D6, 2E1, 3A7 and 4A11 are virtually inactive with this substrate (Chang et al., 1993; May-Manke et al., 1999; Roy et al., 1999). Up to 95% of CPA *N*-dechloroethylation is catalyzed by CYP3A (Huang et al., 2000; Yu and Waxman, 1996).

A high degree of inter-patient variation in the kinetics and metabolism of CPA has been observed in both adults and children, which reflects differences in expression level of CYP enzymes and genetic polymorphisms in some of the enzymes, e.g. CYP2C9 and 2C19 (Chang et al., 1997a). In addition, drug–drug interactions also contribute.

Several mechanisms of resistance to cyclophosphamide have been proposed, including increased levels of intracellular thiols and DNA repair (Dong et al., 1996), upregulated glutathione *S*-tranferase activity (Chen and Waxman, 1995), deficient CYP enzymes, increased aldehyde dehydrogenase activity (Giorgianni et al., 2000; Sladek, 1999) and overexpression of anti-apoptotic genes (Hill et al., 1996; Munker et al., 1998). The p-glycoprotein (p-gp)-overexpressed *mdr-1* phenotype has not been reported as one of the resistant mechanism.

In rats, marked suppression of liver microsomal CYP activities has been reported following CPA treatment (Chang and Waxman, 1993; McClure and Stupans, 1992). CYP enzymes involved in CPA metabolism as well as other CYPs were found to be downregulated at the protein and/or mRNA level (Kraner et al., 1996).

In contrast to the suppressive effects described above, an opposite phenomenon was observed in cancer patients who received high-dose CPA as chemotherapy or pretreatment prior to bone marrow transplantation. Repeated or continuous administration of CPA over several days resulted in a decrease of the elimination half-life and an increase in total body clearance (Schuler et al., 1987; Chen et al., 1995), which was explained by an increase in CYP-dependent metabolism. However, few and partly conflicting studies have been done in vitro to verify that. One study in human primary hepatocyte culture (Chang et al., 1997b) reported that CYP3A4, CYP2C8 and CYP2C9 but not CYP2B6 protein levels were increased by exposure to CPA (50 μM), which thereby increased the rate of 4-hydroxylation. In another study, however, no detectable change in CYP3A4 protein expression was reported after CPA exposure, whilst CYP2B6 was induced on both mRNA and protein level (Gervot et al., 1999).

In view of the conflicting reports and the importance of the bioactivation step for the clinical outcome, we investigated the effect of CPA directly in human tumor cell lines. A human hematopoietic (promyelocytic leukemia) cell line, HL-60S, and its multidrug-resistant (MDR) phenotype HL-60R (Jonsson et al., 1995) were used. Our aim was to study whether CYP autoinduction or suppression is observed at

the mRNA level under continuous exposure to CPA. We also investigated the possible CPA cell toxicity and effect on the *mdr-1* gene under the same conditions.

2. Materials and methods

2.1. Chemical

Cyclophosphamide was purchased from Sigma (Stockholm, Sweden) and dissolved in PBS.

2.2. Cell culture

The human promyelocytic leukemia cell line HL-60S (ATCC, USA) and its classic MDR phenotype, p-gp and *mdr-1* overexpressed subline HL-60R was used. The latter was established by continuous exposure to increasing concentrations of doxorubicin and has previously been characterized (Jonsson et al., 1995). Both cell lines were kept in a medium consisting of RPMI Glutamax supplemented with 10% fetal bovine serum. The cells were cultured at 37 °C in a humidified incubator containing 5% CO_2.

2.3. In vitro drug cytotoxicity assay

Cytotoxicity effect of CPA in HL-60 was determined by two parallel methods. (1) ATP-bioluminescence assay. The number of viable cells is measured as well as their cellular ATP content. The correlation of bioluminescence assay cell viability and its accuracy for in vitro assays has previously been described (Rhedin et al., 1993). The bioluminescence assay was performed automatically in a luminometer (Lucy 1, Anthos, Austria). The ATP monitoring reagent and the ATP standard used were both supplied by Bio Thema, Stockholm, Sweden. The results were given as nmol ATP/sample. The percentage of ATP in a sample when compared to the drug-free control was calculated (Mollgard et al., 2000). (2) Trypan blue exclusion. After staining with trypan blue, viable cells were counted in a microscopic counting chamber.

