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# Identification of human reductases that activate the dinitrobenzamide mustard prodrug PR-104A: A role for NADPH:cytochrome P450 oxidoreductase under hypoxia

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ABSTRACT

Hypoxia is a common trait found in many solid tumours and thus represents a therapeutic target with considerable potential. PR-104, a hypoxia-activated prodrug currently in clinical trial, is a water-soluble phosphate ester which is converted *in vivo* to the corresponding alcohol, PR-104A. This 3,5-dinitrobenzamide-2-nitrogen mustard is activated by reduction to the corresponding 5-hydroxylamine (PR-104H) and 5-amine (PR-104M) in hypoxic cells. The clinical effectiveness of PR-104 will depend in part on the expression of reductases within tumours that can effect this reduction. Here, we evaluate the roles of NADPH:cytochrome P450 oxidoreductase (CYPOR; E.C.1.6.2.4) and NAD(P)H:quinone oxidoreductase (NQO1; E.C.1.6.99.2) as candidate PR-104A reductases. A weak correlation was observed between NQO1 activity and aerobic cytotoxicity in a panel of eight tumour cell lines. However, overexpression of human NQO1 did not increase cytotoxicity of PR-104A or the formation of PR-104H/M, showing that PR-104A is not a substrate for NQO1. Overexpression of human CYPOR did, however, increase the hypoxic cytotoxicity of PR-104A, and its metabolism to PR-104H and PR-104M, demonstrating it to be a PR-104A reductase. To assess the contribution of CYPOR to overall activation of PR-104A in hypoxic SiHa cells, a combination of siRNA transfection and antisense expression were used to suppress CYPOR protein by 91% (±3%), a phenotype which conferred 45% (±7%) decrease in cytotoxic potency of PR-104A. Regression analysis of all CYPOR depletion data was found to correlate with cytoprotection and metabolism ($p < 0.001$). Residual PR-104A reductase activity could be inhibited by the flavoprotein inhibitor diphenyliodonium. We conclude that CYPOR is an important PR-104A reductase, but that other flavoenzymes also contribute to its activation in hypoxic SiHa cells.



## 1. Introduction

Hypoxia in tumours represents an important therapeutic target for cancer chemotherapy for three reasons. The first is that hypoxic cells are refractory to radiotherapy and chemotherapy through multiple mechanisms [1,2]. The second is that hypoxia enhances the biological aggressiveness of tumours, increasing genetic instability, invasiveness [3] and metastasis [4]. A third reason for considering hypoxia as a therapeutic target is that it is more common and more severe in tumours than in normal tissues [5]. Thus, even if hypoxic cells were not an impediment to treatment success, it would

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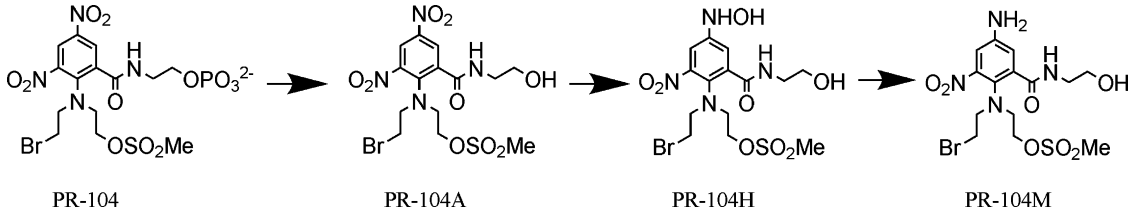


**Fig. 1 – Pathway of PR-104 metabolism.**

nonetheless represent a target that might be exploited to achieve tumour selectivity. For these reasons there is interest in development of prodrugs that can be activated by metabolic reduction under hypoxic conditions, and several such agents (e.g. tirapazamine, banoxantrone, EO9, PR-104, NLCQ-1, KS-119W and TH-302 [5,6]) have recently been or are currently in development in this context.

PR-104 (Fig. 1), the first hypoxia-activated nitrogen mustard alkylating agent to enter clinical trial, is a water-soluble phosphate ester which is hydrolysed rapidly *in vivo* to the corresponding alcohol PR-104A [7]. PR-104A, a 3,5-dinitrobenzamide-2-mustard, is a hypoxia-activated prodrug by virtue of metabolic reduction of its 5-nitro group to the corresponding hydroxylamine (PR-104H) and further reduction to the 5-amine (PR-104M) (Fig. 1). The biotransformation of the electron-withdrawing nitro group to the electron-donating hydroxylamine and amine results in direct activation of the nitrogen mustard moiety, as previously described for the 2,4-dinitrobenzamide-5-mustard prototype SN 23862 [8,9], and results in hypoxia-selective cytotoxicity in cell culture [7]. PR-104A penetrates efficiently into hypoxic regions of tumours [10] where it forms the same reduced metabolites as in hypoxic cell cultures [11]. PR-104 provides very efficient killing of hypoxic cells in human tumour xenografts and is also surprisingly active against aerobic cells as demonstrated by its marked antitumour activity as a single agent [7]. This may be a manifestation of a bystander effect resulting from diffusion of reduced metabolites out of hypoxic zones, as demonstrated for SN 23862 in studies with multicellular layer cultures [12], although oxygen-insensitive reduction to PR-104H at low rates in aerobic tumour cells might also contribute to this activity [7].

Identification of the reductases capable of activating PR-104A in tumour tissues is important for the optimal clinical development of PR-104 since the sensitivity of individual tumours is likely to depend on the activity of these reductases, as well as the severity and spatial distribution of hypoxia. This approach, termed as enzyme-directed bioreductive drug development, seeks to individualise treatment as well as predict normal tissue toxicities [13]. Many ''bioreductive drugs'' (prodrugs activated by reduction, whether selective for hypoxia or not) are known to be activated by both one-electron and two-electron reductases [14,15]. The former add a single electron to the prodrug to form a radical intermediate which, if it is readily reoxidised to the original prodrug by dioxygen, ensures that further reduction is confined to hypoxic cells. In contrast, two-electron reductases bypass this oxygen-sensitive intermediate and are thus capable of reducing and activating quinones and nitroarenes in the presence of oxygen.

