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Cytochrome P450 CYP1B1 protein expression: a novel mechanism of anticancer drug resistance

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Abstract

The overexpression of human cytochrome P450 CYP1B1 has been observed in a wide variety of malignant tumours, but the protein is undetectable in normal tissues. A number of cytochrome P450 enzymes are known to metabolise a variety of anticancer drugs, and the consequence of cytochrome P450 metabolism is usually detoxification of the drug, although bioactivation occurs in some cases. In this study, a Chinese hamster ovary cell line expressing human cytochrome P450 CYP1B1 was used to evaluate the cytotoxic profile of several anticancer drugs (docetaxel, paclitaxel, cyclophosphamide, doxorubicin, 5-fluorouracil, cisplatin, and carboplatin) commonly used clinically in the treatment of cancer. The MTT (3-[4,5-dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide) assay was used to determine the levels of cytotoxicity. The key finding of this study was that on exposure to docetaxel, a significant decrease in sensitivity towards the cytotoxic effects of docetaxel was observed in the cell line expressing CYP1B1 compared to the parental cell line ($P = 0.03$). Moreover, this difference in cytotoxicity was reversed by co-incubation of the cells with both docetaxel and the cytochrome P450 CYP1 inhibitor alpha-naphthoflavone. This study is the first to indicate that the presence of CYP1B1 in cells decreases their sensitivity to the cytotoxic effects of a specific anticancer drug.

Keywords: Cytotoxicity; Cytochrome P450; Docetaxel; Drug resistance; Neoplasm

1. Introduction

We have demonstrated that human cytochrome P450 CYP1B1 is overexpressed in a wide variety of cancers [1–4]. This finding raises the question as to what the functional role of CYP1B1 is in tumours. CYP1B1 has been shown to be capable of the 4-hydroxylation of oestradiol [5–7], and a significant increase in the 4-hydroxylation of oestradiol has been demonstrated in breast cancer [8]. Several cytochrome P450 enzymes are involved in the metabolism of a range of anticancer drugs, including cyclophosphamide, paclitaxel, and docetaxel [9–14]. Cytochrome P450-mediated metabolism usually results in reduced activity or inactivation of the anticancer drugs, but in some cases bioactivation to a more cytotoxic metabolite occurs. One example of detoxification of anticancer drugs is shown by the taxanes. The major pathway of metabolism of paclitaxel, an anticancer drug used in the treatment of breast, ovarian, and non-small cell lung cancer, is catalysed by CYP2C8 and involves the hydroxylation of position 6 on the taxane ring [15]. The metabolite 6-hydroxytaxol is 30-fold less cytotoxic than the parent compound paclitaxel [16], and this metabolite is further metabolised by CYP3A4 [13]. Docetaxel, a semisynthetic taxane currently under going phase II and phase III trials for use in first-line therapy of ovarian cancer, is metabolised by CYP3A to apparently less cytotoxic metabolites [12,13].

Several anticancer drugs are metabolically activated by

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Abbreviations: CYP, cytochrome P450; ANF, alpha-naphthoflavone; MTT, 3-[4,5-dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide; and 5-FU, 5-fluorouracil.

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P450s. In particular, cyclophosphamide, an inactive prodrug used in the treatment of a number of different cancers, including ovarian, must first undergo a 4-hydroxylation reaction to 4-hydroxycyclophosphamide by cytochrome P450 enzymes (CYP2B6 and CYP3A4) [17] before becoming cytotoxic [18]. CYP3A4 has also been shown to be involved in the metabolic activation of doxorubicin to the more cytotoxic morpholino doxorubicin [19,20].

In this study, the influence of CYP1B1 on the cytotoxic profile of several anticancer drugs commonly in clinical practice was evaluated in a Chinese hamster ovary cell line expressing human CYP1B1.

