

ORIGINAL ARTICLE

Effects of hypoxia and limited diffusion in tumor cell microenvironment on bystander effect of P450 prodrug therapy

M Günther¹, DJ Waxman², E Wagner¹ and M Ogris¹

¹Pharmaceutical Biology-Biotechnology, Department of Pharmacy, Ludwig-Maximilians-Universität, Munich, Germany and ²Department of Biology, Division of Cell and Molecular Biology, Boston University, Boston, MA, USA

Cytochrome P450 (CYP) enzyme 2B1 metabolizes the anticancer prodrug cyclophosphamide (CPA) to 4-hydroxy-CPA, which decomposes to the cytotoxic metabolites acrolein and phosphoramidate mustard. We have evaluated the bystander cytotoxicity of CPA in combination with CYP2B1 gene-directed enzyme prodrug therapy using a cell culture-based agarose overlay technique. This method mimics the tumor microenvironment by limiting the diffusion of metabolites and by reducing the oxygen concentration to levels similar to those found in solid tumors. Under these conditions, the CYP activity of CYP2B1-expressing tumor cells was decreased by 80% compared to standard aerobic conditions. Despite this decrease in metabolic activity, a potent bystander effect was observed, resulting in up to 90% killing by CPA of a tumor cell population comprised of only ~20% CYP-expressing tumor cells. Similarly, transient transfection of a small fraction (~14%) of a human hepatoma Huh7 cell population with a CYP2B1 expression plasmid followed by short-term treatment with CPA (5 h) led to an eradication of 95% of the cells. No such bystander effect was observed without the agarose overlay. These findings suggest that the agarose overlay technique is very useful as an *in vitro* test system for investigation of the bystander effect of CYP/CPA and other enzyme/prodrug combinations under conditions that mimic the hypoxic conditions present in solid tumors *in vivo*.

Cancer Gene Therapy (2006) 13, 771–779. doi:10.1038/sj.cgt.7700955; published online 17 March 2006

Keywords: GDEPT; cyclophosphamide; cytochrome P450; agarose overlay

Introduction

Tumor cell delivery of genes coding for prodrug activation enzymes is a promising approach for the treatment of solid tumors. A broad range of such enzyme–prodrug combinations have been extensively researched for their utility in cancer therapy leading to clinical trials.¹ These gene-directed enzyme prodrug therapies (GDEPT) aim to increase antitumor activity by conversion of an inactive prodrug into an active, cytotoxic metabolite after delivery of the therapeutic gene to the tumor using either viral or non-viral gene transfer methods. High concentrations of active, cytotoxic drugs may thus be generated within a tumor, which may ultimately allow for a reduction in drug dosage and an associated reduction in side effects associated with systemic exposure to toxic drug metabolites.²

Cyclophosphamide (CPA) is an alkylating agent widely used in cancer therapy. Cyclophosphamide is activated in

the liver in a reaction catalyzed by specific cytochrome P450 (CYP) enzymes (for review, see Roy and Waxman³). The primary active metabolite, 4-OH-CPA, is transported from the liver to the tumor through the blood stream. Tumor cells infected with a viral vector encoding a CPA-activating CYP gene are highly sensitized to the cytotoxic actions of CPA, as are surrounding tumor cells, through their exposure to diffusible, cytotoxic metabolites produced by the CYP-expressing tumor cells.^{4,5} Thus, CYP gene transfer can increase antitumor activity by providing for the activation of CPA at the target site.⁶

Drug diffusion and concentration effects within a tumor play an important role in the action of drugs, such as CPA, owing to the short half-life of its active metabolites,⁷ especially within the context of a GDEPT strategy. In contrast to other GDEPT-based enzyme/prodrug combinations, such as herpes simplex virus thymidine kinase/ganciclovir, 4-OH-CPA readily diffuses across cell membranes and is not dependent on direct cell–cell contact for bystander killing. Tumor cell uptake of CPA and 4-OH-CPA is facilitated by the low extracellular pH associated with tumors, which may increase intracellular accumulation of this weakly acidic drug and its metabolite by an ion trapping mechanism.⁸

The tumor microenvironment has a substantial impact on the response of tumor cells to cytotoxic agents,⁹

Correspondence: Dr M Ogris, Pharmaceutical Biology-Biotechnology, Department of Pharmacy, Ludwig-Maximilians-Universität, Butenandtstrasse 5-13, D-81377 Munich, Germany.

E-mail: Manfred.Ogris@cup.uni-muenchen.de

Received 24 November 2005; accepted 4 February 2006; published online 17 March 2006

including CPA. In addition to the level of *CYP* gene expression, the extent of CPA activation is highly dependent on the availability of O₂, which is a co-substrate for all CYP-catalyzed monooxygenation reactions. Low oxygen pressure resulting from an insufficient blood supply is a hallmark of solid tumors.¹⁰ Despite the limited oxygen supply, excellent antitumor activity was reported in mouse and rat models of GDEPT using CYP2B enzymes^{1,11} with some indications of efficacy reported in initial clinical trials after local delivery of a *CYP* gene.¹²

In this study, we use an agarose overlay technique to study the effect of limited diffusion and reduced oxygen supply on the bystander cytotoxicity of CYP-activated CPA. Studies carried out with a tumor cell line that stably expresses CYP2B1 demonstrate that the bystander cytotoxicity of CYP-activated CPA can be substantially enhanced using this technique. Moreover, transient transfection of the *CYP2B1* gene led to eradication of tumor cells subsequently treated with CPA.

Materials and methods

Reagents, chemicals and plasmids

Agarose ultrapure, geneticin, cell culture medium and fetal bovine serum (FBS) were purchased from Invitrogen GmbH (Karlsruhe, Germany). *Xho*I restriction enzyme and ×10 buffer D were obtained from Promega (Mannheim, Germany). Plasmids pCR3.1-CYP2B1 (rat CYP2B1 under the control of the cytomegalovirus (CMV) enhancer/promoter⁵) and pEGFP-N1 (Clontech, Palo Alto, CA, USA) were produced and purified by ELIM Biopharmaceuticals (San Francisco, CA) with endotoxin levels <0.1 EU/50 µg DNA. pCMV-Luc plasmid was purified with the Qiagen Endo Free Giga kit (Qiagen, Hilden, Germany). Linear polyethylenimine (PEI) with an average molecular weight of 22 kDa (PEI22) was purchased from PolyPlus (Illkirch, France) and used at a 1 mg/ml working solution, neutralized with HCl. All other reagents were purchased from Sigma-Aldrich (Munich, Germany).