2.4. CPA IC_{50}

Six different CPA concentrations were used in IC_{50} determination: 50 μg/ml (0.179 nM), 100 μg/ml (0.358 nM), 300 μg/ml (1.1 mM), 500 μg/ml (1.79 mM), 1 mg/ml (3.58 mM), 5 mg/ml (17.9 mM), as well as drug-free control. One-fifth milliliter CPA was added to 1.8 ml of cell suspension (0.55×10^5 cells/ml culture medium) to make the desired final concentrations. CPA exposure experiments were made in duplicate at each concentration, except the controls that were made in quadruplicate. At incubation days 0, 1, 2, 3 and 4, 100 μl of cell suspension was removed, followed by addition of 100 μl of 2.5% trichloracetic acid for the bioluminescence assay. A 100-μl sample of cell suspension was also taken for Trypan blue exclusion assay. These tests were made three times for each of the cell lines.

2.5. Treatment with CPA

Final concentrations of 100 and 500 μg CPA/ml were used in the medium after dilution in 75-cm^2 culture flasks with 0.5×10^6 cells/ml. Control cultures contained the corresponding volume of PBS only. CPA-treated cells were incubated up to 72 h. At the time points 0, 24, 48 and 72 h, samples were removed for transcriptase–polymerase chain reaction (RT-PCR), bioluminescence and Trypan blue assays.

2.6. Extraction of RNA and cDNA synthesis

Total RNA was isolated with QIAamp RNA Mini Kit (Qiagen) from the cells. The concentration and the purity of all RNA samples were determined by measuring their absorbance at 260 and 280 nm with correction for background at 320 nm in a spectrophotometer (Beckman DU® 530 Life Science UV/Vis, USA). RNA concentrations ranged from 450 to 1130 ng/μl, and the A_{260}/A_{280} ratio was between 1.6 and 1.9. Reverse transcription was performed by the First Strand cDNA Synthesis Kit (Pharmacia Biotech, Sweden). Total RNA (1.5 μg) was used to make a final 15-μl cDNA. The reaction was incubated at 37 °C for 1 h, and heat denatured at 95 °C for 5 min. Samples were either used immediately for PCR or stored at −70 °C.

2.7. RT-PCR for CYP enzyme profiles

Ten CYP enzymes were investigated: CYP1A1, 1A2, 1B1, 2B6, 2D6, 2E1, 2C9, 2C19, 3A4 and 3A5. The housekeeping gene β-actin was used as cDNA template control. "No template" controls, as well as human normal blood samples and specific CYP-positive liver samples were included in parallel for control of reactions. The primers used for CYP-specific PCR amplification were designed and made by Finnstrom et al. (2001a). The primers were constructed to span at least one intron to exclude genomic DNA contamination. PCR reactions were carried out in a final volume of 50 μl containing: 4 μl cDNA, 5 μl 10× PCR buffer (Mg^{2+} free), 2 mM $MgCl_2$, 40 pmol forward and reverse primers and 0.5 U Taq Polymerase (Saveen 5 U/μl). The PCR was performed in a "GeneAMP® PCR system 9700 land" as follows: 95 °C for 5 min; 32–35 cycles at 95 °C for 30 s, 53 °C for 30 s and 72 °C for 1 min; with a single final extension at 72 °C for 10 min. A 20-μl sample of PCR product was electrophoresed on a 2% agarose gel visualized by ethidium bromide. A 100-bp DNA ladder (Promega) was included as size marker. The picture was scanned and photographed by "Bio-RAD® CHEMI DOC" UV camera, with data analyzed by "Quantity One software, Version 4.1 USA".