2. Materials and methods

2.1. Cell lines and cell culture

A Chinese hamster ovary fibroblast cell line (V79MZ) and a clone expressing human CYP1B1 (V79MZh1B1) [21] were grown at 37°, 5% CO_2, and 90% saturated humidity in DMEM (Dulbecco's modified Eagle's medium) high glucose type supplemented with 1 mM sodium pyruvate, 10% fetal bovine serum, 100 U/mL of penicillin, and 100 μg/mL of streptomycin. Neither cell line expresses endogenous P450s, although cytochrome P450 reductase is present in both cell lines [22–24]. The parental and CYP1B1-expressing cell lines double in cell number every 10–12 hr [23] and were subcultured at 1:50 ratio (i.e. 1 mL of cells to 50 mL of fresh media) every 4–5 days. The cells were not allowed to reach confluence at any time to ensure optimal cell physiological conditions and maximal cytochrome P450 activity [23]. Cells were routinely used at 3rd–5th passage for all experiments [21].

2.2. Immunoblotting of CYP1B1 protein in V79 total cellular homogenate

Both cell lines (V79MZ and V79MZh1B1) were grown to 60–80% confluence and a total cellular homogenate from each prepared. Cellular protein was determined according to the method of Bradford [25]. Samples of cellular homogenates were then resolved by SDS–PAGE on a 10% polyacrylamide gel using a Hoefer SE 600 (Amersham Pharmacia Biotech) vertical gel electrophoresis system. This was followed by transfer to nitrocellulose membrane (Hybond-ECL, Amersham Pharmacia Biotech). Sites of immunoreactivity were detected with a monoclonal antibody to human CYP1B1 which we have developed [3]. This antibody was raised against a 15-amino-acid peptide corresponding to amino acid residues 437–451 of the human CYP1B1 protein [3]. CYP1B1 immunoreactivity was visualised by an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech). Microsomes prepared from human lymphoblastoid cells that had been transfected to stably express human CYP1B1 (Gentest) were used as the positive control [26].

2.3. Cell viability assay

Paclitaxel, cyclophosphamide, doxorubicin, 5-FU, cisplatin, carboplatin, and ANF were purchased from Sigma. Docetaxel was a generous gift from Rhone-Poulenc Rorer. Optimal growth conditions were established. The same conditions were used for both cell lines: V79MZ and V79MZh1B1 cells at 60–80% confluence were harvested and seeded at 0.5–1 $\times$ 10^3 cells per well in 96-well culture plates. Cells were grown for 48 hr, media were then removed, and each drug added at increasing concentrations in the appropriate solvent (0.1% ethanol for docetaxel, paclitaxel, cyclophosphamide, and carboplatin, 0.1% DMSO for cisplatin, sterile water for 5-FU and doxorubicin). Cells treated with solvent alone acted as a negative control.

Enzyme inhibition studies were undertaken by co-administration of docetaxel and the known P450 CYP1 inhibitor ANF (Sigma) [27] at different concentrations in the media. Stock solutions of ANF (1, 10, and 100 mM) were dissolved in DMSO and added to the media to give final concentrations of 1, 10, and 100 μM ANF (in each case the final concentration of DMSO in media was 0.1%). Following 24-hr exposure to each drug (with or without inhibitor), the media were removed and replaced with fresh media without drug. In this study, cells were then grown for three doubling times (36 hr) with the media changed at 24 hr. Cell viability was then assessed using the MTT assay, which is comparable to using a clonogenic assay [23,28,29]. Media were removed from the wells and replaced with 200 μL of fresh media, followed by addition of 50 μL of MTT solution (50 mg/mL of MTT [Sigma] in sterile PBS). The cells were incubated at 37° in a humidified atmosphere with 5% CO_2/95% air for 4 hr. The MTT-containing media were then removed and 200 μL of DMSO plus 25 μL of glycine buffer (0.1 M glycine/0.1 M NaCl pH 10.5) added to the cells in each well. This procedure overcomes any effect that cell density or culture medium may have on the absorption spectrum [28]. The absorbance of the formazan produced by the viable cells was measured once for each well at 540 nm on a Labsystems EMS microplate reader (Life Sciences International). To calculate the cell viability, cells treated with solvent alone were assigned a value of 100% absorbance indicating zero cytotoxicity, i.e. 100% viability. The cytotoxic profile of each drug was evaluated in triplicate 96-well plates. Each plate included several controls (media only, cells only, and cells treated with solvent alone). The 96-well plate format allowed eight concentrations of the appropriate drug per plate, with eight replicates (i.e. eight separate wells) per drug concentration (i.e. 8 measurements of absorbance for each concentration of drug per plate). There were three triplicate plates per experiment, resulting in a total of 24 measurements of absorbance per concentra-