Cell culture

Murine neuroblastoma Neuro2A cells (ATCC CCI-131), murine colon adenocarcinoma CT26 cells (ATCC CRL-2638) and the stable, CYP2B1-expressing cell clone X39 were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS. The human hepatoma cell line Huh7 (JCRB 0403; Tokyo, Japan) was cultured in DMEM/HAM's F12 medium, supplemented with 10% FBS, all at 37°C in 5% CO₂ and a humidified atmosphere.

Generation of a CYP2B1-expressing clone X39

Plasmid pCR3.1-CYP2B1 was linearized by digestion with *Xho*I, followed by agarose gel electrophoresis and Qiaprep[®] Spin MiniprepKit 250 (Qiagen) purification according to the manufacturer's instructions. CT26 cells were seeded and transfected in 24-well plates. The

transfection complex of linearized pCR3.1-CYP2B1 plasmid with PEI was generated at an N/P ratio (nitrogen in PEI/phosphate in DNA) of 6 in HBS (HEPES-buffered saline: 20 mM HEPES pH 7.1, 150 mM NaCl) at a final DNA concentration of 20 µg/ml.¹³ Forty-eight hours after transfection, cells were selected with 0.5 µg geneticin per ml culture medium. To obtain subclones, the surviving cells were re-seeded in a 96-well plate at 1 cell/well after 2 weeks of geneticin selection. Subclones were analyzed and characterized for CYP2B1 activity using pentoxylresorufin as substrate. Clone X39 gave the highest P450 activity and was used in all subsequent experiments.

Agarose overlay method

Cells were seeded either in 24-well (Hoechst 33258 diffusion experiment) or 48-well plates (all other assays) 24 h before addition of the agarose overlay. Culture medium was removed and replaced with 345 µl medium (24-well plates) or 200 µl medium (48-well plates) containing 0.5% (w/v) agarose. The agarose-containing medium was obtained by stepwise dilution of complete medium with melted agarose (10% agarose in phosphate-buffered saline (PBS), w/v). Before applying the agarose-containing medium to the seeded cells, the medium was allowed to cool to 37°C. After solidification of the agarose, 1040 µl (24-well plate) or 600 µl (48-well plate) of complete culture medium without agarose (±CPA) was added to the cells.

Pentoxylresorufin assay

A modified assay was used to assay CYP2B1 activity.¹⁴ Cells were incubated in 48-well plates with 200 µl OptiMem I medium containing 1.7 µM 7-pentoxylresorufin and 100 µM 3,3'-methylene-bis(4-hydroxycoumarin) ('substrate solution') for 20–120 min. Following incubation, the cells were subject to a freeze–thaw cycle to stop the enzymatic reaction. The supernatant was transferred to a 1.5 ml reaction tube, centrifuged at 15 000 g for 10 min and 150 µl of the clear liquid was assayed for fluorescence using a Cary Eclipse fluorimeter (Cary, Mulgrave, Australia) with excitation and emission wavelengths set to 562 and 585 nm, respectively. Measurements were performed in triplicate and enzyme activity was expressed as relative fluorescence units.

When measuring CYP activity under the agarose layer (Figure 3b, '+ gel'), 200 µl substrate solution was injected between the adherent cells and the agarose layer after solidification of the 200 µl agarose overlay. After incubation at 37°C for 40 min, the agarose layer was removed and the resorufin content in the supernatant was quantified. In the '-gel' samples, 200 µl substrate solution was added to the cells under standard conditions, which were incubated for 40 min at 37°C followed by resorufin content quantification. In the case of the 'gel removed' samples, cells were overlaid with 200 µl agarose for 1 h. Thereafter, the gel was removed, 200 µl substrate solution added to the cells and after incubation at 37°C for 40 min resorufin was quantified in the supernatant. No significant amounts of resorufin were found in the agarose layer, indicating that the majority of resorufin is localized in the solution between the cells and the agarose overlay.

When performing the pentoxyresorufin assay in a hypoxia chamber, 100 000 X39 cells were seeded in a 3.5 cm culture dish. Twenty-four hours after seeding, the cells were incubated in the hypoxia chamber for 40 min with 500 μ l of the substrate solution either under normoxia (21% O₂) or under various decreased O₂ concentrations, with O₂ replaced by a mixture of N₂ and CO₂. Oxygen partial pressure was measured with a digital oxymeter (GMH 3690, Greisinger Electronic, Germany). Relative humidity and temperature (37°C) were similar under all conditions.

DNA content assay

To assay for DNA content, the culture medium and the agarose layer were removed and cells were lysed with Millipore water followed by a freeze–thaw cycle. Cell lysis buffer (1 mM Tris-EDTA, pH 7.4, 200 mM NaCl) containing 0.2 ng/ml Hoechst 33258 was applied to each well followed by another freeze–thaw cycle. The DNA content was measured by quantifying fluorescence with a plate reader (Tecan, Grödig, Austria) equipped with filters for excitation at 360 nm and emission at 465 nm. Results obtained with the DNA content assay correlate well with those of the 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (MTT) assay (described below) under normoxic culture conditions, but not under long-term hypoxic conditions (e.g., under an agarose overlay), where the results of the MTT assay were artificially low, perhaps owing to adaptation mechanisms.¹⁵ As the DNA content assay always correlates with cell numbers and, in the presence of oxygen, with the MTT assay as well (M Günther, unpublished observations), cytotoxicity assays with long-term agarose overlay were evaluated by the DNA content assay, whereas for short-term agarose overlay (transient transfection experiments, see below), the MTT assay was used.