The key reductases that activate bioreductive drugs in human tumours are poorly defined, although aerobic activation of quinones such as mitomycin C (MMC), EO9 and RH1 is catalysed by the two-electron reductase DT-diaphorase (NQO1) [16–18] which is over-expressed in some human tumours [19–21]. CB 1954, a dinitrobenzamide prodrug with some structural similarity to PR-104A, is activated by rat NQO1 [22–24] suggesting that NQO1 should be considered as a candidate PR-104A aerobic reductase. The flavoprotein NADPH:cytochrome P450 oxidoreductase (CYPOR) is a one-electron reductase with a key physiological role in reducing CYPs but is also a major contributor to activation of quinone [25–27], aromatic *N*-oxide [28] and nitroarene compounds [25,29].

In this study we evaluate the roles of NQO1 and CYPOR in the metabolic activation of PR-104A by human tumour cells under aerobic and hypoxic conditions. This is investigated by testing for correlations between enzyme activity and PR-104A activation across a panel of eight cell lines and by over expressing the enzymes in tumour cell lines. The latter studies implicate CYPOR (but not NQO1) in the activation of PR-104A. In addition, quantitative suppression of CYPOR gene expression at the RNA level (using siRNA and antisense) confirms its role as a PR-104A reductase under hypoxia but demonstrates that it is not the sole reductase responsible for the hypoxic activation of this prodrug.

## 2. Methods

### 2.1. Compounds

PR-104A, synthesized in the Auckland Cancer Society Research Centre (ACSRC) as previously described [30], had a purity of 94–100% by HPLC and was stored at −20 °C. Stock solutions were prepared in DMSO to a final concentration of 100 mM and stored at −80 °C. PR-104H, prepared by zinc dust reduction of PR-104A as previously described [7], was stored in acetonitrile at −80 °C and had a purity of 95% by HPLC. Mitomycin C (10 mM), dicoumarol (10 mM) and diphenyiodonium (100mM) (Sigma–Aldrich) were prepared as DMSO stocks and stored at −80 °C. Reagents for CYPOR and NQO1 activity assays were prepared as described previously [31,32].

### 2.2. Cell lines

Cell lines were passaged in alpha minimal essential medium (αMEM) supplemented with 5% fetal bovine serum (FBS) without antibiotics. Cell lines were obtained from ATCC

(Manassas, VA) except for gifts of A549 cells (Dr. Martin Brown, Stanford U.), H1299 and C33A (Onyx Pharmaceuticals, Richmond, CA), HT29 (Dr. David Ross, U. Colorado), PC3 (Dr. Lynn Ferguson, U. Auckland), and SiHa (Dr. David Cowan, Ontario Cancer Institute). Cell lines were confirmed to be free of mycoplasma by PCR–ELISA (Roche Diagnostics). The vector and methodologies used to construct the CYPOR over expressing cell line, SiHa$^{CYPOR}$, have been described [33]. The NQO1 expressing MDA-231 cell line (MDA-231$^{NQO1}$) was generated using plasmid F397 [34]. Cell lines overexpressing these enzymes were grown in the presence of 3 μM puromycin. The antisense-CYPOR plasmid (pcDNA-aNPR) [35], a gift from Dr S. Imaoka (Osaka City University), was introduced into SiHa and the stable clone SiHa$^{antiCYPOR}$ was isolated in the presence of 1 mg/ml geneticin.

### 2.3. Inhibition of cell proliferation in vitro

Cells were seeded into 96-well plates (typically 1000 cells/0.1 ml) and exposed to compounds for 4 h under aerobic or hypoxic conditions as detailed elsewhere [36]. Media composition was αMEM with 5% FBS (for oxic incubations) or anoxia equilibrated culture media containing 10% FCS, 200 μM 2′ deoxycytidine and additional D-glucose (total 17 mM). Hypoxic cultures were exposed in a $H_2$/Pd-catalyst scrubbed anaerobic chamber, using plastics and liquids pre-equilibrated (>3 days) to remove residual oxygen. Cultures were then grown for 5 days and stained with sulphorhodamine B to measure total cells [37]. The $IC_{50}$ was determined by interpolation as the drug concentration reducing staining to 50% of controls on the same plate.

### 2.4. Determination of enzyme activity

The activity of CYPOR and NQO1 in the cell lines was measured in S-9 fractions prepared as described [31]. Total protein concentration was measured using the BCA assay [38]. CYPOR activity was determined by spectrophotometric assay as the cyanide-resistant reduction of cytochrome *c* in the presence of NADPH, as described [31]. NQO1 activity was detected as the dicoumarol sensitive (0.1 mM), NADH-dependent reduction of cytochrome *c* in the presence of menadione as described [32,39].

### 2.5. Determination of PR-104A metabolism in stirred cell suspensions by HPLC

Sub-confluent cells were harvested from T175 flasks, counted and aliquots of $2.5 \times 10^7$ cells were centrifuged (800 × *g*, 5 min) and resuspended in 8.4 ml oxic or anoxic culture medium (as above), the latter in an anaerobic chamber. 1.4 ml aliquots of cell suspension were transferred to 4 ml glass vials containing stir bars. PR-104A was added to a final concentration of 100 μM, lids were tightly sealed and vials transferred to a 37 °C water bath with a magnetic stirrer and incubated for 1 h. Following incubation, samples were immediately placed on ice, 1 ml was removed and pipetted up and down the walls of a fresh, pre-chilled 4 ml glass vial to reoxygenate before transfer of 900 μl to a 1.5 ml microfuge tube on ice. Cells were pelleted (16,000 × *g*, 15 s, 4 °C), the supernatant immediately aspirated and the cell pellet lysed with 80 μl ice-cold methanol and stored at −80 °C. Prior to HPLC, samples were centrifuged (16,000 × *g*, 5 min, 4 °C) and 30 μl diluted with 70 μl 45 mM ammonium formate (pH 4.5). Samples (80 μl) were analysed by HPLC using a HP 1100 (Agilent Technologies, Walbronn, Germany) equipped with a diode-array detector and an Alltima C8 reverse phase column (150 mm × 2.1 mm, 5 μm; Alltech Associated Inc., Deerfield, IL) at a flow rate of 0.3 ml/min. The mobile phase comprised a gradient of acetronitrile in 45 mM ammonium formate in water (pH 4.5). Absorbance was monitored at 254 nm. PR-104A and PR-104H standards at known concentrations were included in each experiment.