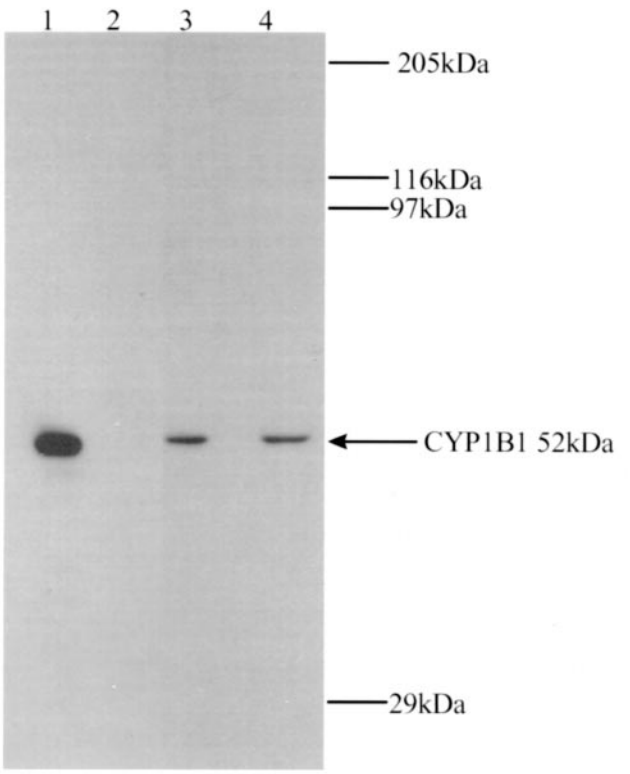

Fig. 1. Immunoblot of parental V79MZ and V79MZh1B1 total cellular homogenate with monoclonal antibody to CYP1B1, demonstrating the presence of CYP1B1 immunoreactivity in the CYP1B1-transfected cells and an absence of CYP1B1 in the parental non-transfected cells. Lane 1, human lymphoblastoid cell line expressing CYP1B1 (10 μg of microsomal protein); lane 2, total cellular homogenate from V79MZ cells (80 μg of total cellular protein); lane 3, total cellular homogenate from V79MZh1B1 cells (40 μg of total cellular protein); and lane 4, CYP1B1-expressing V79MZh1B1 cells total cellular homogenate (80 μg of total cellular protein). Molecular weight markers are shown at right in kDa.

tion of drug. Plate reader variability was found to be negligible.

2.4. Statistics

Statistical analysis was performed using both Statistics for Windows 95™ and SPSS version 7.5 for Windows 95™.

3. Results

3.1. Cytotoxic effects of treatment with anticancer drugs

A protein band of approximately 52 kDa was identified in the cellular homogenate from the V79MZh1B1 cell line, corresponding to the expected molecular size observed with lymphoblastoid cells that also express human CYP1B1 [3]. No immunoreactive band was observed in the parental V79MZ cell line (Fig. 1).