3-(4,5-Dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide assay

After removing the culture medium and, if applicable, the agarose layer, 300 μ l of culture medium containing 0.25% MTT (w/v) was applied to each well after which the plates were incubated for 3 h at 37°C. 3-(4,5-Dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide is converted to a colored, water-insoluble formazan salt by the metabolic activity of viable cells. The culture medium was then removed and the cells were frozen at –80°C. After thawing the cells, 300 μ l of dimethylsulfoxide was added to dissolve the insoluble formazan salt and absorbance at 590 nm was measured with a plate reader (Tecan, Grödig, Austria). A reference absorbance at 630 nm was subtracted from the absorbance at 590 nm for each well.

Bystander cytotoxicity assay

The bystander effect of CYP-activated CPA was assayed by measuring the DNA content and the metabolic activity (MTT) of a cell population composed of different percentages of X39 cells mixed with either Neuro2A cells or CT26 cells, followed by CPA treatment. In total, 1500 cells per well were plated in 48-well plates. Twentyfour

hours after seeding, the culture medium was removed and replaced by either 200 μ l of fresh medium or 200 μ l of medium containing 0.5% (w/w) agarose. Then, 600 μ l of 0.66 mM CPA dissolved in culture medium was added to each well. Control cells were treated with 600 μ l culture medium without CPA. The total volume was 800 μ l with a final CPA concentration of 0.5 mM. Treated cells and controls were incubated for 5 days in a humidified atmosphere containing 5% CO₂ at 37°C. DNA contents were then assayed.

Transgene expression analysis after transient transfection

Huh7 cells were seeded 24 h before transient transfection in a 48-well plate. Cells were transfected with 100 ng pCR3.1-CYP2B1 or pCMV-EGFP-N1 using linear PEI (N/P 6 in HBS). Forty-eight hours after transfection, the cells were washed with PBS and harvested by trypsin treatment. For analysis of enhanced green fluorescent protein (EGFP) expression, the cells were analyzed as described.¹⁶ For analysis of CYP2B1 expression, cells were fixed with 2% paraformaldehyde in PBS, followed by permeabilization with 0.1% (w/v) Triton X-100 in PBS. Cells in 5% FBS in PBS were incubated with mouse monoclonal anti-rat CYP2B1 (Oxford Biomedical Research, Oxford, MI) or, as a control, with nonspecific mouse isotype control antibody. Alexa488-conjugated goat antimouse IgG (Molecular Probes) was used as a secondary antibody. Cells were analyzed on a Cyan MLE flow cytometer (DakoCytomation, Copenhagen, Denmark). As an additional control, non-transfected cells were similarly stained and analyzed.

Bystander cytotoxicity assay after transient transfection

Huh7 cells were seeded 24 h before transient transfection in a 48-well plate. Cells were transfected with 100 ng reporter gene plasmid (pCMV-Luciferase) or pCR3.1-CYP2B1 using linear PEI (N/P 6 in HBS). Forty-eight hours after transfection, the cells were treated for 5 h with 0.5 mM CPA with or without an agarose overlay. The culture medium and agarose were then removed and replaced with fresh medium. Cell viability was measured using the MTT assay after further culture for 3 days.

Results

Stable transfection of the murine colon carcinoma cell line CT26 with plasmid encoding CYP2B1 was carried out and a clone exhibiting high CYP activity (clone X39) was isolated. Cytochrome P450 activity was ~300-fold higher in X39 cells compared to the parental CYP-deficient tumor cell line CT26, based on the CYP-catalyzed conversion of pentoxyresorufin to resorufin (Figure 1a). The neuroblastoma cell line Neuro2A also exhibited very low CYP activity, whereas the human hepatoma cell line Huh7 had an endogenous CYP activity that was significantly higher, but still substantially lower than X39 cells (Figure 1a).

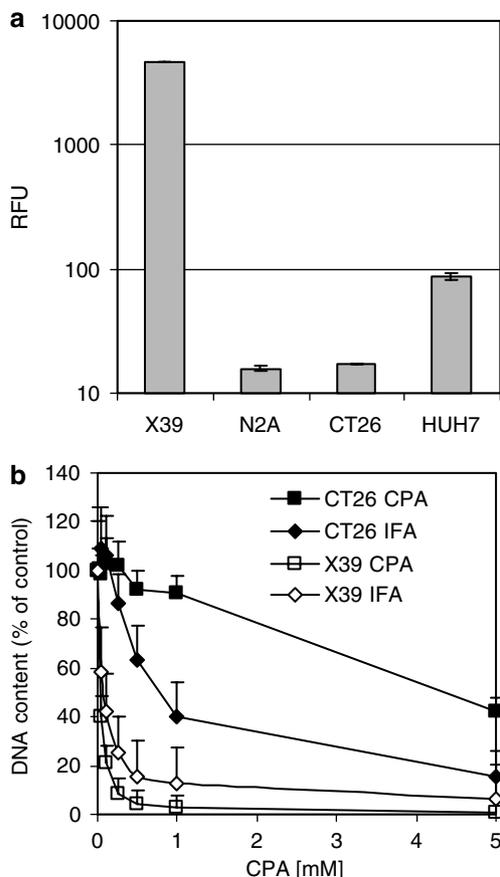


Figure 1 Comparison of different cell lines in terms of CYP2B1 activity and sensitivity toward cyclophosphamide (CPA) or ifosfamide (IFA) treatment. (a) The indicated cell lines were seeded 24 h before cytochrome P450 (CYP) enzyme assay, measured using pentoxerysorufin as substrate, as described in Materials and methods. Cell number was determined in parallel samples by counting, and enzyme activity (expressed as relative fluorescence units (RFU)) was normalized to cell number. The experiment was carried out in triplicate, with mean values \pm standard deviation (s.d.) shown. (b) CT26 cells and X39 cells were treated for 5 days with increasing concentrations of CPA or IFA beginning 24 h after seeding. Cell proliferation was then determined by the DNA content assay as described in Materials and methods. Mean values from triplicates \pm s.d. are shown.