### 2.6. Determination of PR-104A metabolism in confluent monolayers by HPLC

Sub-confluent cells were harvested from T175 flasks, counted and aliquots of $8 \times 10^6$ cells were centrifuged as above and resuspended in 4 ml oxic medium ±100 μM DPI or anoxic medium ±100 μM DPI, the latter in an anaerobic chamber as above. Cells were plated in 6-well plates giving $8 \times 10^6$ cells/well, incubated for 2 h at 37 °C before addition of an equal volume of the same medium containing PR-104A to give a final concentration of 45 μM and then returned to the incubator for 1 h. Anoxic samples were taken out of the anaerobic chamber and processed immediately. Medium (7 ml) was quickly removed and the cells dislodged from the wells using the remaining 1 ml before transfer into a microfuge tube on ice. Samples were prepared for HPLC as described for the metabolism in stirred cell suspensions above.

### 2.7. Optimisation of short interfering RNA

Transfection conditions for SiHa cells were optimised in 24-well plates using oligofectamine complexed FITC-labelled siRNA (Silencer FAM-Labelled Negative Control #1 siRNA, Ambion). CYPOR knockdown was evaluated using four individual pre-designed siRNAs (Invitrogen and Ambion) and a pool consisting of a further four pre-designed siRNAs (Dharmacon). Sequences of the siRNAs are provided in Table S1. Knockdown of CYPOR was assessed by Western blot analysis and quantified using ImageJ (version 1.36 of the public domain Java image processing program) with normalisation against GAPDH expression levels.

### 2.8. Clonogenic assays

Cell killing was assessed by clonogenic assay (loss of colony forming potential). Non-transfected or siRNA-transfected cells were harvested and counted with an electronic particle counter (Z2 Coulter Particle Analyzer, Beckman Coulter, Florida, USA). $5 \times 10^6$ cells were pelleted (800 × *g*, 5 min) and overlaid with 100 μl of oxic culture medium. Pellets were transferred into the anaerobic chamber and resuspended in 900 μL of anoxia-equilibrated culture medium (as above) and transferred to anoxia-equilibrated tubes containing a further 1.5 ml of media. Cells were plated into a 96-well plate at $3 \times 10^5$ cells/well (150 μL of cell suspension), and incubated at 37 °C for 2 h. PR-104A drug dilutions were prepared in anoxic media at two times final concentration; 150 μl of drug was added to appropriate wells and the plate returned to 37 °C. After 1 h drug exposure, the plate was removed from the

anaerobic chamber. Using a 12-channel pipette, media above the cells was collected and transferred to 1 mL microtubes. Monolayers were rinsed with 100 μL PBS, which was also transferred to microtubes, and cells were harvested into the same tubes using 100 μL trypsin. Six-fold serial dilutions were carried out in microtubes using a 12-channel pipette, and transfer pipettes were used to plate into 60 mm cell culture dishes containing 5 ml culture media (αMEM with 5% FBS). The plates were incubated at 37 °C for 14 days, stained with methylene blue and colonies with >50 cells were counted. Surviving fraction (SF) was calculated as the ratio of colonies from treated/control wells.

## 3. Results

### 3.1. Relationships between reductase activity and PR-104A cytotoxicity

CYPOR and NQO1 are candidate one- and two-electron reductases for the metabolism of PR-104A to cytotoxic species under hypoxic and aerobic conditions, respectively. Initially, we evaluated the relationship between PR-104A cytotoxicity and the activities of these two enzymes using *in vitro* human tumour cell cultures comprising one prostate (PC3), two colorectal (HCT116, HT29), two cervical (C33A and SiHa) and three non-small cell lung cancer cell lines (H1299, A549, H460). There was a 4.5-fold range in CYPOR activities in S9 preparations from these cell lines, and a weak correlation with hypoxic PR-104A sensitivity ($1/IC_{50}$) ($r^2 = 0.47$) which did not reach statistical significance ($p = 0.06$) (Fig. 2A). NQO1 activity spanned a 14.7-fold range across the eight cell lines, and appeared to be correlated with aerobic PR-104A sensitivity ($r^2 = 0.73$, $p = 0.01$) (Fig. 2B).

Given that variations in intrinsic cellular sensitivity to PR-104A cytotoxic metabolites is an undefined variable across the cell line panel, we stably over expressed human CYPOR and NQO1 to provide a more direct gain-of-function assessment of their potential roles. Transfection and stable expression of CYPOR cDNA in SiHa cells provided the clone $SiHa^{CYPOR}$ with cytochrome c reduction activity in S-9 preparations 13.7-fold higher than the parental line (158 ± 10 (S.E.M.) versus 11.5 ± 0.3 nmol/mg protein/min). As illustrated in Fig. 3A, over-expression of CYPOR increased the hypoxic sensitivity to PR-104A by 6.0-fold (measured as the reciprocal of the $IC_{50}$; $p = 0.027$), without significant modulation of aerobic sensitivity. Similar results were obtained in a second genetic background, an A549 CYPOR overexpressing clone previously described [33], with a 9.0-fold increase in CYPOR activity providing comparable cytotoxicity enhancement ratios (1.6-fold aerobic and 7.4-fold hypoxic sensitisation by WT/CYPOR $IC_{50}$ ratio) as those for $SiHa^{CYPOR}$.

