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Cytochrome P450 reductase dependent inhibition of cytochrome P450 2B1 activity: Implications for gene directed enzyme prodrug therapy

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ABSTRACT

Cytochrome P450 (P450) enzymes are often used in suicide gene cancer therapy strategies to convert an inactive prodrug into its therapeutic active metabolites. However, P450 activity is dependent on electrons supplied by cytochrome P450 reductase (CPR). Since endogenous CPR activity may not be sufficient for optimal P450 activity, the overexpression of additional CPR has been considered to be a valuable approach in gene directed enzyme prodrug therapy (GDEPT). We have analysed a set of cell lines for the effects of CPR on cytochrome P450 isoform 2B1 (CYP2B1) activity. CPR transfected human embryonic kidney 293 (HEK293) cells showed both strong CPR expression in Western blot analysis and 30-fold higher activity in cytochrome *c* assays as compared to parental HEK293 cells. In contrast, resorufin and 4-hydroxy-ifosfamide assays revealed that CYP2B1 activity was up to 10-fold reduced in CPR/CYP2B1 cotransfected HEK293 cells as compared to cells transfected with the CYP2B1 expression plasmid alone. Determination of ifosfamide-mediated effects on cell viability allowed independent confirmation of the reduction in CYP2B1 activity upon CPR coexpression. Inhibition of CYP2B1 activity by CPR was also observed in CYP2B1/CPR transfected or infected pancreatic tumour cell lines Panc-1 and Pan02, the human breast tumour cell line T47D and the murine embryo fibroblast cell line NIH3T3. A CPR mediated increase in CYP2B1 activity was only observed in the human breast tumour cell line Hs578T. Thus, our data reveal an effect of CPR on CYP2B1 activity dependent on the cell type used and therefore demand a careful evaluation of the therapeutic benefit of combining cytochrome P450 and CPR in respective *in vivo* models in each individual target tissue to be treated.

1. Introduction

Cytochrome P450 (P450) enzymes are widely expressed in prokaryotes and eukaryotes. P450 is involved in the metabolic oxidation and reduction of a variety of compounds including xenobiotics used in tumour therapy [1]. Mammalian P450 enzymes are mainly expressed in the liver and only at low levels in other tissues or tumours [2]. Thus, delivery of exogenous P450 genes to tumours is used in gene-directed enzyme prodrug therapy (GDEPT) to enable the hydroxylation

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of chemotherapeutic prodrugs including cyclophosphamide and ifosfamide (IFO) into their active forms [3,4].

The flavoprotein NADPH cytochrome P450 reductase (CPR) interacts with P450 as an electron donor and catalyses P450 monooxygenase reactions [5]. Most tested drugs are activated more efficiently in microsomes containing P450 and CPR as compared to P450 alone [6,7]. Recently, the influence of CPR on P450 has also been shown in transgenic mice, which carry a liver-specific deletion of CPR leading to a reduced P450 activity in this organ [8].

Since the amount of P450 often exceeds that of CPR in microsomal membranes, especially when P450 is overexpressed in GDEPT, the CPR/P450 ratio has been increased by coexpression of CPR to provide an enhanced supply of electrons, thereby enhancing P450 function and thus resulting in a superior therapeutic effect [9]. For instance, the human P450 isoform CYP3A4 was overexpressed in V79 Chinese hamster lung fibroblast cells and CYP3A4 enzymatic activity was measured by the dealkylation and deethylation of 7-alkoxyresorufins [10]. CPR coexpression in these cells stimulated P450 activity 5–10-fold suggesting a lack of endogenous CPR [11]. In other studies the CYP3A4 cDNA was amplified in Chinese hamster ovary cells to obtain high level expression of CYP3A4. Here coexpression of CPR increased CYP3A4 activity 15-fold, although P450/CPR coexpression was accompanied by a decrease in the amount of immunologically and spectrally detectable P450 [12]. CPR overexpression was assumed to increase heme-oxygenase activity, which decreases intracellular heme and thereby P450 protein levels [12].

Coexpression of CPR in P450 mediated GDEPT has been described previously to be effective in 9L rat glioma derived cells, where cell killing upon cyclophosphamide treatment was increased in rat CPR/CYP2B1 coexpressing cell clones as compared to cells expressing CYP2B1 alone [7]. CPR overexpression increased the concentration of toxic derivatives of cyclophosphamide in the presence of CYP2B1 leading to a better therapeutic effect [7].

In this study we evaluated the effect of CPR coexpression on CYP2B1 activity in different cell lines such as NIH3T3 mouse fibroblast cells and HEK293 human embryonal kidney cells, as well as in two human breast cell lines (T47D, Hs578T) and two human pancreatic tumour cell lines (Pan02, Panc-1). Cell lines with and without CYP2B1 and/or CPR expression cassettes were analyzed for the levels of respective protein expression. CPR function analysed in cytochrome *c* assays and CYP2B1 activity determined in metabolic resorufin, 4-hydroxylation and cell viability assays revealed no or even a dose dependent inhibiting effect of CPR on CYP2B1 activity in the majority of cell lines tested.