Next, we evaluated the sensitivity of X39 and CT26 cells to the CYP prodrugs, CPA and ifosfamide (IFA), which are converted into their active metabolites by CYP2B1 (Figure 1b). Cells were treated with increasing concentrations of each prodrug for 5 days, followed by measurement of the cell content of each culture using a DNA-based assay.¹⁷ This assay is particularly suitable for evaluation of cytotoxicity under hypoxic conditions, as in the case of the agarose overlay technique described below (see Materials and methods). Highly efficient (90–95%) killing of X39 cells was achieved at 0.25–0.5 mM CPA, whereas no toxicity was seen under the same conditions with the CYP-deficient CT26 cells. Ifosfamide also induced a strong killing of X39 cells (85% toxicity at 0.5 mM IFA); however, some toxicity toward the CYP-

negative CT26 cells was also apparent, especially at high drug concentrations. This difference is likely owing to differences in the intrinsic cytotoxicity of each prodrug. All further experiments were performed with CPA.

Diffusion of drugs is far more limited within a tumor *in vivo* than in tumor cells cultured *in vitro*. In addition, tumor cells are often subject to a hypoxic environment. These factors are both likely to impact the efficacy of CYP prodrug activation and bystander cytotoxicity. To better mimic the microenvironment within tumors, tumor cells seeded in tissue culture plates were covered with a thin layer of agarose dissolved in cell culture medium. Oxygen, nutrients and drugs can reach the cells by diffusion through the agarose layer.¹⁸ We anticipated that the activated metabolites of CPA would form a concentration gradient in the vicinity of the drug-activating cells, similar to the situation in a solid tumor. To test this hypothesis, a small amount of the membrane-permeable DNA stain Hoechst 33258 was added to the cells (Figure 2). When the stain was added to normal culture medium, the concentration was too low for detection of fluorescent cell nuclei. However, when a similar amount of Hoechst stain was injected in the agarose layer covering the cells, bright fluorescent nuclei were observed near the injection site. More distant cells were less fluorescent but still visibly stained. Thus, the agarose layer is likely to limit diffusion of low molecular weight drugs and their metabolites, thereby establishing a concentration gradient. Hoechst 33258 is a good model for CPA in terms of diffusion within a gel, as both drugs have a low molecular weight (M_r (Hoechst 33258) = 624 Da; M_r (CPA) = 250 Da) and both drugs are hydrophilic.

Our next goal was to determine if the agarose overlay imposes a hypoxic environment. We first investigated the extent to which CYP activity is oxygen-dependent in X39 cells (Figure 3). Cells were incubated with the CYP2B1 substrate pentoxerysorufin in a hypoxia chamber under defined ambient oxygen concentrations. The culture medium was assayed for enzymatic conversion of the substrate pentoxerysorufin to the fluorescent metabolite resorufin. Cytochrome P450 activity was directly dependent on the oxygen content, as indicated by the near-linear correlation between O_2 concentration and CYP activity (Figure 3a). Based on this calibration curve, CYP activity was reduced to \sim 25% of the normoxic control level at 5% O_2 , and to 7% in the absence ($<$ 0.1%) of O_2 . In a separate experiment (Figure 3b), X39 cells cultivated under standard conditions or under an agarose layer were assayed for CYP activity. The substrate, pentoxerysorufin, (1) was added directly to the culture medium ('-gel'), (2) was carefully injected into the space between the cells and the agarose overlay ('+gel') or (3) was added to cells cultured under agarose after the gel was removed ('gel removed'). The DNA content of each well was quantified to ensure that similar cell numbers were present in the individual wells. The CYP activity of cells grown under standard conditions was similar to that of cells cultured under agarose with removal of the agarose before substrate incubation. In contrast, CYP activity assayed in cells under the agarose layer was reduced to

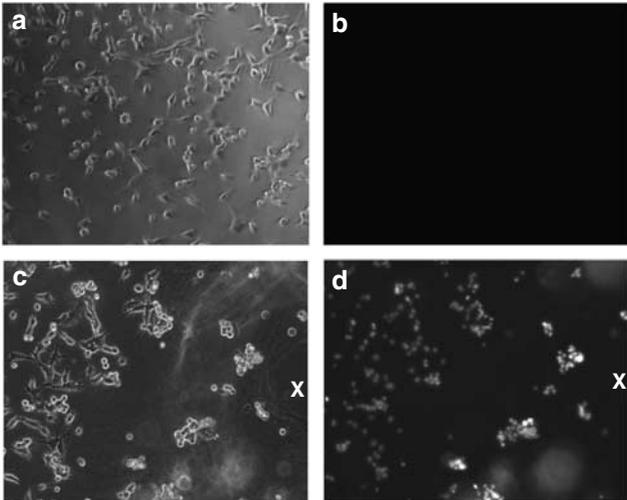


Figure 2 Diffusion and concentration effects of an agarose overlay using Hoechst 33258. CT26 cells were seeded at 40 000 cells per well of a 24-well plate 24 h before addition of an agarose overlay. The agarose was added as described in Materials and methods. Hoechst 33258-containing solution (1 μ l at 0.02 ng/ml in phosphate-buffered saline) was injected into the agarose layer and cellular fluorescence was monitored 30 min after the injection using a Zeiss Axiovert 200 microscope equipped with appropriate excitation (365 nm) and emission (420 nm long pass) filters. Cells were viewed with a $\times 10$ objective and phase contrast pictures were obtained for transmitted light. A control experiment was performed without agarose overlay, with the same amount of Hoechst 33258 dye added to a similar volume of culture medium. Application of Hoechst 33258 without agarose overlay ((a) transmitted light, (b) fluorescence); injection of Hoechst 33258 into the agarose layer ((c) transmitted light, (d) fluorescence). Similar exposure times were used for (b) and (d) (X: point of dye injection).

12% compared to cells without the agarose (Figure 3b), despite the presumed substrate concentrative effect of the agarose overlay. This CYP activity level corresponds to an oxygen concentration of about 1–2% based on the calibration curve shown in Figure 3a.