MDA-231 cells have undetectable NQO1 activity (measured as <20 nmol cytochrome c reduction/mg s9/min) due to a homozygous point mutation (P187S) [40] and were relatively resistant to PR-104A under aerobic conditions ($IC_{50}$ 53 ± 12 μM). Overexpression of NQO1 in clone $MDA\text{-}231^{NQO1}$ provided enzyme activity of 1518 ± 115 nmol/mg/min (>75-fold increase), which resulted in no significant change in PR-104A sensitivity under either aerobic or hypoxic conditions (Fig. 3A). NQO1 biological activity in $MDA231^{NQO1}$ was confirmed by aerobic sensitisation to mitomycin C [41], with a 12-fold increase in sensitivity relative to the parent line ($IC_{50}$ 1.25 ± 1.04 μM versus 0.103 ± 0.01 μM; $p < 0.001$).

### 3.2. PR-104A metabolism in reductase overexpressing cell lines

The cytotoxicity results indicate that PR-104A is a substrate for CYPOR but not NQO1. To test whether these enzymes can metabolise PR-104A we developed an HPLC assay for its major cytotoxic intracellular metabolites, which we have recently identified as the 5-hydroxylamine (PR-104H) and 5-amine (PR-104M) reduction products [7] (Fig. 1). These more polar metabolites were well resolved from PR-104A in methanol extracts from $SiHa^{WT}$ cell pellets as illustrated in Fig. 3B. To

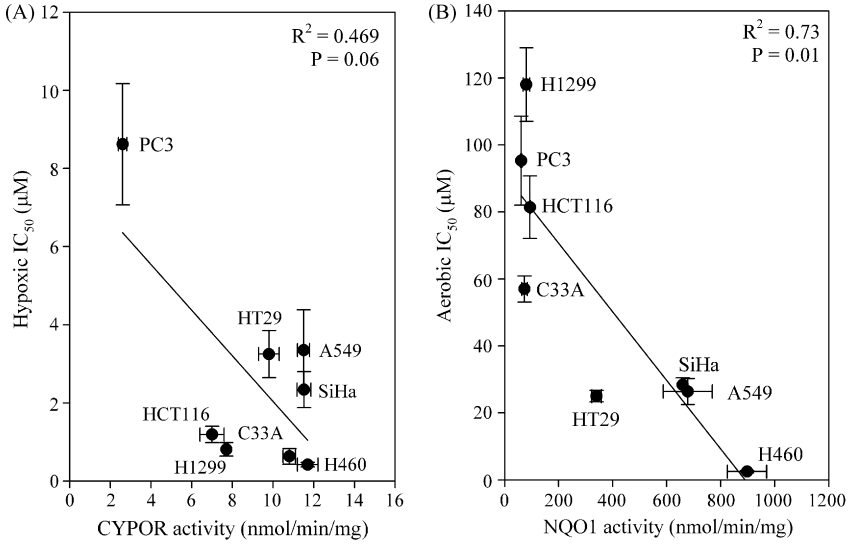


**Fig. 2 – Relationships between anti-proliferative activity (IC50) and reductase activity for a panel of eight tumour cell lines. (A) Hypoxic $IC_{50}$ and CYPOR activity. (B) Aerobic $IC_{50}$ and NQO1 activity.**