2. Material and methods

2.1. Plasmid construction

Construction of plasmids pCMV-CYP2B1 encoding the CYP2B1 gene under control of the immediate early cytomegalovirus (CMV) enhancer/promoter region, as well as the retroviral vector plasmid pPCCMm1, which drives CYP2B1 expression from mouse mammary tumour virus (MMTV) promoter in infected cells have been described elsewhere [13,14]. CPR cDNA was cloned from rat liver by isolating mRNA using the RNeasy Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, and reverse transcribed using primer 5′-CTG ATC TAG ACT CGA GCT AGC TCC ACA CAT CTA GTG AGT AGC GGC CCT TG-3′. The obtained cDNA was PCR amplified using additionally the forward primer 5′-CGC CAA GCT TCT CGA GCA CCA TGG GGG ACT CTC ACG AAG ACA CCA GTG CCA C-3′, and the resulting fragment was inserted into the expression plasmid pcDNA3.1 (Invitrogen, Lofer, Austria) via *Hind*III/*Xba*I restriction sites resulting in plasmid pCMV-CPR. To construct the retroviral vector plasmid pLCSN, the CYP2B1-fragment was released from plasmid pc3/2B1 [3] using *Eco*RI and *Sma*I restriction enzymes and inserted into the large *Eco*RI/*Hpa*I fragment of plasmid pLXSN [15].

2.2. Cell lines, transfection and infection

Human 2GP19Talf amphotropic retroviral packaging cells [16], human HEK293 [17], Pan02 [18], Panc-1 [19] and murine NIH3T3 [20] cells were grown in Dulbecco's modified Eagle's medium/Glutamax (DMEM, Invitrogen) supplemented with 10% fetal calf serum (FCS, Invitrogen). DMEM containing 10% FCS and 10 mg/l insulin (Sigma, Schnelldorf, Germany) was used to cultivate Hs578T cells [21]. T47D cells [22] were grown in RPMI 1640 medium (Invitrogen) supplemented with 10% FCS and 10 mg/l insulin.

Transfections were performed using Lipofectamin 2000 as recommended by the supplier (Invitrogen). In dose-dependent experiments, 100 fmol of plasmid pCMV-CYP2B1 and 0, 12.5, 25, 50, 100 and 200 fmol of plasmid pCMV-CPR and 20 fmol of plasmid pCMV-DsRed-Express (Becton-Dickinson, Schwechat, Austria) as transfection control, respectively, were supplemented with plasmid pcDNA3.1 to a final amount of 320 fmol plasmid DNA, which was then transfected into the respective cell lines. Forty-eight hours after transient transfection, cells were subjected to fluorescence activated cell sorting (FACS), cytochrome c, resorufin and 4-hydroxy-ifosfamide hydroxylation assays.

Infection of 4×10^5 target cells with virus supernatant from 2×10^6 virus producing cells was performed as described previously [16]. For the generation of stable CYP2B1 expressing cell populations, cells infected with vectors LCSN and PCCMm1, respectively, were selected and maintained in the presence of 0.4 mg/ml geneticin (G418, Invitrogen). Cells stably transfected with plasmid pCMV-CPR were selected and cultivated in the presence of 0.3 mg/ml hygromycin B (Sigma).

2.3. FACS analysis

Cells were trypsinised and washed twice with phosphate-buffered saline (PBS). Then, 10,000 cells per sample were analysed for red fluorescence by FACS (FACSCalibur, Becton-Dickinson), by exciting at a wavelength of 488 nm and detecting in the spectrum of 585 ± 21 nm. The percentage of red fluorescing cells determined indicates the transfection efficiency. For determination of green fluorescence, cells were excited at a wavelength of 488 nm and emission was detected in the spectrum of 530 ± 15 nm. Experiments were performed

twice in triplicates and mean values with corresponding standard errors were calculated.

2.4. Western blot analysis

Briefly, cell monolayers were washed with cold PBS, lysed in buffer (25% glycerol, 0.1 M Tris–HCl pH 6.8, 1% Triton X-100, 1 mM PMSF) and sonicated. Protein concentration of lysate was measured using the DC protein assay kit (Bio-Rad, Vienna, Austria). Thirty micrograms of the protein extract and 10 μl of a prestained protein standard (GE Healthcare, Freiburg, Germany), respectively, were separated using SDS polyacrylamide gel electrophoresis and blotted onto a polyvinylidene fluoride membrane (Amersham Biosciences, Little Chalfont, United Kingdom). The membrane was blocked using 5% milk powder in PBS supplemented with 0.1% Tween 20 and probed using a polyclonal goat anti-CYP2B1 antibody (Becton-Dickinson) diluted 1:5000 and a polyclonal goat anti-CPR antibody (Daiichi Pure Chemicals, Tokyo, Japan) diluted 1:10000 in PBS. After three washing steps with PBS, the membrane was incubated with a horseradish-peroxidase-linked immunoglobulin G anti-goat antibody (Dako, Glostrup, Denmark). Membranes were treated with the enhanced chemiluminescence system (ECL; Amersham Biosciences) and signals were visualized by exposure on X-ray films (Amersham Biosciences). Signal intensities were quantified using the Adobe Photoshop 7.0 programme. Additionally, load of equal amounts of protein per lane was verified in respective Coomassie staining.