2.8. Semiquantitative RT-PCR for CYP1B1 and β-actin

We re-optimized the RT-PCR conditions for CYP1B1 and β-actin in order to set up a semiquantitative method. For both genes, PCR reactions were carried out in a final volume of 25 μl containing: 2.5 μl 10× PCR buffer (Mg^{2+}-free), 2 mM $MgCl_2$, 40 pmol forward and reverse primers and 0.5 U Taq Polymerase (Saveen 5 U/μl). For CYP1B1, 10 μl 1:10 diluted cDNA was used as template, whereas for β-actin, only1 μl was used. To make sure that the PCR amplification for CYP1B1 and β-actin was operating in the exponential range, we performed a linearity test. On the basis of this test, 32 cycles for CYP1B1 and 30 cycles for β-actin were chosen for PCR condition. The other conditions were followed as described above.

The between-reaction coefficient of variation (CV) was calculated as 11.5% from duplicate PCR on the same cDNA preparation of 26 pairs (HL-60R).

In order to get reliable and precise results, we cultured the cells from three batches. From each culture, we prepared cDNA at least twice on different days and the total RNA concentration was measured each time. From each cDNA sample, PCR was run in duplicate and the PCR procedure was repeated at least twice.

2.9. Real-time PCR in TAQman

The results from RT-PCR were confirmed with real-time PCR in an ABI Prism 7700 machine (Applied Biosystems, Foster City, CA, USA). Using 1:30 diluted solutions from cDNA samples, the CYP1B1 mRNA were quantified. In addition, we quantified CYP3A4 and *mdr-1* mRNA alteration under CPA treatment with both concentrations. The primers and probes were designed and optimized (Finnstrom et al., 2001b).

3. Results

3.1. In vitro drug cytotoxicity assay

High correlation between the Trypan blue exclusion assay and the ATP bioluminiscent assay was observed ($r^2 = 0.97$). Since the ATP assay provides more precise and reliable values, we used the data from this method in the results.

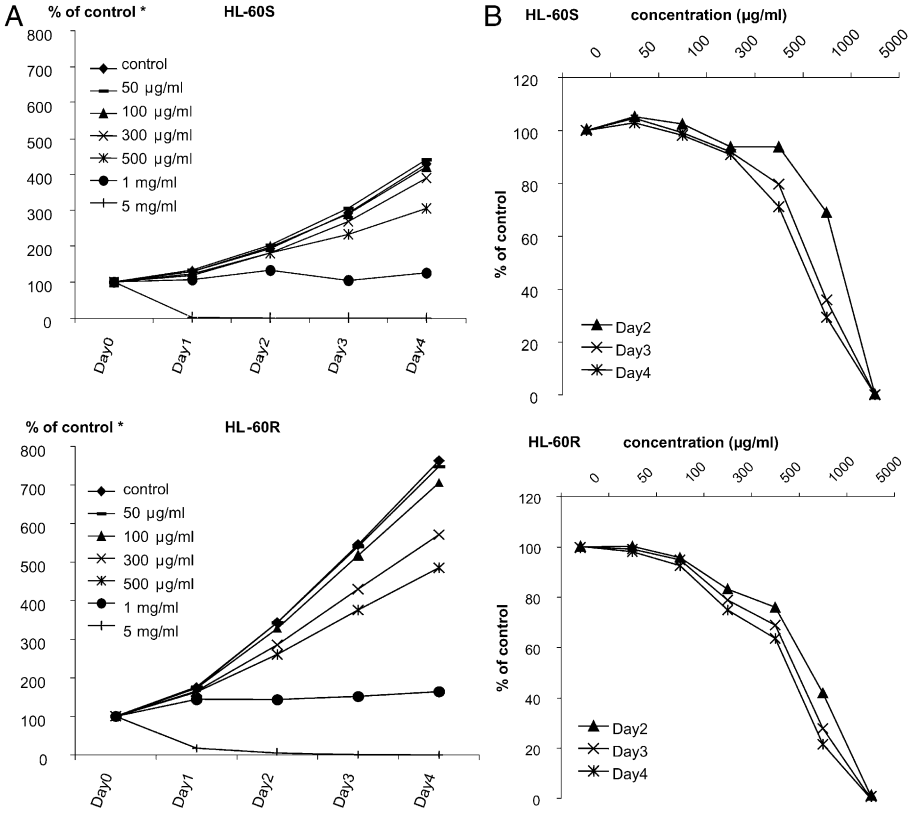

Fig. 2. (A) Cell growth curves. HL-60S and HL-60R cells were treated with six different concentrations of cyclophosphamide over 4 days, together with untreated control. * The percentage of ATP when compared to drug-free control at day 0. (B) Concentration–response curves. At days 2, 3 and 4, cytotoxicity of CPA (represented as percentage of drug-free control) was plotted versus concentrations.