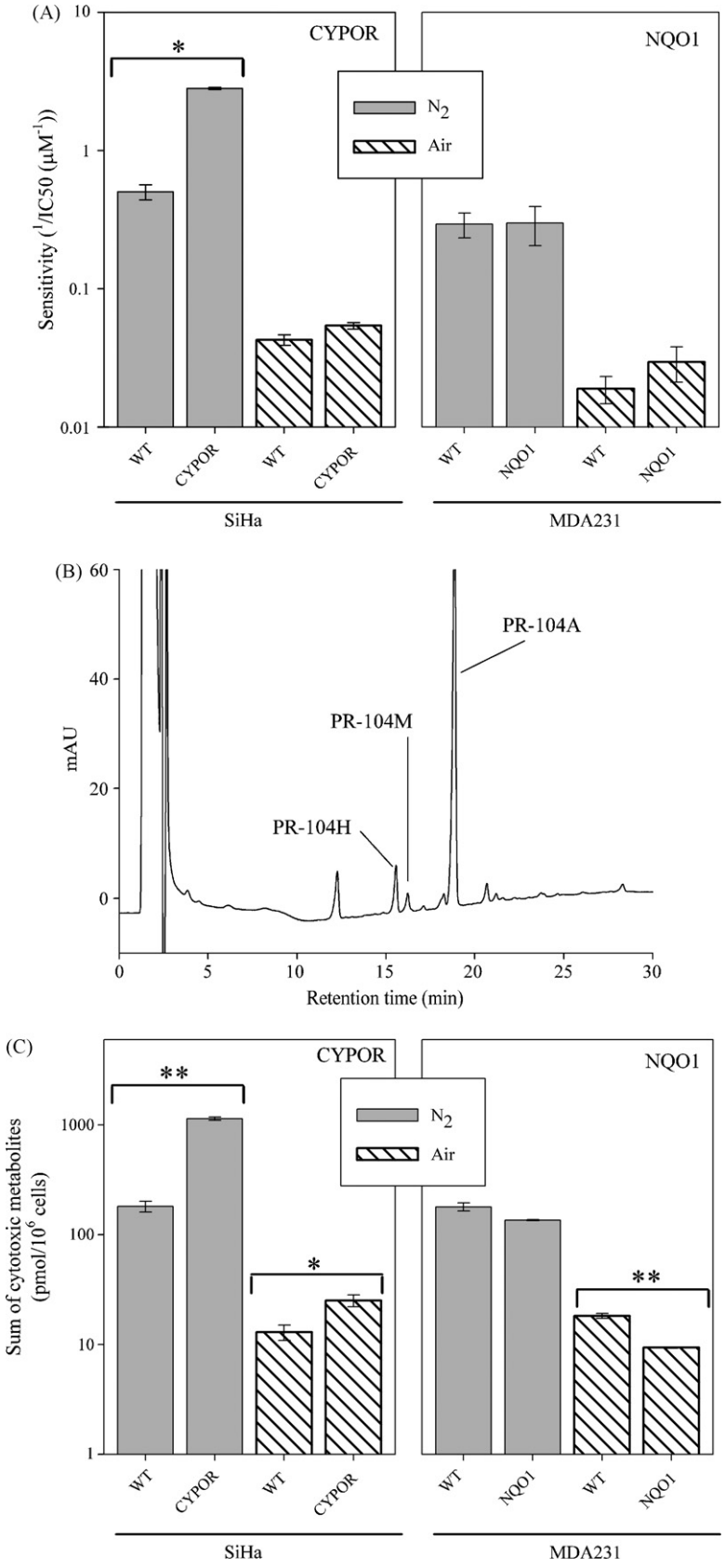


**Fig. 3 – A comparison of PR-104A activation in parent cell lines and in cell lines overexpressing CYPOR or NQO1. (A) Effect of over-expression of CYPOR and NQO1 on the sensitivity of cells to PR-104A. Sensitivity represents the reciprocal of the $IC_{50}$ for the various cell lines as determined by cytotoxicity assays. Error bars represent the S.E.M.; (*) $p < 0.05$, (**) $p < 0.01$ (B) Representative HPLC chromatogram showing the intracellular metabolism of PR-104A into PR-104H and PR-104M. $8 \times 10^6$**

assess the intracellular concentration of activated PR-104A metabolites we developed a rapid sample processing method (cell chilling to 4 °C with rapid centrifugation (16,000 × *g*, 15 s) and immediate extraction with ice-cold methanol). Method development experiments showed that these precautions are necessary to avoid artifactual metabolism of PR-104A in cell pellets due to the development of hypoxia during/after centrifugation (data not shown). Hypoxic exposure conditions resulted in a 10.5-fold increase in total reduced metabolites (PR-104H + PR-104M) in SiHa$^{WT}$ cells (Fig. 3C), broadly consistent with the 13-fold aerobic/hypoxic $IC_{50}$ differential, whilst intracellular concentrations of total intracellular metabolites (PR-104H + M) were increased 45.1-fold by hypoxia exposure of SiHa$^{CYPOR}$ cells. This provided a significant 4.2-fold greater concentration of total reduced metabolites in SiHa$^{CYPOR}$ $_{over\ SiHa}$$^{WT}$ cells under hypoxic conditions ($p < 0.001$). A more modest 1.9-fold increase in total reduced metabolites was also observed in aerobic SiHa$^{CYPOR}$ cells ($p < 0.05$). In contrast, overexpression of NQO1 in MDA-231$^{NQO1}$ cells did not increase PR-104A reductive metabolism, and in fact demonstrated a small but significant decrease relative to WT cells (Fig. 3C).

### 3.3. *Depletion of CYPOR by small interfering RNA and antisense gene expression*

These gain-of-function results clearly identify CYPOR as capable of metabolising PR-104A under hypoxia. However, the increase in cytochrome *c* reduction in the CYPOR overexpressing cell line (13.7-fold) seemed to be greater than the relative increase in cytotoxicity (6.0-fold) or formation of reduced metabolites (4.2-fold) suggesting that enzymes other than CYPOR contribute to hypoxic activation of PR-104A in SiHa cells. To test this possibility we used loss-of-function RNAi knockdown to evaluate the contribution of CYPOR to the hypoxic cytotoxicity and metabolism of PR-104A in SiHa cells. Maximal transfection efficiency (90% of SiHa cells) required 100 nmol small interfering RNA duplex (siRNA) complexed with 2 μl oligofectamine (invitrogen) applied to $5 \times 10^4$ cells/1.9 cm$^2$ (24-well plate). Of eight candidate siRNAs the greatest knockdown was achieved with the 25 nucleotide I-2 stealth siRNA (5′-GUCAGAGA-GAGCAGCUUUGUGGAAA-3′), which provided suppression of CYPOR to 23% of control levels in SiHa$^{WT}$ cells at 96 h, as determined by Western blot analysis (Fig. 4A). Scrambled negative control duplex was without effect. Transfected cells were transferred to an anaerobic chamber, incubated under anoxia for 2 h and then exposed to a range of PR-104A concentrations for 1 h. Clonogenic survival curves (Fig. 4B) showed only a minor increase in $C_{10}$ (concentration for 10% survival) from 16 to 20 μM relative to oligofectamine-treated controls or SiHa cells transfected with scrambled siRNA, corresponding to a 20% decrease in sensitivity ($1/C_{10}$) despite the 77% reduction in CYPOR protein.

Given the incomplete suppression of CYPOR by siRNA we evaluated expression of antisense-CYPOR. SiHa cells were

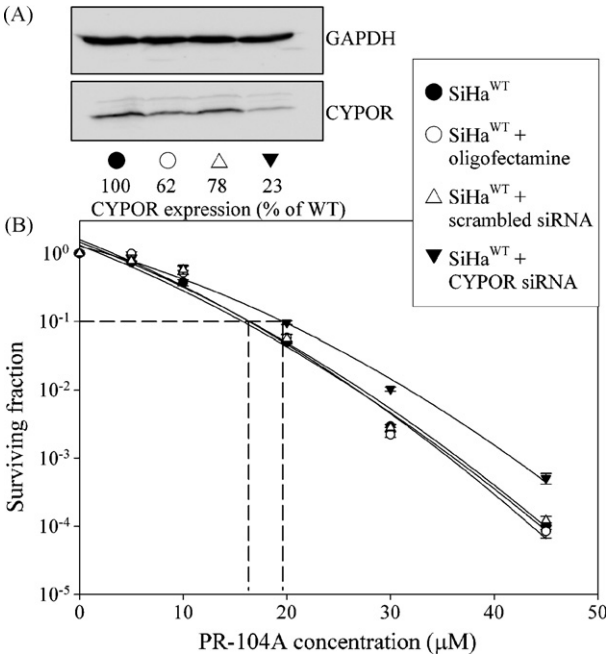


**Fig. 4 – Effect of CYPOR siRNA on CYPOR expression and anoxic sensitivity to PR-104A in SiHaWT cells. (A) Western blot analysis of CYPOR following transfection with CYPOR siRNA. Percentage CYPOR knockdown was quantified from band intensity and normalised against GAPDH expression levels. (B) Clonogenic survival curves. Anoxia-equilibrated cells were exposed to a range of PR-104A concentrations for 1 h and plated to determine clonogenic survival. Survival curves represent mean values of clonogenic assays performed in duplicate with error bars indicating the range.**