2.5. NADPH-cytochrome P450 reductase assay

Cells were collected in PBS supplemented with 1% Triton X-100 (Sigma) and lysed by sonication to generate whole-cell extracts. Fifty micrograms of protein samples were incubated at room temperature using the Genetest NADPH regenerating system according to the manufacturer's instructions (Becton Dickinson, Heidelberg, Germany). The chromophore of cytochrome c (Sigma–Aldrich) which is reduced by CPR in the presence of NADPH absorbs at 550 nm with $\varepsilon = 21{,}000\ M^{-1}\ cm^{-1}$. The rate of cytochrome c reduction was monitored over a 5 min interval on a U-1100 spectrophotometer (Hitachi, Düsseldorf, Germany). Data are shown as absolute values unless indicated otherwise.

2.6. Resorufin assay

For determination of CYP2B1 activity 1×10^5 HEK293, Panc-1, NIH3T3, Pan02, T47D, and Hs578T cells were seeded 72 h after transfection. Then, an excess of 7-pentoxyresorufin was added to a final concentration of 1.5 mM and the cells were incubated for 4 h at 37 °C. Using an excitation wavelength of 520 nm, fluorescence was detected at an emission wavelength of 590 nm in a microplate reader (Tecan, Salzburg, Austria). Resorufin formation was determined by comparing the fluorescence of samples to the resorufin standards. Experiments were performed twice in quadruplicates. Data are shown as absolute resorufin values and expressed as mean values with bars reflecting the standard error unless indicated otherwise. All analyses were conducted within linear ranges in respect to cell number and incubation time.

2.7. Measurement of 4-hydroxy-ifosfamide metabolites

Cells treated with IFO were monitored for their ability to release 4-hydroxy-ifosfamide into the culture media according to the method described by Schwartz et al. [23]. Briefly, 1×10^6 cells were incubated for 8 h in 2 ml DMEM with 10% FCS containing 1 mM IFO and 5 mM semicarbazide to trap and stabilize the 4-hydroxy metabolites of IFO. Then, a 125 μl aliquot of this medium was incubated with 67 μl of 1 M HCl solution supplemented with 6 g/l 3-aminophenol and 6 g/l hydroxylamine for 30 min at 90 °C. Using an excitation wavelength of 350 nm, fluorescence was detected at an emission wavelength of 515 nm in a microplate reader (Tecan). 4-Hydroxy-ifosfamide formation was determined according to 4-hydroperoxyifosfamide standards (Baxter, Vienna, Austria). Experiments were performed twice in triplicate. Data are normalized to pCMV-CYP2B1-transfected HEK293 and Panc-1 cells, respectively, and expressed as mean values with bars reflecting the standard error.

2.8. Cytochrome P450 CO difference spectrum

Briefly, 5×10^6 HEK293 cells were transfected in 10 cm tissue culture dishes with 800 fmol of plasmid pCMV-CYP2B1, 160 fmol of plasmid pCMV-DsRed-Express and 1600 fmol either of plasmid pCMV-CPR or pcDNA3.1 using Lipofectamin 2000 as described above. Forty-eight hours after transfection, cells were harvested with a rubber policeman into 1 ml Et_3N buffer (250 mM sucrose, 10 mM triethanolamine, 1mM EGTA, pH 7.4) per dish. Cells were lysed in a 5 ml Potter tube with a tight Teflon pestle for 4 min and subsequently centrifuged at $9700 \times g$ for 10 min to remove nuclei, mitochondria and cell debris. CO difference spectra were performed with minor modifications as described previously [12]. Briefly, a few grains of sodium dithionite were dissolved in the cell lysate supernatant. Spectrum 1 was measured and recorded, the same sample was gently bubbled with carbon monoxide for 1 min and spectrum 2 was measured and recorded. Then, the difference spectrum (spectrum 2 minus spectrum 1) was calculated [24].

2.9. Cell viability assay

Five $\times 10^3$ HEK293 cells, HEK293 cells cotransfected with 200 fmol plasmid pCMV-CRP and 100 fmol plasmid pCMV-CYP2B1, and HEK293 cells cotransfected with 200 fmol plasmid pcDNA3.1 and 100 fmol plasmid pCMV-CYP2B1 were seeded in the wells of a 96-well plate and exposed once to 1, 0.5, 0.25 and 0 mM ifosfamide, respectively. Five days later, cells were incubated for 4 h in XTT solution of the Cell Proliferation Kit II (Roche, Mannheim, Germany) and subsequently analysed in a microplate reader (Tecan) at an excitation wavelength of 490 nm and an emission wavelength of 690 nm. Experiments were performed in quadruplicates and standard errors calculated.

3. Results

3.1. Expression and function of CPR and CYP2B1

The influence of CPR on CYP2B1 activity in various human cell lines was first investigated in transient cotransfection analyses.

Table 1 – Comparison of CPR and CYP2B1 activity in parental cells and cells transfected with respective CPR and CYP2B1 expression vectors

	Cytochrome c reduction (nmol/(min mg)) CPR		Resorufin assay (μM) CYP2B1	
	−	+	−	+
[a]HEK293	$7.8 \pm 2.0^{***}$	$201.5 \pm 40.4^{***}$	0	$4.1 \pm 0.2^{***}$
Panc-1	$2.1 \pm 1.0^{***}$	$16.3 \pm 0.6^{***}$	0	$2.4 \pm 0.2^{***}$

[a] HEK293 and Panc-1 cells were transiently transfected with expression plasmids pCMV-CPR (CPR+), pCMV-CYP2B1 (CYP2B1+) and the non-coding control vector pcDNA3.1 (CPR−, CYP2B1−), respectively. The CPR activity of cell lysates was determined in cytochrome c reduction assays and the CYP2B1 activity in resorufin assays. Number of cytochrome c reduction is $n \geq 5$ and resorufin assay is $n = 4$. $^{***}P < 0.001$, compared to parental cells