To rule out artifacts due to diffusion of resorufin into the agarose gel, a control experiment with resorufin injected under the agarose layer in the absence of cells was carried out. Approximately, 83% of the fluorescent dye was found in the solution under similar incubation conditions (data not shown). CYP2B1 protein levels were not influenced by the agarose overlay, as determined by antibody staining and flow cytometry analysis (data not shown). Hence, the agarose layer does not influence expression of CYP2B1. We conclude that the agarose overlay technique apparently mimics the tumor environment in terms of its restriction of oxygen supply available to the CYP-expressing tumor cells.

Co-culture experiments were carried out to determine the impact of the conditions imposed by the agarose overlay, that is, limited drug and metabolite diffusion and hypoxic conditions, on bystander antitumor activity. Different ratios of X39 and CT26 cells (Figure 4a and b) or X39 and murine neuroblastoma Neuro2A cells (Figure 4c and d) were seeded in multiwell plates.

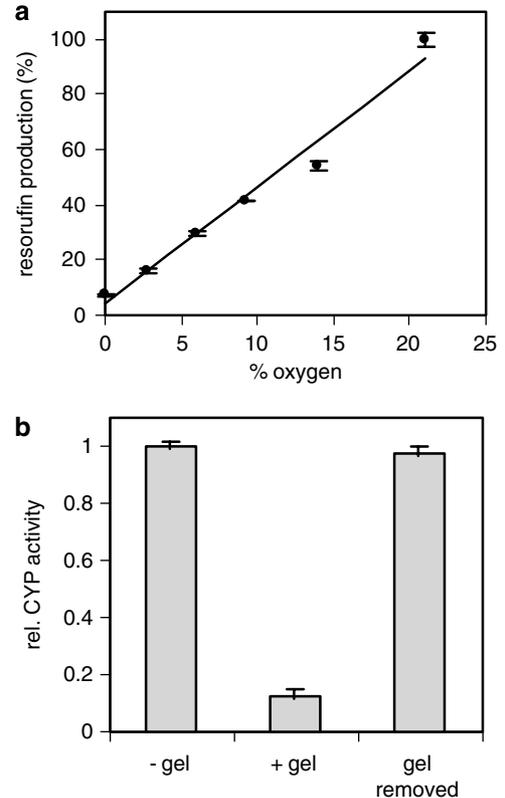


Figure 3 Influence of oxygen concentration on cytochrome P450 (CYP) metabolic activity. (a) Cytochrome P450 activity was measured in X39 cells under different concentrations of O₂ as described in Materials and methods. Resorufin production determined at 21% O₂ was set to 100%; mean values from triplicates \pm s.d. are shown. (b) At 24 h before agarose overlay, 30 000 X39 cells were seeded per well of a 48-well plate. Cytochrome P450 activity was measured as described in Materials and methods ('-gel'). When performing the pentoxoresorufin assay under the agarose overlay ('+gel'), 200 μ l of the incubation solution was injected under the agarose layer 1 h after solidification of the agarose overlay. After the incubation, fluorescence measurements were performed in the same way as described above. In 'gel removed' samples, CYP activity with pentoxoresorufin was determined for cells cultured under agarose after removal of the agarose overlay. Resorufin production by cells without agarose overlay ('-gel') was set to 100%. Mean values from triplicates \pm s.d. are shown.

The cells were treated with 0.5 mM CPA in standard cell culture medium (a and c) or after coating the cells with a layer of 0.5% agarose (b and d). After CPA treatment for 5 days, cell survival was measured using a DNA content assay. Under standard culture conditions, CT26 cells were quite resistant to the cytotoxic effects of CPA activated by X39 cells. Thus, 50% killing of the overall cell population was only achieved when the population was comprised of 75% CYP2B1-expressing (X39) cells (Figure 4a). Neuro2A cells exhibited somewhat greater sensitivity to activated CPA, with 50% cell killing achieved in co-cultures containing \sim 60–65% X39 cells (Figure 4c). By contrast, a remarkable bystander effect was achieved when agarose overlaid co-cultures were treated with CPA. In particular, CPA killed \sim 80% of the Neuro2A + X39

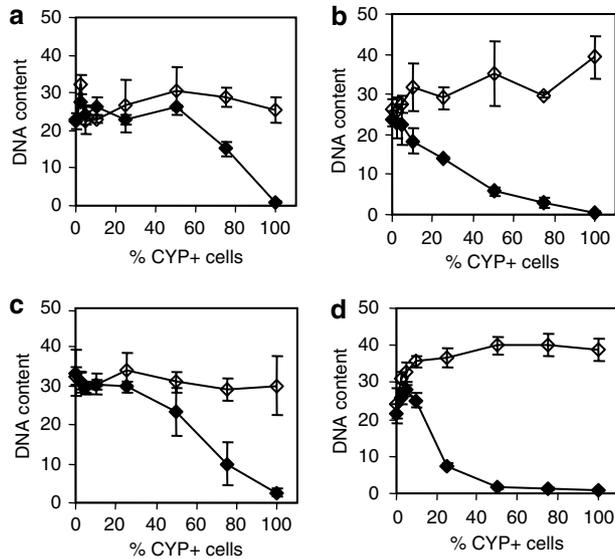


Figure 4 Impact of agarose overlay on bystander effect in mixed cell populations: Different ratios of CT26 cells (a and b) or Neuro2A cells (c and d) mixed with the CYP2B1-expressing X39 cells were treated for 5 days with 0.5 mM cyclophosphamide (CPA) as described in Materials and methods. The DNA content of each well was assayed, and absolute DNA content values expressed as $\text{RFU} \times 1000$ are shown on the y axis. (a and c) conventional cell culture; (b and d) agarose overlay cell culture; mean values from triplicates \pm s.d. are shown. Open symbols, no CPA treatment; closed symbols, treatment with 0.5 mM CPA.

mixed cell population in cultures containing 25% CYP-expressing cells, and almost complete eradication of the Neuro2A + X39 mixed cell population was achieved in cultures containing 50% CYP-expressing cells (Figure 4d; also see CT26 cells in Figure 4b).