The influence of CYP1B1 on cytotoxicity was evaluated for all the anticancer agents investigated. The range of concentrations for each drug used in this study was based on previous experiments with other P450s [23] and additional experiments performed in this laboratory. A significantly greater (at least fourfold) decreased sensitivity to docetaxel was observed in cells expressing CYP1B1 compared with non-CYP1B1-expressing cells (Fig. 2A and Table 1). The cytochrome P450 CYP1 inhibitor ANF was used at serial concentrations (1, 10, and 100 μM) to determine if the differential cytotoxicity demonstrated in the cells expressing CYP1B1 was due to metabolism by CYP1B1 (Fig. 3). Neither cell line (parental or CYP1B1-expressing) showed cytotoxicity on exposure to any of the concentrations of ANF. In contrast to the decreased sensitivity observed with docetaxel, no significant difference in cytotoxicity was observed between CYP1B1-expressing and non-expressing cells after exposure to paclitaxel (Fig. 2B and Table 1). No cytotoxicity was observed in either cell line after exposure to cyclophosphamide (Fig. 2C and Table 1). In addition, no significant difference in cytotoxicity was observed between V79MZ and V79MZh1B1 cells after exposure to doxorubicin, 5-FU, carboplatin, or cisplatin (Fig. 2, D–G; Table 1).

4. Discussion

This is the first study to indicate that CYP1B1 may be a mechanism of anticancer drug resistance. In this study, a Chinese hamster cell line that stably expresses human CYP1B1 [21] was used as a bioassay to assess the effect of CYP1B1 on the cytotoxicity of a range of anticancer drugs. Although several of the drugs used in this study are clinically relevant in the treatment of ovarian cancer, i.e. cisplatin, carboplatin, and 5-FU [30,31], they have no known interactions with cytochrome P450 enzymes. However, these drugs were used to provide controls to assess the validity of the cytotoxicity assay. The prodrug cyclophosphamide provided an appropriate negative control (i.e. non-cytotoxic to either parental or CYP1B1-expressing cells), as it requires activation by other cytochrome P450 enzymes (CYP2B6 and CYP3A4) before becoming cytotoxic, although we showed previously that it does not interact with CYP1B1 [32]. Doxorubicin is known to be metabolised to more cytotoxic compounds by the action of other cytochrome P450 enzymes (CYP3A) [20], whereas the taxanes paclitaxel and docetaxel are both metabolised to pharmacologically less active metabolites by cytochrome P450 enzymes (CYP2C8 and CYP3A4 for paclitaxel and CYP3A4 for docetaxel) [12–15,33]. Previous studies have demonstrated that V79MZ cells, when stably transfected with other cytochrome P450 enzymes, exhibited appropriate cytotoxicity when exposed to individual anticancer drugs, establishing this cell line as an appropriate model for investigating anticancer drug cytotoxicity [23].