For this the CPR coding sequence was isolated using RT-PCR techniques and inserted into an expression plasmid under control of the CMV immediate early enhancer/promoter region to allow constitutive and high level gene expression. Transient transfections of 200 fmol of the resulting plasmid pCMV-CPR into HEK293 cells revealed a CPR-mediated reduction of cytochrome c at 201.5 ± 3.5 nmol/(min mg). This is an almost 30-fold higher CPR activity as compared to the CPR activity of non-transfected HEK293 cells, indicating high-level, functional CPR expression (Table 1). Western blot analysis of these cells using an anti-CPR specific antibody showed a strong CPR specific signal at its expected size of 78 kD (Fig. 1A, upper panel, lane 6), whereas endogenous CPR protein was not detectable in non-transfected HEK293 cells (Fig. 1A, upper panel, lane 7). Panc-1 cells transiently transfected with plasmid pCMV-CPR revealed a strong CPR expression as well, as shown in Western blot analysis (Fig. 1B, upper panel, lane 6). Corresponding CPR-mediated reduction of cytochrome c was observed at 16.3 ± 0.17 nmol/(min mg), which is eight-fold higher as compared to parental Panc-1 cells, but significantly lower than in transfected HEK293 cells (Table 1). In non-transfected Panc-1 cells and Panc-1 cells transfected with plasmid pCYP2B1 only, no or a very weak endogenous CPR expression could be detected (Fig. 1B, upper panel, lanes 1 and 7).

Transient transfection of HEK293 and Panc-1 cells with the CYP2B1 expression plasmid pCMV-CYP2B1 and subsequent Western blot analysis using an anti-cytochrome P450 specific antibody revealed a strong signal specific for cytochrome P450 at the expected size of 56 kD (Fig. 1, lower panels, lanes 1–6). No endogenous cytochrome P450 expression was observed in respective non-transfected cells (Fig. 1, lower panels, lane 7). In pCMV-CYP2B1 transfected HEK293 and Panc-1 cells, respectively, 4.1 ± 0.2 and 2.4 ± 0.2 μM resorufin were converted from an excess of pentoxyresorufin substrate (Table 1), whereas, as expected, in non-transfected cells no conversion of resorufin was observed (Table 1).

3.2. *Dose dependent CPR-mediated inhibition of CYP2B1 activity*

To analyze the effect of CPR on CYP2B1 activity, we transiently transfected HEK293 and Panc-1 cells with constant amounts of plasmid pCMV-CYP2B1 and increasing amounts of plasmid pCMV-CPR. Differences in the amount of transfected DNA were adjusted by adding respective quantities of a non-coding plasmid. The accumulation of CPR protein in these cell lines upon transfection with increasing amounts of the CPR expression plasmid is illustrated in Western blot analysis (Fig. 1, upper

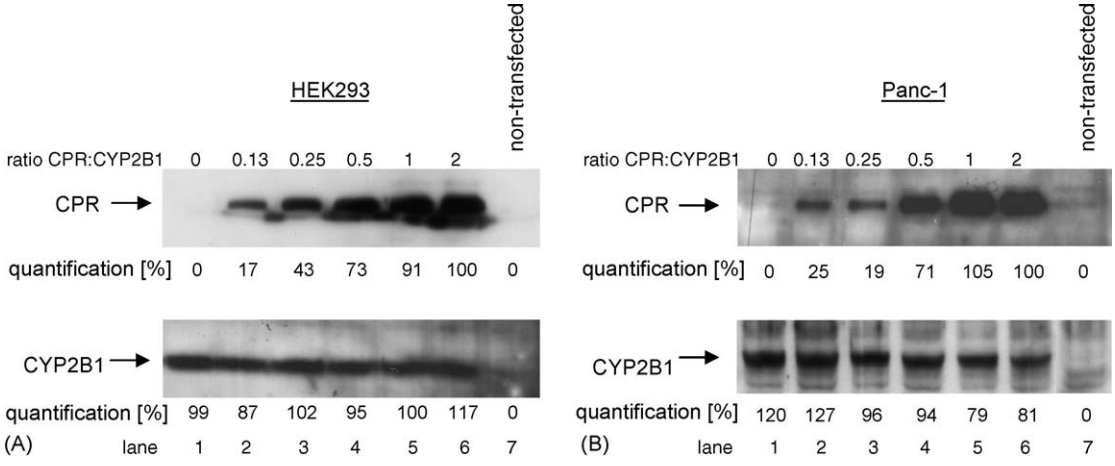

Fig. 1 – Determination of CYP2B1 and CPR levels in transfected cells. HEK293 cells (A) and Panc-1 cells (B) were transiently transfected with constant amounts of the CYP2B1 expression plasmid pCMV-CYP2B1 and variable amounts of the CPR expression plasmid pCMV-CPR as indicated (lanes 1–6) or remained non-transfected (lane 7). Two days later, similar amounts of total protein from cell lysates and analysed using anti-CPR (upper panels) and anti-CYP2B1 (lower panels) specific antibodies, respectively. Signal intensities were quantified using the Adobe Photoshop 7.0 programme.