stably transfected with an antisense construct, providing the clone SiHa$^{antiCYPOR}$ with a decrease in CYPOR/GAPDH ratio to 67% relative to the parental line (Fig. 5A). Subsequent transfection of I-2 siRNA into SiHa$^{antiCYPOR}$ cells provided efficient suppression of CYPOR protein to 3% of controls and concurrently raised the PR-104A $C_{10}$ value from 11.5 to 27 μM (Fig. 5B); a 57% decrease in hypoxic cell sensitivity. This effect was reproducible in two further experiments which confirmed the average antisense effect (66% ±3% of WT level) and additional siRNA knockdown of CYPOR to 14% and 9% of the WT level, providing a 34% and 43% decrease in hypoxic cytotoxicity, respectively (Supplementary Fig. S2). In these two experiments we also measured the levels of PR-104H and PR-104M by HPLC at the end of the 1 h PR-104A exposure, demonstrating a 62% and 61% inhibition relative to SiHa$^{WT}$. Overall, the combined knockdown approach (siRNA plus antisense) was highly efficient, yet appeared to provide a less than proportional decrease in sensitivity to and metabolism of PR-104A.

To confirm the apparent relationship between CYPOR expression and cytotoxicity, we evaluated this by repression

**SiHa$^{WT}$ cells were exposed to 100 μM PR-104A under hypoxic conditions for 1 h and cell pellets were extracted with methanol for HPLC analysis. (C) Effect of overexpression of CYPOR and NQO1 on the formation of intracellular metabolites of PR-104A; (*) $p < 0.05$, (**) $p < 0.01$.**

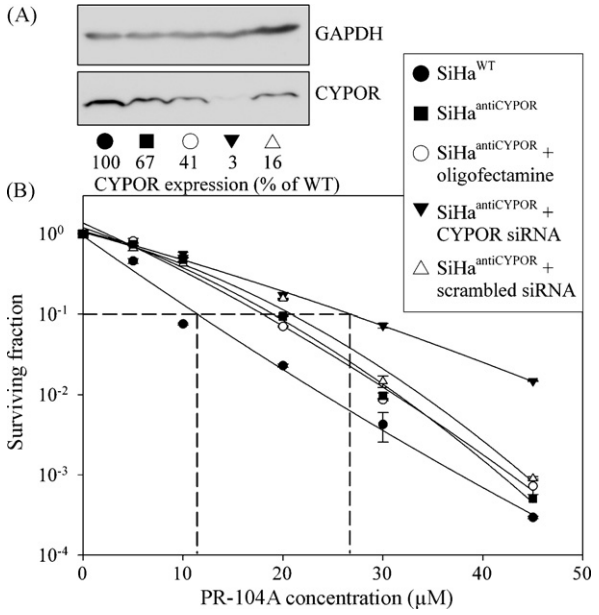


**Fig. 5 – Effect of CYPOR siRNA on CYPOR expression and anoxic sensitivity to PR-104A in SiHa cells stably transfected with a CYPOR antisense construct. (A) Western blot analysis of CYPOR following transfection with CYPOR siRNA. Percentage CYPOR knockdown was quantified from band intensity and normalised against GAPDH expression levels. (B) Clonogenic survival curves. Anoxia-equilibrated cells were exposed to a range of PR-104A concentrations for 1 h and plated to determine clonogenic survival. Survival curves represent mean values of clonogenic assays performed in duplicate with error bars indicating the range.**

analysis across four independent experiments in which both parameters were measured. The combined data showed a highly significant ($p < 0.001$) first order regression for cytotoxicity ($C_{10}$ values) versus CYPOR expression, with an extrapolated intercept at $C_{10}$ = 22.7 μM PR-104A (Fig. 6A). This represents only a 44% decrease in cytotoxic potency relative to the unmodified $C_{10}$ control value (12.8 ± 0.7 μM). Importantly, the extrapolated $C_{10}$ at zero CYPOR is much lower than the aerobic $C_{10}$ for SiHa cells (270 μM), consistent with additional hypoxic PR-104A reductase activity not accounted for by CYPOR. Similarly, Fig. 6B shows the regression of PR-104A metabolism against CYPOR/GAPDH for all groups in the two experiments in which this was measured. This again provided a significant ($p < 0.001$) linear relationship with a non-zero intercept (28.2% of SiHa$^{WT}$ metabolism when extrapolated to zero CYPOR). Taken together, these data clearly demonstrate that PR-104A is a CYPOR substrate under hypoxic conditions, but that other hypoxic reductases also contribute to its activation in SiHa cells.

### 3.4. *Inhibition of PR-104A metabolism by DPI*

To further characterise the enzymology of PR-104A activation in hypoxic SiHa cells, we tested whether the uncompetitive flavoenyme inhibitor dipenyliodonium chloride (DPI) [42] could inhibit cytotoxicity and metabolism to a greater extent than that achieved by eliminating CYPOR expression alone. Fig. 7 shows that SiHa$^{CYPOR}$ was 5.4-fold more sensitive to PR-104A under hypoxia than SiHa$^{WT}$ by clonogenic assay ($C_{10}$ 4.3 μM and 23.4 μM, respectively), consistent with the 6.0-fold intra-experimental difference in the $IC_{50}$ assay. Addition of 100 μM DPI provided essentially 100% protection of both SiHa$^{WT}$ and SiHa$^{CYPOR}$ cells from PR-104A cytotoxicity under anoxia, with barely detectable cell killing at the highest PR-104A exposure (45 μM; surviving fractions of 0.8 and 0.7, respectively).