The CPA concentration achieved in tumor tissue *in vivo* can be quite low ($<0.5 \text{ mM}^{19}$) owing to rapid clearance of the drug from the bloodstream (half-life $<30 \text{ min}^{20}$). We therefore investigated if a bystander effect of activated CPA can also be obtained at low CPA concentrations (Figure 5). CT26, Huh7 and X39 cells, and mixtures of X39 cells and either CT26 cells or Huh7 cells (25/75%) were treated for 5 days with 0.01–10 mM CPA in culture medium, with or without an agarose overlay. The CYP-deficient CT26 and Huh7 cells were quite resistant to CPA, both in the absence and presence of the agarose layer, and significant cell killing was only observed at CPA concentrations $\geq 3 \text{ mM}$ (Figure 5a and b). No beneficial effect of the agarose layer was observed in cultures comprised of X39 cells alone, as all the cells express CYP2B1 and generate 4-OH-CPA intracellularly, and thus no bystander effect is required for cell killing (Figure 5c). In co-cultures of 25% X39 cells and 75% CT26 cells, the presence of an agarose overlay markedly increased CPA-induced cell killing (Figure 5d). At 0.05 mM CPA, $\sim 40\%$ cell killing was observed, both with and without the agarose overlay. As the X39 cells grow slightly faster than the parental cell line (data not shown), the 40% reduction in DNA content most

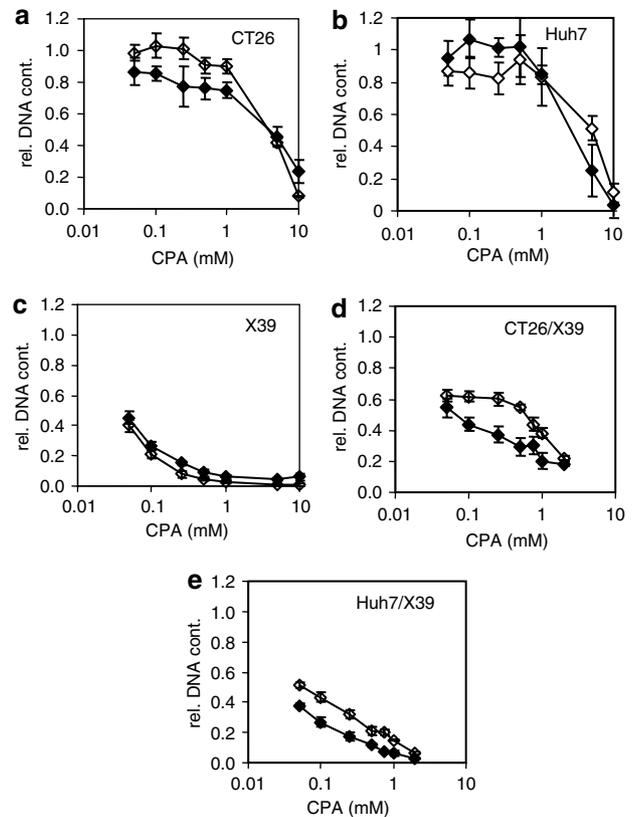


Figure 5 Effect of cyclophosphamide (CPA) concentration on tumor cell proliferation in co-cultures with CYP2B1-expressing cells. Cells (a) CT26; (b) Huh7; (c) X39; (d) CT26/X39 75/25%; (e) Huh7/X39 75/25%) were treated with different concentrations of CPA for 5 days and the DNA content was measured thereafter. Relative DNA content is shown, with the cells without CPA treatment set to 100%. Black symbols: cells cultured with agarose overlay; white symbols: cells cultured without agarose overlay; mean values from triplicates \pm s.d. are shown.

probably reflects the killing of the 25% X39 cells seeded initially. A clear bystander effect was observed for the cells under the agarose layer at CPA concentrations between 0.1 and 1 mM, which corresponds to a pharmacologically relevant concentration range. In co-cultures of X39 cells with Huh7 hepatoma cells, 50% cell killing was achieved at very low concentrations of CPA (0.05 mM) and this effect was further enhanced by the agarose overlay (Figure 5e).

Next, we investigated the impact of the agarose overlay on bystander activity under conditions where CYP2B1 cDNA is delivered to the tumor cells by transient transfection, to mimic CYP gene delivery to tumor cells *in vivo*. Huh 7 cells were transfected with CYP2B1 cDNA using linear PEI, which resulted in $\sim 13\%$ transfection efficiency, as shown by using EGFP as a reporter gene and confirmed by antibody staining for CYP2B1 (Figure 6a). Using similar conditions, Huh7 cells were transfected with plasmid encoding CYP2B1, or luciferase, followed by treatment with 0.5 mM CPA (Figure 6b). In cells transfected with CYP2B1 and overlaid with

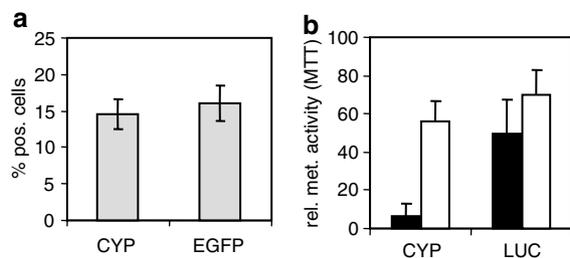


Figure 6 (a) Transfection efficiency on HUH7 cells. Huh7 cells were transfected with polyethylenimine (PEI)-DNA polyplexes containing either pCMV-EGFP-N1 (enhanced green fluorescent protein (EGFP)) or pCR3.1-CYP2B1 (cytochrome P450 (CYP)) as described in Materials and methods. Forty-eight hours after transfection, the percentage of EGFP-positive cells was determined by flow cytometry, and the percentage of CYP2B1-expressing cells was determined 48 h after transfection by flow cytometry by antibody staining as described in Materials and methods. Data shown are mean values for duplicates \pm s.d., based on cells pooled from 4 wells. (b) Influence of agarose overlay on cytotoxicity of cyclophosphamide (CPA) toward transiently transfected tumor cells. Huh7 cells were transfected with PEI-DNA polyplexes containing either pCMV-Luc (Luc) or pCR3.1-CYP2B1 (CYP) as described in Materials and methods. Forty-eight hours after transfection, the cells were treated for 5 h with 0.5 mM CPA either with agarose overlay (black bars) or under standard culture conditions (white bars). Culture medium and agarose overlay were then removed and replaced by CPA-free medium. Three days after CPA treatment, cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide assay as described in Materials and methods. Data are normalized to control cells treated in the same way but without CPA. Data shown are mean values for triplicates, \pm s.d.

agarose, >90% of the cells were killed after only a 5-h CPA treatment, as judged in a cell growth inhibition assay scored 3 days later. The control cells, transfected with luciferase, showed only moderate toxicity.