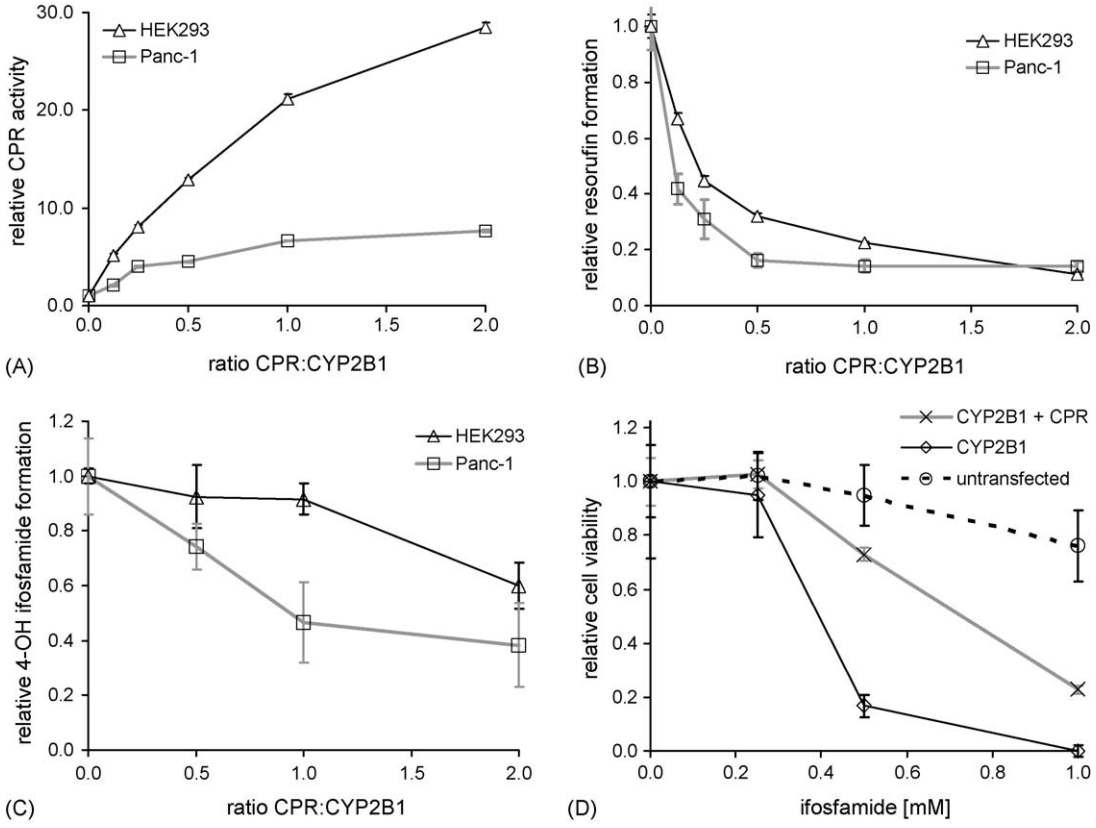

Fig. 2 – Dose dependent effects of CPR on CYP2B1 function. HEK293 and Panc-1 cells were transiently cotransfected with constant amounts of plasmid pCMV-CYP2B1 and variable amounts of plasmid pCMV-CPR. (A) The CPR activities of the cotransfected cells relative to parental HEK293 and Panc-1 cells were determined in cytochrome c reduction assays. The CYP2B1 activity of the cotransfected cells as determined by the formation of (B) resorufin and (C) 4-hydroxy-ifosfamide is depicted relative to the activities in the respective cell lines transfected with plasmid pCMV-CYP2B1 only. (D) After treatment with different concentrations of IFO the relative cell viability of HEK293 cells, which were either transfected with plasmid pCMV-CYP2B1 (100 fmol) and spacer DNA (pcDNA3.1, 200 fmol) or with plasmids pCMV-CYP2B1 (100 fmol) and pCMV-CPR (200 fmol), respectively, was determined.

panels, lanes 1–6). In HEK293 cells, the elevated amount of CPR protein results in an increase of CPR activity as reflected by the amount of reduced cytochrome *c* (Fig. 2A). Immune-detected amounts of CPR protein in Panc-1 cells also increased with increasing ratios of cotransfected plasmids pCMV-CPR and pCMV-CYP2B1 until both expression plasmids are cotransfected in equal amounts (Fig. 1B, upper panel, lanes 1–5). Then saturation of the CPR amount occurs (Fig. 1B, upper panel, lanes 5 and 6). CPR activity was increased up to 28-fold at highest amounts of plasmid pCMV-CPR transfected in HEK293 cells as compared to endogenous CPR activities in cells non-transfected with this plasmid (Fig. 2A, triangles). In Panc-1 cells, CPR activity was increased only eight-fold at highest amounts of plasmid pCMV-CPR transfected, where saturation was reached (Fig. 2A, squares). These data are in accordance with the data shown in Table 1. Surprisingly, cotransfection of these cells with similar amounts of the CYP2B1 expression plasmid pCMV-CYP2B1 and subsequent resorufin assays revealed a dramatic, up to 10-fold decrease in CYP2B1 activity with increasing amounts and activity of CPR in these cells (Fig. 2B).