To confirm that PR-104A cytoprotection by DPI was due to inhibition of reductive metabolism, intracellular metabolites were measured by HPLC after drug treatment in monolayers under the same conditions as for the clonogenic assays. Pre-treating SiHa$^{WT}$ cells with DPI inhibited PR-104A metabolism by 90% with PR-104M metabolite being undetectable (Table 1). SiHa$^{CYPOR}$ cells yielded a 12.5-fold increase in total metabolite formation (PR-104H + PR-104M), an increase

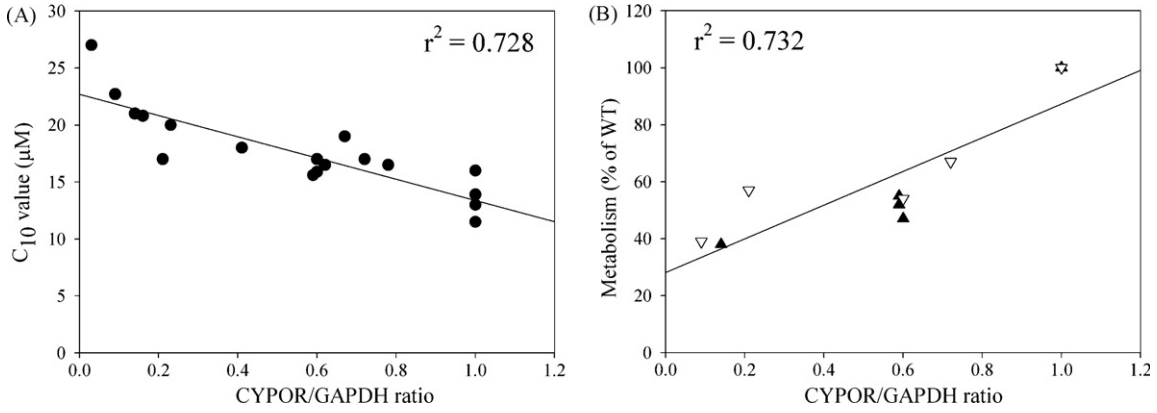


**Fig. 6 – Regression analysis of CYPOR/GAPDH ratio for untreated, oligofectamine-treated, scrambled siRNA-treated and I-2 siRNA-treated SiHa$^{antiCYPOR}$ cells with the SiHa$^{WT}$ control in the same experiment. (A) Linear regression of extent of reductive metabolism of PR-104A versus the CYPOR/GAPDH ratio from the experiments shown in Fig S2A (closed triangles) and Fig. S2B (open triangles). (B) Linear regression of PR-104A cytotoxicity ($C_{10}$ values) vs. the CYPOR/GAPDH ratios taken from the experiments shown in panels A and B of supplementary Fig. S2, Fig. 4 and Fig. 5.**

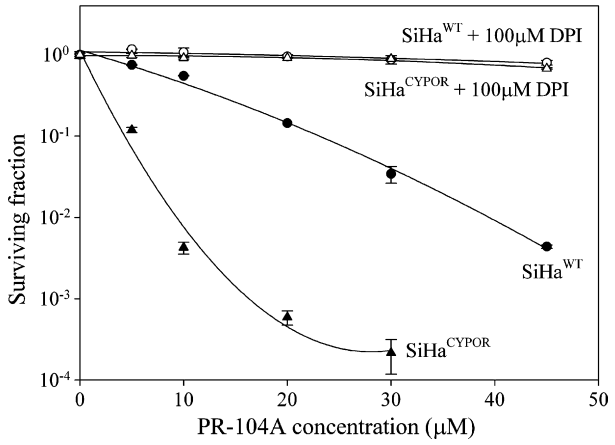


**Fig. 7 – Inhibition of the anoxic cytotoxicity of PR-104A by diphenyliodonium (DPI). Anoxia-equilibrated SiHa$^{WT}$ and SiHa$^{CYPOR}$ cells were pre-incubated in the presence or absence of 100 μM DPI for 2 h prior to exposure to a range of PR-104A concentrations for 1 h. Cells were plated to determine clonogenic survival. Survival curves represent mean values of clonogenic assays performed in duplicate with error bars indicating the range.**

that was inhibited strongly by DPI. The modest change (1.6-fold, $p = 0.04$) in aerobic PR-104H formation apparently conferred by CYPOR overexpression was refractory to DPI inhibition.

## 4. Discussion

PR-104 is a phosphate ester pre-prodrug currently in clinical trials as a hypoxic cytotoxin [7]. The prodrug PR-104A is released *in vivo* through the action of systemic phosphatases, whereupon unknown cellular reductases can metabolise PR-104A to its 5-hydroxylamine and 5-amine reduction products, activating the latent mustard moiety (Fig. 1). This biotransformation is largely inhibited by oxygen, presumably reflecting the predominance of one versus two electron reduction. PR-104A is the first 3,5-dinitrobenzamide-2-nitrogen mustard pharmacophore to undergo human evaluation and as yet no information is available on the potential oxidoreductase enzymes that can catalyse its metabolism in human tissues. This study examines the roles of two candidate enzymes, NADPH:cytochrome P450 oxidoreductase (CYPOR) and NAD(P)H:quinone oxidoreductase (NQO1; DT-diaphorase), both well known mediators of quinone and nitroarene prodrug metabolism [43].