Table 1
Cyclophosphamide IC_{50} in HL-60 cell line

	IC_{50} (μg/ml)[a]		RF[b]	P[c]
	HL-60S	HL-60R		
Day 2	5507 (2082)	1690 (404)	0.31	0.04
Day 3	1186 (28)	1017 (127)	0.86	0.18
Day 4	861 (34)	829 (79)	0.96	0.64

[a] Cyclophosphamide IC_{50} values (μg/ml) at days 2, 3 and 4 in HL-60S and HL-60R cell lines are presented as mean and (S.D.). Triplicate experiments were run in parallel for both cell lines, with six concentrations from 50 to 5000 μg/ml. GraphPad PRISM software was used for calculating the IC_{50} values.

[b] RF (Resistance Factor) = IC_{50} in HL-60R/IC_{50} in HL-60S.

[c] Statistical analysis was done using STATISTICA software for the comparison of IC_{50} between HL-60S and R cell lines.

3.2. IC_{50} determination

IC_{50} values reflect the concentration at which CPA causes 50% cell growth inhibition and were calculated on the basis of "cell growth curves" (Fig. 2A). Cytotoxicity of CPA to HL-60S and HL-60R cells was expressed as "response over time curve". The response at 48, 72 and 96 h, was plotted versus concentrations as "concentration–response curve" (Fig. 2B) and IC_{50} was calculated (Dhar et al., 1996, 1998) as can be seen in Table 1. The IC_{50} value was not calculated at 24 h since the cells had not yet recovered from the lag phase. The IC_{50} was significantly higher ($p < 0.05$) at 48 h for HL-60S compared to HL-60R. The resistance factor (RF) was defined as the IC_{50} value obtained from the resistant subline divided by the IC_{50} from the parental cell line. RF of the p-gp-related MDR phenotype HL-60R against CPA was 0.31 (48 h), 0.86 (72 h) and 0.96 (96 h), with a mean of 0.71 (Table 1). Our results demonstrate that: (a) the resistant cell line grows faster than its sensitive cell line; (b) exposure of HL-60 sensitive and resistant cell lines to CPA leads to suppression of cell growth and proliferation in a concentration and time-dependent manner. HL-60R, the classical multidrug-resistant phenotype, was not cross-resistant to CPA. On the contrary, HL-60R exhibits a higher sensitivity to CPA over time, compared to its parental cell line.

3.3. Profile of CYP enzyme gene expression

Among the 10 investigated CYPs, only CYP1B1 was expressed in both HL-60S and HL-60R cell lines. CYP1A1 was weakly expressed in HL-60R. None of the other CYPs was expressed at detectable levels in the cell lines.

3.4. CPA induction studies

No induction of CPA metabolism or mRNA specific for CYP2B6 and CYP 3A4/5 was observed at 100 or 500 μg/ml concentration of CPA.

3.5. Effects of CPA on CYP1B1 and β-actin gene expression

CYP1B1 gene expression in the HL-60S and R cell lines was suppressed at both concentrations of CPA (Figs. 3, 4). The effect was more pronounced at the higher concentration of CPA. The nadir of CYP1B1 mRNA was observed after 24 h at 100 μg/ml and after 48 h at 500 μg/ml. Compared to sensitive cells, the resistant cells were more suppressed by CPA and recovered more slowly (Fig. 4). Suppression on β-actin in both HL-60 sensitive and resistant cells was observed, except at 100 μg/ml CPA in HL-60S cells (Fig. 4). The extent of suppression was less on β-actin in both cell lines. The CPA effect on β-actin was more pronounced at 500 μg/ml CPA and more suppressed in HL-60R than in HL-60S cells similar to the effects on CYP1B1.