The observed decrease of CYP2B1 activity in HEK293 and Panc-1 cells was confirmed using other assay systems: CYP2B1, for example, is able to metabolise the prodrug ifosfamide into its toxic form 4-hydroxy-ifosfamide. The enhanced CPR activity in HEK293 and Panc-1 cells transfected with increasing amounts of plasmid pCMV-CPR led to up to 25% and 60% reduction in CYP2B1 activity as measured by 4-hydroxy-ifosfamide formation as compared to the CYP2B1 activity in cells transfected with the CYP2B1 expression plasmid alone (Fig. 2C). Interestingly, the saturation of CPR amounts in Panc-1 cells is reflected in saturation of their minimal CPR-dependent CYP2B1 activity (Fig. 1B, upper panel, Fig. 2B and C, squares). Cell viability assays, in addition, revealed a more effective ifosfamide mediated cell killing of HEK293 cells transfected with pCMV-CYP2B1 only as compared to cells cotransfected with pCMV-CPR and pCMV-CYP2B1 at a ratio of two (Fig. 2D). The determined IC_{50} values of 0.78 ± 0.02 mM ifosfamide for CPR and CYP2B1 expressing cells and of 0.38 ± 0.05 mM ifosfamide for only CYP expressing HEK293 cells differ significantly (Student-*t* test: $^{***}p \leq 0.01$; $n \geq 3$). In parental HEK293 cells, where no conversion of ifosfamide occurs, an only marginal unspecific cell killing even at high doses of ifosfamide was observed (Fig. 2D).

Remarkably, the CPR mediated reduction of CYP2B1 activity in HEK293 and Panc-1 cells is not dependent on reduced amounts of expressed CYP2B1 protein, since Western blot analyses of these cells showed similar CYP2B1 specific signal intensities irrespective of the amount of the CPR expression plasmid transfected (Fig. 1, lower panels, lanes 1–6). Interestingly, CO difference spectra of microsomes derived from HEK293 cells transfected with plasmids pCMV-CYP2B1 and pCMV-CPR, and pCMV-CYP2B1 alone, respectively, revealed a 4 ± 0.6-fold decrease in absorbance at 450 nm as maximum with CYP2B1/CPR expressing HEK293 cells as compared to HEK293 cells expressing CYP2B1 alone. These data suggest a significantly drop in the available prosthetic heme group pool in CYP2B1/CPR coexpressing HEK293 cells (data not shown).

3.3. *The CPR-mediated decrease of CYP2B1 activity is cell type independent*

To evaluate whether the observed CPR-mediated inhibition of CYP2B1 in HEK293 and Panc-1 cells is a general phenomenon, additional cell lines were investigated. The murine fibroblast cell line NIH3T3 and the human pancreatic tumour cell line Pan02 were stably infected with the retroviral vector LCSN, in which expression of the CYP2B1 gene is driven from the CMV enhancer/promoter region, whilst the human breast tumour cell lines T47D and Hs578T were transduced with the retroviral vector PCCMm1 [14], in which the CYP2B1 gene in infected cells is under expression control of the MMTV promoter. Subsequently, all infected cell lines were stably transfected with plasmid pCMV-CPR. Western blot analysis of the resulting cell populations revealed a strong expression of the introduced CPR gene (Fig. 3, lower panel, lanes 4, 6 and 8),

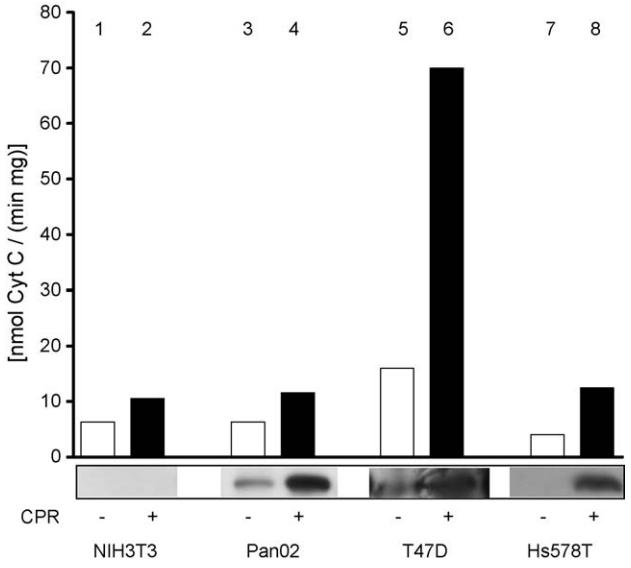

Fig. 3 – CPR activity in various cell lines. NIH3T3, Pan02, T47D and Hs578T cells were stably transfected with plasmid pCMV-CPR. CPR expression and activity was determined in Western blot analysis using an anti-CPR specific antibody (lower panel) and in cytochrome *c* reduction assays (graph; white bars: non-transfected cells, black bars: pCMV-CPR transfected cells).