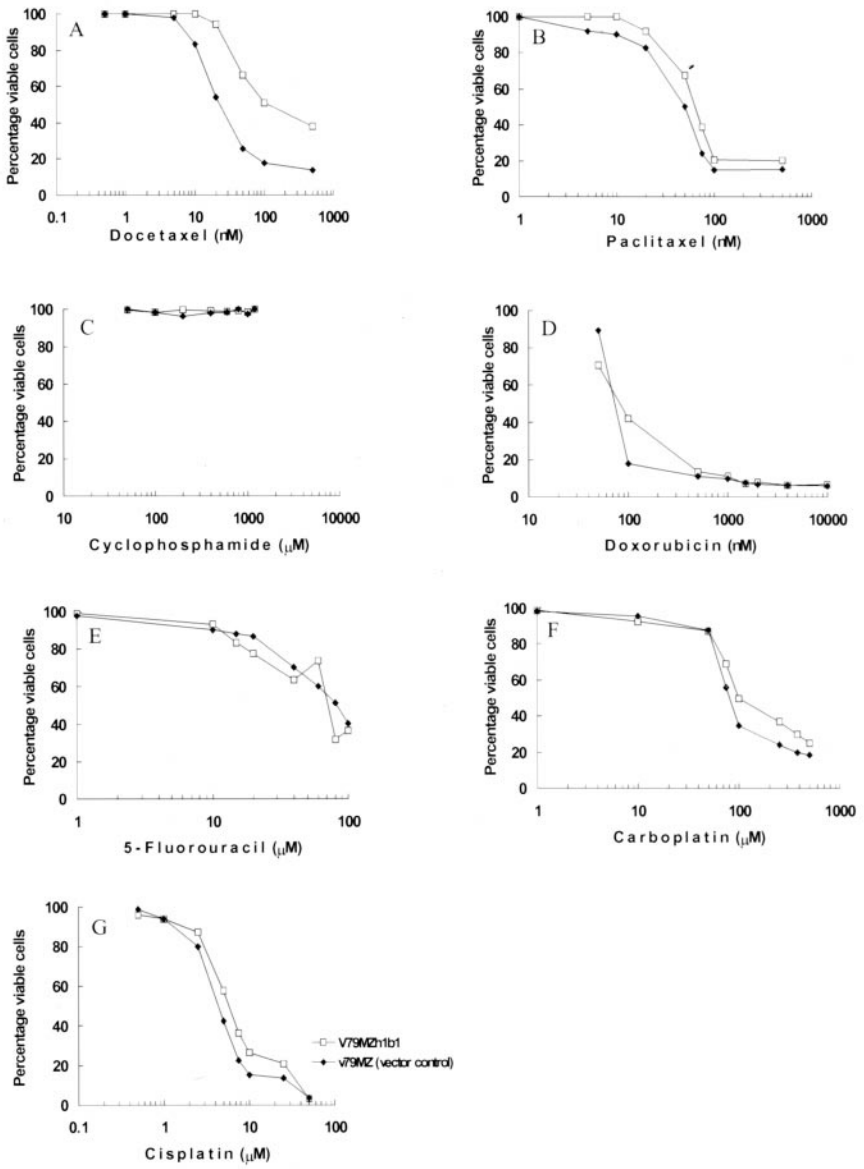

Fig. 2. Survival curves of cells treated with: A, docetaxel; B, paclitaxel; C, cyclophosphamide; D, doxorubicin; E, 5-FU; F, carboplatin; and G, cisplatin. Parental V79MZ cells and those transfected with CYP1B1 (V79MZh1B1) were incubated with increasing concentrations of the appropriate drug for 24 hr. Cell viability was then determined by the MTT assay, and the percentage of surviving cells relative to the respective controls (cells treated with solvent only) was calculated for each drug concentration. There was significantly different cytotoxicity for docetaxel (A) in CYP1B1-expressing and non-expressing cell lines, whereas with the other drugs a similar amount of cytotoxicity was observed in both parental and transfected cells (B, D, E, F, and G). There was no cytotoxicity observed in cells exposed to cyclophosphamide (C). Each 96-well plate allowed eight concentrations of the appropriate drug per plate, with eight replicates (i.e. eight separate wells) per concentration (i.e. 8 measurements for each concentration of drug per plate). There were three triplicate plates per experiment, resulting in a total of 24 measurements of absorbance per concentration of drug. The standard deviation was less than 5% of the mean absorbance for all drugs used, and at each concentration of drug.

It is widely known that both primary cultures and human tumour cell lines rapidly lose the ability to constitutively express cytochrome P450 enzymes in culture. Indeed, we have shown that the MCF-7 human breast carcinoma- and PEO4 ovarian adenocarcinoma-derived cell lines do not express CYP1B1,[1] even though we have shown overexpression of CYP1B1 in both breast and ovarian tumours [1]. The lack of constitutive expression of cytochrome P450s in tumour cell lines was overcome by the use of a stably transfected cell line expressing CYP1B1. CYP1B1 activity in these cells was previously shown to be approximately 10 pmol/min/mg of protein by the EROD assay [21], and is likely to be comparable with that observed in human tumours.