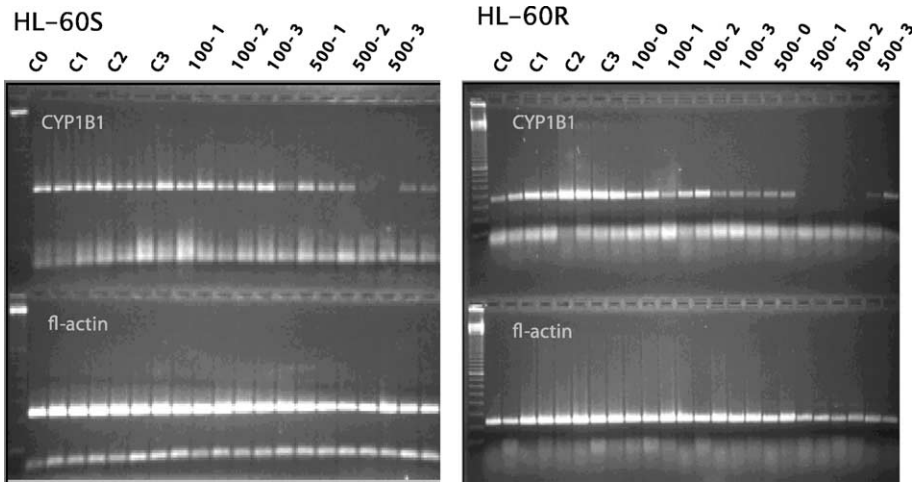

Fig. 3. RT-PCR image. Lanes C0 to C3 represent control cells at days 0 to 3; Lanes 100-0 to 100-3 represent 100 μg/ml CPA-treated cells at days 0, 1, 2 and 3, respectively; Lane 500-0 to 500-3 represent 500 μg/ml CPA treated cells at days 0, 1, 2 and 3, respectively.

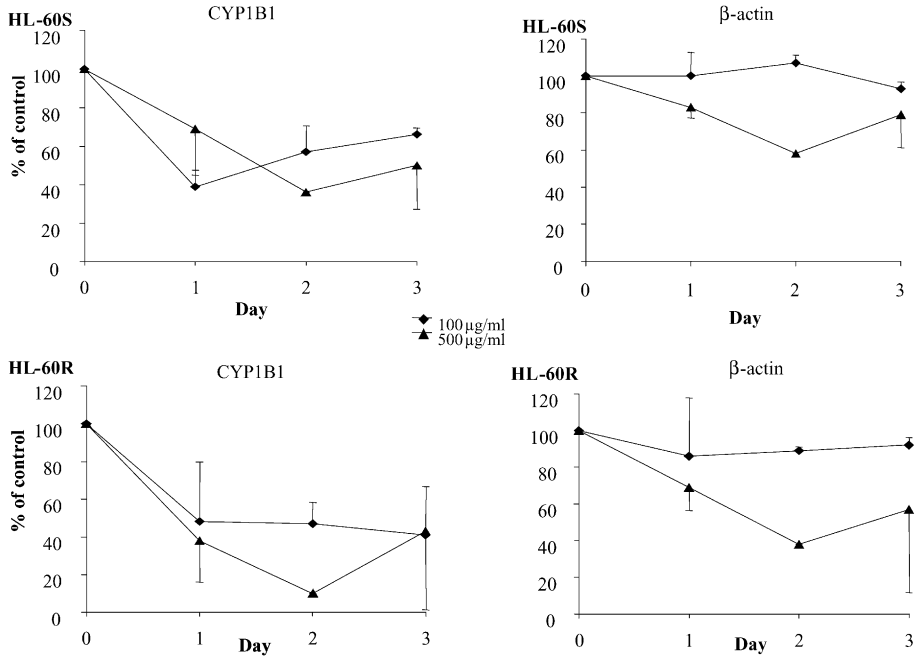

Fig. 4. Effect of CPA on CYP1B1 and β-actin mRNA in HL-60S and HL-60R cell lines. *Y*-axis represents percentage of mRNA values in CPA-treated cells compared to untreated control. *X*-axis represents time points for cell culture at days 1, 2 and 3. Standard deviation at day 2 (500 μg/ml CPA) ranged from 1–3%.