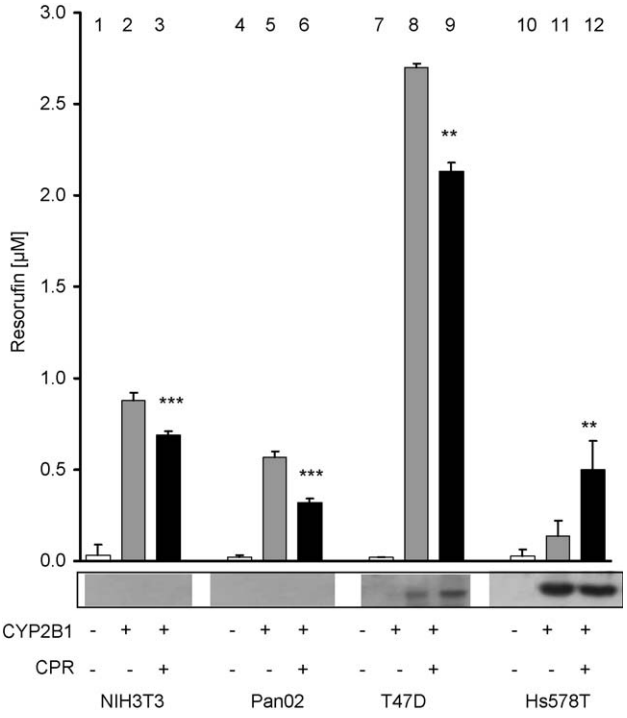

Fig. 4 – CPR effects on CYP2B1 activity in various cell lines. NIH3T3 and Pan02 cells were stably infected with the retroviral vector LCSN, and T47D and Hs578T cells were stably infected with the retroviral vector PCCMm1 both encoding the CYP2B1 gene. Cells expressing the CYP2B1 gene were subsequently transfected with the CPR expression plasmid pCMV-CPR. CYP2B1 expression was analysed in Western blot experiments using an anti-CYP2B1 specific antibody (lower panel). Cytochrome P450 activity dependent on the presence of CYP2B1 and CPR was determined in resorufin assays (graph; white bars: non-transfected cells, grey bars: CYP2B1 expression vector infected cells, black bars: CYP2B1 expression vector infected and pCMV-CPR transfected cells). A significant difference in P450 activity of cells expressing CYP2B1 and CPR to cells expressing CYP2B1 alone is indicated (Student-*t* test, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$).

but was not sensitive enough to detect CPR in CPR expression plasmid transfected NIH3T3 cells (Fig. 3, lower panel, lane 2). Interestingly, in parental T47D and Pan02 cells a weak endogenous CPR expression could be detected (Fig. 3, lower panel, lanes 3 and 5). Endogenous CPR activities as measured for all cell lines in cytochrome *c* assays were observed to be high in T47D cells (16.2 ± 2.3 nmol cytochrome *c*/(min mg)), medium in Pan02 (6.3 ± 1.5 nmol cytochrome *c*/(min mg)), HEK293 (7.8 ± 2.0 nmol cytochrome *c*/(min mg)) and NIH3T3 cells (6.4 ± 1.0 nmol cytochrome *c*/(min mg)), and low in Hs578T (4.1 ± 0.8 nmol cytochrome *c*/(min mg)) and Panc-1 cells (2.1 ± 1.0 nmol cytochrome *c*/(min mg)) (Table 1 and Fig. 3, open bars). In accordance with the CPR expression data, cytochrome *c* assays revealed a two- to four-fold increase in CPR activity in pCMV-CPR transfected cells as compared to non-transfected, parental cells (Fig. 3, graph, lanes 2 versus 1, 4 versus 3, 6 versus 5 and 8 versus 7).

In T47D and Hs578T cells stably infected with the retroviral CYP2B1 expression vector, a CYP2B1 protein specific signal appeared in Western blot analysis (Fig. 4, lower panel, lanes 8+9 and 11+12), whereas unfortunately no cytochrome P450 protein could be detected in infected Pan02 and NIH3T3 cells (Fig. 4, lower panel, lanes 2+3 and 5+6) as well as in all of the non-infected parental cell lines (Fig. 4, lower panel, lanes 1, 4, 7 and 10). In resorufin assays P450 activities of the parental cell lines NIH3T3, Pan02 and T47D were slightly above background (Fig. 4, graph, lanes 1, 4 and 7), in the infected cell populations high level CYP2B1 activity could be detected (Fig. 4, graph, lanes 2, 5 and 8). However, in the respective cell lines expressing both the CYP2B1 and the CPR gene, CYP2B1 activity was reduced up to 40% as compared to cells expressing CYP2B1 alone (Fig. 4, graph, lanes 3 versus 2, 6 versus 5 and 9 versus 8). The observed decrease of CYP2B1 activity measured in the stable CYP2B1 and CPR expressing cell populations is in accordance to data from the transient transfection experiments using HEK293 and Panc-1 cells, as a moderate increase of CPR activity (Fig. 2A, ratio 0–0.13) leads to a moderate decrease in CYP2B1 activity as measured in resorufin assays in these cells (Fig. 2B). Interestingly, in Hs578T cells coexpression of exogenous CPR led to a significant increase in CYP2B1 activity (Fig. 4, graph, lane 12 versus 11). This might be due to the fact that these cells reveal a low endogenous CPR level (Fig. 4, graph, lane 10) and, even after stable infection using an CYP2B1 expression vector, a very modest exogenous CYP2B1 activity, although a lot of CYP2B1 protein is produced (Fig. 4, lower panel, lane 11).

Thus, our data, in summary, indicate that in the majority of the cell lines investigated coexpression of CPR decreases rather than increases CYP2B1 activity, which might have implications *in vivo*. Therefore, in our opinion, the coexpression of CPR to increase the efficiency of cytochrome P450 mediated GDEPT has to be carefully evaluated for each cell type in the respective *in vivo* model systems, before considering this treatment regime for use in clinical studies.