Discussion

The diffusion of nutrients, drugs and metabolites within a solid tumor is limited. Moreover, most solid tumors are characterized by extended hypoxic regions and areas with an acidic microenvironment that are resistant to chemotherapy and radiotherapy.¹⁰ Intratumoral activation of CYP prodrugs, such as the oxazaphosphorine CPA, is an oxygen-dependent reaction and can therefore be impacted by the lack of oxygen within a tumor. Nevertheless, CPA and IFA have been shown to be efficiently activated within tumors expressing CYP enzymes in the context of a GDEPT strategy, leading to enhanced cytotoxic responses.²¹

Using viral vectors for gene delivery, only a fraction of the cells within a tumor can typically be transfected with a therapeutic gene.^{6,12} Non-viral gene delivery systems suffer from an even lower transfection efficiency. Nevertheless, they offer the possibility of safe and repeated administration of the transgene without the immunological

complications associated with adenovirus and certain other viral vectors. In order for GDEPT to be therapeutically effective, a potent bystander effect is required so that a high fraction of tumor cells may be exposed to the tumor cell-generated cytotoxic metabolites.²² Bystander studies are often carried out by mixing tumor cells transfected with a therapeutic gene with unmodified tumor cells.^{22,23} However, the specific therapeutic effect induced by a therapeutic gene *in vivo* may overlap with other effects that occur *in vivo*, including antiangiogenic effects, enzyme induction and immunogenic effects. On the other hand, results obtained in bystander experiments carried out *in vitro* may be non-physiological and reflect the unnatural environment of conventional cell culture. The present study was designed to address this issue by assessing the bystander activity of CYP-activated CPA in cell culture using an agarose overlay method that mimics the tumor microenvironment.

To evaluate the bystander activity of CYP/CPA, and potentially other enzyme/prodrug combinations, we devised a simple method that uses an agarose overlay technique to mimic *in vitro* two important features of the microenvironment of solid tumors, namely, limited diffusion of active drug metabolites and tumor hypoxia. The limitation in diffusion is especially important in the case of unstable, short-lived drug metabolites, such as those derived from CPA. Agarose has been described as a suitable material for simulation of diffusion within a solid tumor.²⁴ This effect was demonstrated by injecting the membrane-permeable DNA dye Hoechst 33258 into the agarose layer. The limited diffusion of this dye led to the formation of a concentration gradient and the staining of tumor cell nuclei in the immediate vicinity of the injection site (Figure 2). The agarose overlay also imposed an apparent hypoxic environment on the cells, as shown by the 88% decrease in CYP metabolic activity of X39 tumor cells cultured under an agarose layer, which corresponds to the CYP activity at an ambient oxygen content of 2% (v/v). Despite the reduced activity, however, the CYP-expressing tumor cells cultured with the agarose overlay exhibited an enhanced bystander cytotoxic effect. The increased bystander activity was seen in experiments with co-cultures of CYP-expressing cells and CYP-deficient cells, and in experiments where the CYP cDNA was introduced by transient transfection.

Thus, with the agarose overlay, a population of 25% CYP-expressing Neuro2A cells killed 80% of the overall tumor cell population as compared to only 13% tumor cell killing in the absence of the overlay. Of note, X39 cells exerted greater bystander toxicity toward Huh7 hepatoma cells compared to CT26 cells. The enhanced sensitivity of the Huh7 cells toward CPA may in part be owing to the intrinsic CYP activity of Huh7 cells, which is ~2% of that seen with the X39 clone (Figure 1). Short-term treatment (5 h) with 0.5 mM CPA should mimic the *in vivo* situation, where short exposures to CPA at peak levels of <1 mM can be found in tumor tissue, even after a high-dose bolus of CPA.²⁵ In the transient transfection studies, where only a limited number of cells expressed CYP2B1 (13%, estimated based on an CYP2B1 antibody staining

and EGFP as reporter gene; Figure 6a), a clear CPA-induced killing effect was only observed for cells cultured under an agarose layer.

In conclusion, the present studies highlight the utility of an agarose overlay method that mimics the hypoxic nature of the tumor microenvironment and the restricted diffusion of activated drug metabolites. In the case of CYP2B1-dependent CPA activation, the restricted diffusion of activated metabolites out from the tumor cell microenvironment is likely to prolong the exposure to elevated concentrations of active metabolites that play a crucial role in enhancing bystander cytotoxicity. Despite the fact that CYP activity, and therefore prodrug activation, is substantially reduced under the hypoxic conditions imposed by the agarose overlay, a strong bystander effect could nevertheless be observed as a consequence of the restricted diffusion of active metabolites. This bystander effect becomes even more important at low oxygen concentrations and under conditions where only a limited fraction of the tumor cells express the CYP gene, as is likely to be the case in any *in vivo* gene delivery situation. The agarose overlay method described here may also be suitable for studies of other prodrug activation enzymes and other conditions that involve responses to hypoxic conditions, limited diffusion of drugs and metabolites and paracrine effects.

Abbreviations

CPA, cyclophosphamide; CYP, cytochrome P450; GDEPT, gene-directed enzyme prodrug therapy; FBS, fetal bovine serum; HBS, 150 mM NaCl, 20 mM HEPES pH 7.4; IFA, ifosfamide; MTT, (3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide); PEI, polyethylenimine.