3.6. Assays of mRNA by RT-real-time PCR

Results from real-time PCR confirmed the previous data. The reproducibility between duplicates was high, with an inter-assay CV of 1.6%. No inducting effect on CYP3A4 could be detected and no upregulated *mdr-1* expression was observed in either cell line (Fig. 5).

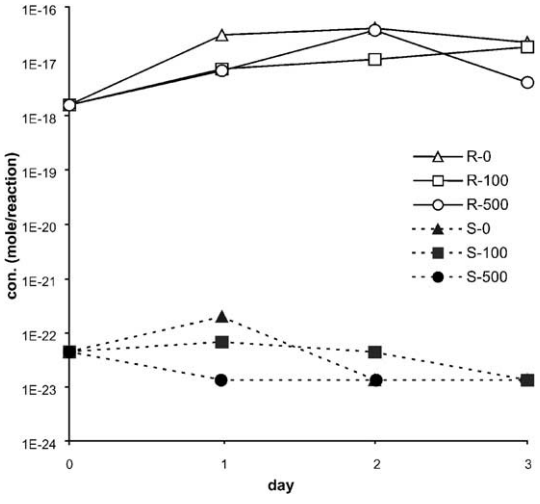

Fig. 5. Real-time PCR quantification of *mdr-1* mRNA in HL-60S and HL-60R cells under 3 days' cyclophosphamide treatment. Open symbols represent resistant cells; closed symbols represent sensitive cells. Concentrations of CPA (μg/ml) are indicated after each symbol.

4. Discussion

Cyclophosphamide can modulate hepatic cytochromes *P*450 in the rats and humans (Chang and Waxman, 1993; Laslett et al., 1995; McClure and Stupans, 1992, 1995). Several groups have independently investigated the role of CPA as a modulator of various rodent CYPs. The suppressing effect of CPA on hepatic CYPs in vivo and/or in vitro has been shown on the functional, the protein, as well as the mRNA levels. In particular, the CYP2C11 and CYP2C12 that are male and female specific, respectively, were suppressed (Kraner et al., 1996).

In view of the clinical importance of the bioactivation reaction of CPA, we have studied the effect of CPA on the CYP enzyme gene expression profile in the promyelocytic sensitive and resistant HL-60 cell line. Furthermore, the only two studies (Chang et al., 1997b; Gervot et al., 1999) have given different results. One study demonstrated that CYP3A4, CYP2C8 and CYP2C9 protein were increased by CPA in primary human hepatocyte cell cultures (Chang et al., 1997b). In the other study (Gervot et al., 1999), however, evidence was presented that CPA could induce both CYP2B6-specific protein and mRNA and the CYP3A4 protein but not CYP3A4-specific mRNA. At present, no confirming data on these matters from in vivo studies have been reported.

Among our 10 investigated cytochromes, only CYP1B1 was clearly expressed in both S and R cell lines. Under CPA treatment, no inducing effect on either of CYP

enzymes was observed. In contrast, CYP1B1 expression was suppressed.

Since its discovery in 1994 (Savas et al., 1994), CYP1B1 was found in at least 15 human tissues, especially in extrahepatic tissues such as lung, kidney, blood, brain, steroidogenic and mammary tissues (Murray et al., 2001). Interestingly, CYP1B1 was also detected in many carcinomas, such as mammary, lung, uterine and prostatic tumors, but not in the normal tissues adjacent to the tumor cells (Mollerup et al., 2001; Murray et al., 2001; Rochat et al., 2001; Zheng et al., 2000). This CYP may play a role in tumor-selective drug inactivation and resistance-related mechanism (Murray et al., 2001; Rochat et al., 2001). In our experiments, we also observed the CYP1B1 high expression in yet another tissue: hematopoietic tumor cells. This shows that CYP1B1 may be overexpressed also in hematological malignant cells. Further studies are needed to verify these data.

CYP1B1 plays an important role not only in bioactivation of a number of environmental carcinogens, but also in the metabolism of hormones. The activation of both carcinogenic polycyclic aromatic hydrocarbons and aryl amines can be catalyzed by CYP1B1. The estradiol 4-hydroxylase activity and hydroxylation of 17β-estradiol at the C-4 position has recently been investigated (Spink et al., 2000). In addition, this CYP also plays an important role in testosterone metabolism (Hudson et al., 2001), but the possible role of CYP1B1 as a catalyst of chemotherapeutic drugs is still being investigated. According to a report (Roy et al., 1999), CYP1B1 is not involved in the metabolism of CPA.