4. Discussion

Here we describe the effect of CPR on the enzymatic activity of CYP2B1. We observed a CPR dose-dependent inhibition of CYP2B1 enzymatic activity in HEK293 human embryonic kidney cells and Panc-1 pancreatic tumour cells. This observation is in line with corresponding cell viability assays, where HEK293 cells showed better survival after exposure to the prodrug ifosfamide when coexpressing CYP2B1 and CPR than cells expressing CYP2B1 alone. The inhibitory effect of CPR upon CYP2B1 enzymatic activity was confirmed in the fibroblast cell line NIH3T3, in the pancreatic tumour cell line Pan02 and in the breast tumour cell line T47D. Only in Hs578T breast tumour cells was CPR overexpression observed to increase the enzymatic activity of CYP2B1.

Interestingly, a more profound effect of CPR was observed on CYP2B1 mediated resorufin metabolism than that of ifosfamide. This might be due to the fact that different substrates also differ in their reaction kinetics and that the sensitivity of a cell to the activated ifosfamide leading to cell killing is a multistep process involving multiple different parameters. The 4-hydroxy-ifosfamide mediated cell toxicity, for example, depends on the drug resistance of the individual cell line, which has been described to vary, dependent on the endogenous aldehyde dehydrogenase levels [25–27].

The activity of recombinant CPR in HEK293 and T47D cells is significantly higher, when compared to Panc-1, Pan02, NIH3T3 and Hs578T cells expressing recombinant CPR. Nevertheless, in most cell lines tested CPR is well expressed and its gene product is functional in all cell lines. The presence of endogenous CPR activity observed in the analysed cell lines corresponds to the respective EST expression profiles (UniGene Hs.354056) [28]. CYP2B1 overexpression led to a massive increase in CYP2B1 activity in all tested cell lines. A similar observation was made by Chen and co-workers, who sensitized tumour cells to the cytotoxic action of the alkylating prodrug cyclophosphamide by overexpression of CYP2B1 [27]. The endogenous P450 activity which is close to background level in the cell lines tested in our study again correlates with data obtained from P450 expression profiles, where ESTs were counted at marginal levels in tissues of breast and pancreas (UniGene) [28].

Experiments using isolated yeast microsomes revealed that CPR overexpression is able to stimulate CYP1A activity and facilitate the metabolism of various drugs [6]. In rat 9L gliosarcoma cells an increase of P450 activity accompanied by the respective therapeutic effect was detected after introduction and expression of the CPR gene [7]. In this study P450 activity was significantly increased in the presence of coexpressed CPR in one cell line, the human breast tumour cell line Hs578T. However, all three assay systems used reveal comparably low levels of endogenous CPR activity in this cell line as, for example, compared to the other cell lines investigated here, resulting in P450 interaction with insufficient amounts of CPR and thus a suboptimal electron supply for P450 function [2,5,29]. Therefore, CPR coexpression in the HS578T cells is able to support CYP2B1 activity. However, to allow a clear estimation, whether the addition of reductase to P450-mediated GDEPT is beneficial or detrimental for the treatment of cancer patients, further detailed analyses of these effects in *in vivo* model systems are strongly required.

Ding and colleagues [12] observed the stimulation of CYP3A4 activity in Chinese hamster ovary cells. However, in these cells the CYP3A4 cDNA was amplified by increasing the selection pressure, which led to a large excess in CYP3A4 protein requiring an excess supply of electrons. Although CPR coexpression increased CYP3A4 activity by providing electrons, which was suggested to be the limiting factor, a decrease of immunologically and spectrally detectable P450 was observed in these experiments [12]. This observation might be explained by an increase in CPR-mediated heme oxygenase activity leading to a reduction of cellular levels of heme, which is the prosthetic group of P450 activity and therefore necessary for P450 enzymatic activity [12]. This hypothesis is supported by our results obtained from reduced CO difference spectra for HEK293 cells either overexpressing CYP2B1 alone or CYP2B1 and CPR together, where the amount of P450 holoenzyme in cell expressing CYP2B1 and CPR was four-fold lower. This is indicative for reduced levels of free prosthetic heme in these cells. Interestingly, levels of immunologically detectable CYP2B1 were similar in these

differently transfected cells. Henderson et al. [30] developed hepatic CPR-null mice, which are characterized by a decrease of P450 activity, but, however, an increase in P450 protein levels in the liver.

Coexpression of the human CYP2B6 and CPR genes has also been analysed in context of a CYP2B6/CPR fusion cassette inserted into an adenoviral vector construct [31]. Infection of HepG2 cells and several pulmonary cell lines with this viral vector resulted in a three-fold increase in CPR activity and a slight improvement in cell killing properties after cyclophosphamide treatment of the pulmonary cell lines [31]. Interestingly, overexpression of CPR in HepG2 cells, which had the highest levels of endogenous CPR activity, did not increase cyclophosphamide-induced, CYP2B6-mediated cytotoxicity, even when CPR and CYP2B6 were expressed at high levels from separated gene cassettes [31].

Taken together, the data presented here clearly show that in the majority of the cell lines tested here, the coexpression of CPR did not improve activity of the therapeutic CYP2B1 protein, but, moreover, had a diametrically opposed effect by inhibiting CYP2B1 function. Thus, combination of P450 and CPR in GDEPT has to be evaluated on a case by case basis in order to avoid compromising the therapeutic benefit of such a treatment.

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