Initial research in our laboratory has indicated that do-

[1] McFadyen MC, Murray GI. Unpublished observations.

Table 1
IC_{50} values[a] for V79MZ and V79MZh1B1 cell lines treated with several anticancer drugs

Drug	V79MZ (control vector)	V79MZh1B1	*P* value[b]
Docetaxel	22 nM	100 nM	0.03
Paclitaxel	35 nM	60 nM	NS
Cyclophosphamide	NC	NC	ND
Doxorubicin	80 nM	90 nM	0.79
5-Fluorouracil	70 μM	80 μM	NS
Carboplatin	80 μM	100 μM	NS
Cisplatin	4.4 μM	6 μM	NS

Statistical comparison of dose–response curves (IC_{50s}) using Mann–Whitney U test. The data represent the means of 24 determinations per drug concentration. NC = no cytotoxicity (no cytotoxicity observed with cyclophosphamide); ND = not determined; NS = not statistically significant for paclitaxel, 5FU, cisplatin, or carboplatin.

[a] IC_{50} = Drug concentration at which 50% of the cells are viable.

[b] The statistical software package we used did not provide *P* values in many cases when the result was non-significant.

cetaxel is metabolised by expressed human CYP1B1 [32]. Docetaxel is a semisynthetic taxane derived from the European yew, and is currently under investigation for use as first-line treatment of ovarian cancer [12,13,34]. The key finding of the current study was the significant differential cytotoxicity observed on exposure to docetaxel, the cytotoxicity observed in CYP1B1-expressing cells being fourfold less than that observed in non-CYP1B1-expressing cells. In addition, co-treatment of these cells with known CYP1 P450 inhibitor ANF [27] resulted in the complete reversal of differential cytotoxicity observed in these cells, i.e. the effect is attributed to metabolism of docetaxel by CYP1B1. The resistance to the cytotoxic effects of docetaxel in those cells expressing CYP1B1 may have important clinical implications.

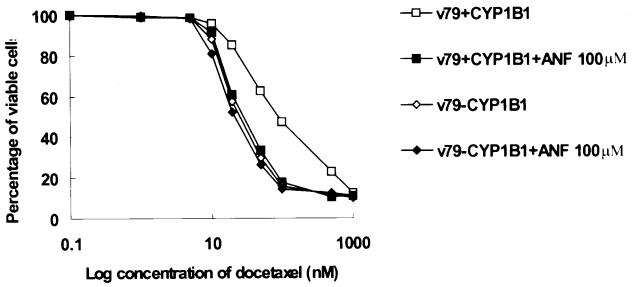

Fig. 3. Survival curve of cells treated with docetaxel in the presence or absence of the cytochrome P450 inhibitor ANF. Parental V79MZ cells and those transfected with CYP1B1 (V79MZh1B1) were incubated with increasing concentrations of docetaxel for 24 hr. ANF was added at a concentration of 1, 10, or 100 μM to the transfected V79MZh1B1 cells. Cell viability was then determined by the MTT assay and the percentage of surviving cells relative to the respective solvent controls was calculated. ANF at a concentration of 100 μM totally abolished the differential cytotoxicity observed in the V79MZh1B1-transfected cells. ANF, itself, exhibited no cytotoxic effects on the parental (V79MZ) and CYP1B1 (V79MZh1B1)-expressing cell lines at any of the concentrations used.

Inhibition of CYP1B1 in tumours may offer a specific mechanism for overcoming the resistance to docetaxel and other drugs. Development of a specific inhibitor to CYP1B1 is clinically important, as ANF also inhibits CYP1A1 and CYP1A2 [27]. Since CYP1B1 is overexpressed in tumour but not in normal tissue, increasing the tumour sensitivity to anticancer drugs by CYP1B1 inhibition would not be expected to have an effect on normal tissues.

In summary, this study provides evidence for the concept that the presence of CYP1B1 in tumour cells may have an important role in drug resistance.

Acknowledgments

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