Here, we report for the first time the CYP1B1 expression in HL-60 sensitive and resistant cells. We also demonstrate in vitro that CPA may modulate the gene expression of a CYP enzyme in humans. In our human hematopoietic cell model, CYP1B1 expression was significantly suppressed by CPA treatment. The same pattern was observed generally in HL-60 and in its p-gp-related MDR subline.

The CYP1B1-related metabolic potential in the cells might play a role in the cellular damage upon exposure to certain drugs or compounds. The suppression caused by CPA on CYP1B1 emphasizes the importance of considering possible drug–drug interactions in the design of clinical protocols.

The β-actin gene is supposed to be constantly expressed in most tissues and cells. The role of its relevant protein product in the function of eukaryotic cells is ubiquitous and essential. Its expression level is relatively stable among tissues and at different stages of cell growth. Therefore, β-actin is often used as an endogenous control for in quantitative studies (Bustin, 2000).

We observed a suppression of β-actin gene expression in HL-60 sensitive and resistant cell lines, which could reflect the cytotoxic effect of this prodrug on the tumor cells, in the absence of an apparent metabolism and bioactivation of CPA. In our present study, we demonstrated that no CPA-related CYP enzyme was detected or inducible, yet it exerted significant cytotoxicity. One possible explanation could be that CPA blocked the cell division during cell cycle by repressing the cellular β-actin gene, thereby leading to cell death. The higher chemosensitivity to CPA treatment in faster growing HL-60R cells implicates that highly proliferating cells can be more attacked by β-actin repression. Thereby, more cells can be blocked in G2 phase due to lack of protein needed for division. The cells would subsequently undergo apoptosis. This hypothesis needs to be further verified. Our findings warrant against a critical use of β-actin as a housekeeping gene.

There is a wide variety of dose regimens of CPA (450 mg/m^2 up to 7 g/m^2) (Boddy and Yule, 2000). It is usually administered as an intravenous infusion over a period of 1 h. Alternatively, the total dose is fractionated over several days. In our cell line model, CPA exerted its cytotoxicity in a dose-dependent and time-dependent manner, which indicates that the use of prolonged infusion time at a slower administration rate might be as effective as a short infusion of a high dose. However, the low dose with prolonged infusion might result in a significant decrease of the treatment-related toxicity.

The well-known major mechanisms of multidrug resistance in cancer patients involves at least six systems including the MDR genotypes, glutathione *S*-transferase detoxification, low expression of topoisomerase II, DNA repair, CYP-related drug activation and inhibition of apoptosis (Ishii and Kitada, 1997). In cancer patients, the mechanisms of resistance to CPA usually involve upregulated glutathione *S*-transferase activity, alteration of DNA repair, absence of relevant CYP catalysts, anti-apoptosis gene expression, increased aldehyde dehydrogenases activity (Boddy and Yule, 2000), etc. However, no correlation between p-gp-related phenotype and resistance to CPA was identified in the present study. By using the myeloma-originated cell line RPMI 8226S and its P-gp-related phenotype 82261R, Dhar et al. (1996) tested the cytotoxicity of eight alkylating agents. The RF (resistance factors) reported in their study ranged from 0.74 to 1.08, these values were calculated at 72 h post-CPA incubation. In our investigation, the RF in HL-60 was 0.86 (at 72 h), which is in agreement with their results. This indicates that p-gp is not involved in the mechanisms of action of CPA. This *mdr-1* gene can be easily induced, which can cause great clinical therapeutic problem. In our study, however, no induction of *mdr-1* mRNA was observed in the cell lines. This suggests that cyclophosphamide is still an important option in the choice of chemotherapeutic strategies for drug-resistant malignancies.

Acknowledgements

This work was supported by grants from the Swedish Cancer Society, the Medical Research Council (04496), the

Swedish Children Cancer Foundation (grant no. PROJ01/059) and Stockholm's Cancer Society (grant no. 02:119).

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