Acknowledgements

This work was supported in part by Grant CA49248 from the NIH (DJW) and by a Grant from the Wilhelm Sander Stiftung (MG and MO).

References

- Dachs GU, Tupper J, Tozer GM. From bench to bedside for gene-directed enzyme prodrug therapy of cancer. *Anticancer Drugs* 2005; **16**: 349–359.
- Günther M, Wagner E, Ogris M. Specific targets in tumor tissue for the delivery of therapeutic genes. *Curr Med Chem Anti-cancer Agents* 2005; **5**: 157–171.
- Roy P, Waxman DJ. Activation of oxazaphosphorines by cytochrome P450: application to gene-directed enzyme prodrug therapy for cancer. *Toxicol in Vitro* 2006; **20**: 176–186.
- Wei MX, Tamiya T, Rhee RJ, Breakefield XO, Chiocca EA. Diffusible cytotoxic metabolites contribute to the *in vitro* bystander effect associated with the cyclophosphamide/cytochrome P450 2B1 cancer gene therapy paradigm. *Clin Cancer Res* 1995; **1**: 1171–1177.
- Chen L, Waxman DJ. Intratumoral activation and enhanced chemotherapeutic effect of oxazaphosphorines following cytochrome P-450 gene transfer: development of a combined chemotherapy/cancer gene therapy strategy. *Cancer Res* 1995; **55**: 581–589.
- Jounaidi Y, Waxman DJ. Use of replication-conditional prodrug-activation genes for cancer therapy. *Cancer Res* 2004; **64**: 292–303.
- Wright JE, Tretyakov O, Ayash LJ, Elias A, Rosowsky A, Frei III E. Analysis of 4-hydroxycyclophosphamide in human blood. *Anal Biochem* 1995; **224**: 154–158.
- Mahoney BP, Raghunand N, Baggett B, Gillies RJ. Tumor acidity, ion trapping and chemotherapeutics. I. Acid pH affects the distribution of chemotherapeutic agents *in vitro*. *Biochem Pharmacol* 2003; **66**: 1207–1218.
- Jahde E, Roszinski S, Volk T, Glusenkamp KH, Wiedemann G, Rajewsky MF. Metabolic response of AH13r rat tumours to cyclophosphamide as monitored by pO_2 and pH semi-microelectrodes. *Eur J Cancer* 1992; **29A**: 116–122.
- Gillies RJ, Schornack PA, Secomb TW, Raghunand N. Causes and effects of heterogeneous perfusion in tumors. *Neoplasia* 1999; **1**: 197–207.
- Jounaidi Y, Waxman DJ. Combination of the bioreductive drug tirapazamine with the chemotherapeutic prodrug cyclophosphamide for P450/P450-reductase-based cancer gene therapy. *Cancer Res* 2000; **60**: 3761–3769.
- Braybrooke JP, Slade A, Deplanque G, Harrop R, Madhusudan S, Forster MD *et al*. Phase I study of MetXia-P450 gene therapy and oral cyclophosphamide for patients with advanced breast cancer or melanoma. *Clin Cancer Res* 2005; **11**: 1512–1520.
- Boeckle S, von Gersdorff K, van der PS, Culmsee C, Wagner E, Ogris M. Purification of polyethylenimine polyplexes highlights the role of free polycations in gene transfer. *J Gene Med* 2004; **6**: 1102–1111.
- Donato MT, Gomez-Lechon MJ, Castell JV. A microassay for measuring cytochrome P450IA1 and P450IIB1 activities in intact human and rat hepatocytes cultured on 96-well plates. *Anal Biochem* 1993; **213**: 29–33.
- Kim MH, Jung YS, Moon CH, Lee SH, Baik EJ, Moon CK. High-glucose induced protective effect against hypoxic injury is associated with maintenance of mitochondrial membrane potential. *Jpn J Physiol* 2003; **53**: 451–459.
- von Gersdorff K, Ogris M, Wagner E. Cryoconserved shielded and EGF receptor targeted DNA polyplexes: cellular mechanisms. *Eur J Pharm Biopharm* 2005; **60**: 279–285.
- Rao J, Otto WR. Fluorimetric DNA assay for cell growth estimation. *Anal Biochem* 1992; **207**: 186–192.
- Yuan F, Krol A, Tong S. Available space and extracellular transport of macromolecules: effects of pore size and connectedness. *Ann Biomed Eng* 2001; **29**: 1150–1158.
- Ding Q, Kestell P, Baguley BC, Palmer BD, Paxton JW, Muller G *et al*. Potentiation of the antitumour effect of cyclophosphamide in mice by thalidomide. *Cancer Chemother Pharmacol* 2002; **50**: 186–192.
- Sladek NE. Metabolism and pharmacokinetic behavior of cyclophosphamide and related oxazaphosphorines. In: Powis G (ed). *Anticancer Drugs: Reactive Metabolism and Drug Interactions*. Pergamon Press Ltd: Oxford, 1994, pp 79–156.
- Chen L, Waxman DJ. Cytochrome P450 gene-directed enzyme prodrug therapy (GDEPT) for cancer. *Curr Pharm Des* 2002; **8**: 1405–1416.

- 22 Denny WA. Prodrugs for gene-directed enzyme-prodrug therapy (suicide gene therapy). *J Biomed Biotechnol* 2003; **2003**: 48–70.
- 23 Jounaidi Y, Waxman DJ. Combination of the bioreductive drug tirapazamine with the chemotherapeutic prodrug cyclophosphamide for P450/P450-reductase-based cancer gene therapy. *Cancer Res* 2000; **60**: 3761–3769.
- 24 Yuan F, Krol A, Tong S. Available space and extracellular transport of macromolecules: effects of pore size and connectedness. *Ann Biomed Eng* 2001; **29**: 1150–1158.
- 25 Ding Q, Kestell P, Baguley BC, Palmer BD, Paxton JW, Muller G *et al*. Potentiation of the antitumour effect of cyclophosphamide in mice by thalidomide. *Cancer Chemother Pharmacol* 2002; **50**: 186–192.