NQO1 is an obligatory two electron reductase known to reduce a variety of bioreductive drugs [19,44]. NQO1 was considered as a candidate nitroreductase since it can activate the related dinitrobenzamide CB1954 [24,45,46]. We observed that SiHa$^{WT}$ and MDA-231$^{WT}$ cells are capable of generating the PR-104A hydroxylamine reduction product under aerobic conditions, albeit at substantially lower steady state levels than in the absence of oxygen (Fig. 3C). The observation of a correlation between the aerobic sensitivity to PR-104A and NQO1 activity across a panel of eight human neoplastic cell lines (Fig. 2B) led us to evaluate this relationship explicitly by expression of human NQO1 in the MDA-231 breast cancer cell line (confirmed by elevated menadione-dependent cytochrome *c* reduction and enhanced sensitivity to MMC). This provided an isogenic cell line pair to assess directly the apparent link between NQO1 activity and oxic PR-104A sensitivity and showed that NQO1 was unable to modify the steady state levels of PR-104A reduced metabolites or the aerobic (or hypoxic) cytotoxicity of PR-104A (Fig. 3). Thus, the enzyme(s) responsible for PR-104A reduction under aerobic conditions is distinct from NQO1, but the apparent correlation with NQO-1 enzyme activity across the cell line panel leaves open the possibility of co-ordinate regulation. This result is consistent with the observation that the 2,4-dinitrobenzamide-5-nitrogen mustard SN 23862, an analogue of PR-104A, is also not a substrate for NQO1 [47].

CYPOR catalyses the single-electron reduction of many electron-affinic substrates [15,25,28,29,48], including the dinitrobenzamide CB 1954 [49], and therefore is a candidate PR-104A nitroreductase. Despite a weak correlation across the eight cell lines *in vitro* (Fig. 2A), as shown in Fig. 3 overexpression of CYPOR in the SiHa$^{CYPOR}$ cell line clearly increased hypoxic sensitivity to PR-104A (6.0-fold) and the concentration of intracellular reduced metabolites (4.2-fold). In contrast CYPOR failed to modify aerobic sensitivity to PR-104A; although a minor increase in aerobic intracellular metabolites was noted, this was refractory to DPI inhibition implying that CYPOR has no direct role. One possible interpretation is that overexpression of CYPOR modulates expression of enzymes [50] with a role in aerobic reduction of PR-104A.

Short interfering RNA (siRNA) was utilised to quantify the role of endogenous CYPOR in SiHa$^{WT}$ cells under anoxia, using a clonogenic assay technique which we adapted for use with small numbers of siRNA-transfected cells in a 96-well format. Despite knockdown of CYPOR to 23% of SiHa$^{WT}$

**Table 1 – Sum of intracellular metabolites and effect of DPI onPR-104A metabolite formation**

| Cell line | DPI | Total PR-104A metabolites (pmol/$10^6$ cells) | | PR-104H proportion of total metabolites (%) | |
|---|---|---|---|---|---|
| | | Aerobic | Hypoxic | Aerobic | Hypoxic |
| SiHa$^{WT}$ | – | 6.8 ± 1.3 | 44.6 ± 7.5 | 100 | 66.4 |
| SiHa$^{WT}$ | + | 6.3 ± 1.6 | 4.5 ± 0.8 | 100 | 100 |
| SiHa$^{CYPOR}$ | – | 11 ± 0.5 | 559 ± 90 | 100 | 87.8 |
| SiHa$^{CYPOR}$ | + | 10.4 ± 0.9 | 9.5 ± 2.5 | 100 | 100 |

control levels only a minor decrease in cellular PR-104A sensitivity was observed. To maximise the CYPOR deficient phenotype, a stable antisense-expressing SiHa cell line clone (with CYPOR levels 66% ±3% of $SiHa^{WT}$) was transiently transfected with CYPOR siRNA (I-2), giving near complete ablation (91% ±3%) of immunoreactive CYPOR protein that was accompanied by a 45% (±7%) decrease in hypoxic PR-104A sensitivity. Regression analysis of the pooled knockdown data indicated that CYPOR levels were highly correlated with both cytoprotection ($p < 0.001$) and reduced metabolism ($p < 0.001$) (Fig. 6). We conclude from this observation that although CYPOR is involved in anoxic PR-104A metabolism, accounting for approximately half of anoxic metabolism in SiHa cells, it is not the sole reductase. This may explain the relatively weak correlation observed between CYPOR and $IC_{50}$ shown in Fig. 2.

The striking cytoprotection observed with DPI in the clonogenic assay in both the $SiHa^{WT}$ and $SiHa^{CYPOR}$ cell lines (Fig. 7), and the almost quantitative inhibition of intracellular metabolism by DPI as determined by HPLC (Table 1), indicates that the remaining hypoxic PR-104A reductase(s) in SiHa cells are likely flavin-containing NAD(P)H-dependent single-electron donors [51]. However, as is appropriate with pharmacological inhibitor studies, we interpret this observation with caution; for example it has been reported that iodonium-mediated active site phenylation does not ubiquitously inhibit all flavin-radical containing enzymes [52] and DPI can inhibit the activity of other NAD(P)-dependent non-flavoenzymes [53]. Nevertheless, the data provides valuable evidence to establish future hypotheses, which we are currently testing. The minor proportion of anoxic PR-104A metabolism that is not inhibited by DPI is quantitatively similar to that observed under oxic conditions, and may reflect the basal activity of an unidentified enzyme(s) responsible for the minor aerobic metabolism.

The results of the present study clearly implicate CYPOR as having an important role in PR-104A activation, a reductase that it is widely but variably expressed in human tumour cell lines [54,55] and surgical tumour biopsies [31,56–59]. Quantitative data on expression of CYPOR (and other reductases yet to be identified), along with their spatial relationship to regions of hypoxia, will be important for identifying tumour types, and individual tumours that are more likely to be responsive to PR-104. Identification of CYPOR as an important reductase involved in hypoxic activation of PR-104A *in vitro*, suggests it is likely to contribute to PR-104A activation in hypoxic regions of human tumours and is thus a possible predictor of individual tumour PR-104A sensitivity. The identification of other reductases (both aerobic and hypoxic) will also be important for optimal clinical use of PR-104A. Currently, a systematic evaluation of human reductases is in progress. Ultimately, monitoring pre-treatment tumour biopsies, particularly in concert with markers of tumour hypoxia, may greatly assist the rational development and optimal clinical use of this novel anticancer agent.

## Conflict of interest statement

WRW is a founding scientist, stockholder and consultant to Proacta Inc., which is undertaking the clinical development of PR-104. AVP is a consultant to and has stock options in Proacta Inc.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2007.06.014.

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