Viral delivery of P450 reductase recapitulates the ability of constitutive overexpression of reductase enzymes to potentiate the activity of mitomycin C in human breast cancer xenografts

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Abstract

Indolequinones such as mitomycin C (MMC) require enzymatic bioreduction to yield cytotoxic moieties. An attractive approach to overcome the potential variability in reductive bioactivation between tumors is to exploit specific enzyme-bioreductive drug combinations in an enzyme-directed gene therapy (GDEPT) approach. To this end, human breast cancer cell lines (T47D, MDA468, and MDA231) that overexpress either DT-diaphorase (DTD) or NADPH:cytochrome P450 reductase (P450R) have been developed. Cytotoxicity of MMC was evaluated in the panel of cell lines following aerobic or anoxic exposure *in vitro*. DTD and/or P450R overexpression sensitized cells to MMC in air with no further increase in the cytotoxicity of MMC under anoxia. The most profound effect was seen in the MDA468 cells, where a 27-fold increase in potency was observed for MMC in the DTD-overexpressing cell line. The MMC sensitization achieved through DTD and P450R overexpression in MDA468 cells was maintained *in vivo*. Xenografts established from the clonal lines exhibited significant tumor control following MMC treatment (treated/control [T/C] 17% and 51% for DTD and P450R xenografts, respectively) that was not seen in wild-type tumors (T/C 102%). Delivery of a clinically relevant adenoviral vector encoding P450R to MDA468 wild-type tumors yielded comparable P450R activity to that seen in the P450R clonal xenografts and resulted in greater MMC sensitization (T/C 46%). The model systems developed will facilitate the identification of novel indolequinone agents that are targeted toward a specific enzyme for bioactivation and are consequently of potential use in a GDEPT approach. (Mol Cancer Ther. 2003;2:901–909)

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Introduction

The physiological abnormality of hypoxia is prevalent in solid tumors, yet seldom seen in normal tissues. The tumor specificity of this condition makes hypoxic tumor cells an attractive target for anti-cancer drug design. This is reinforced by the refractive nature of these cells to most forms of cancer therapeutics in current clinical usage. To this end, we are developing indolequinones for use in cancer chemotherapy. These compounds require reductive activation to yield cytotoxic metabolites, a process that is facilitated by both bioreductive enzymes and low oxygen concentrations prevalent in tumors (1). The prototype of this class of agents is the bioreductive alkylating agent mitomycin c (MMC), the activation of which has been extensively studied (2). MMC is commonly used in combination with other chemotherapy agents in the treatment of numerous tumor types including breast, lung, prostate, and bladder cancer (3).

Through initial studies using MMC, a number of enzymes have been shown to be capable of metabolizing indolequinones to release cytotoxic moieties (1). The most important flavoenzymes are probably the one-electron reducing enzyme, NADPH cytochrome P450 reductase (P450R) and the two-electron reducing enzyme, DT-diaphorase [DTD; NAD(P)H: quinone oxidoreductase, NQO1]. DTD is commonly overexpressed in human malignancies compared with normal tissue (4–8), an observation that is supportive of its exploitation in tumor-selective bioreductive drug activation. However, there are a number of caveats associated with reliance on endogenous enzyme metabolism for bioreductive drug activation. These include the heterogeneous nature of enzyme expression in human tumors that could conceivably result in variability in reductive activation from tumor to tumor. In addition, there is evidence from the evaluation of both experimental and clinical material that suggests an inverse correlation between enzyme level and tumor grade in some neoplasms (7–9). Finally, a direct relationship between endogenous P450R and/or DTD activity and response of clinical biopsy

material to treatment with the prototype agent MMC has been established in very few tumor types [bladder (8); disseminated peritoneal cancer (10)] and studies using xenograft models suggest that this predictive relationship may not be generic for all tumors (11, 12).

Rather than relying on endogenous enzyme expression, an alternative approach is to exploit specific enzyme/bioreductive drug combinations in an enzyme-directed gene therapy (GDEPT) approach. The purpose of the present study was to generate stable human tumor cell lines overexpressing either P450R or DTD that would have utility in the evaluation of novel indolequinones as hypoxia and/or enzyme-specific cytotoxins. We used MMC as our model drug and evaluated response as a function of enzyme overexpression both *in vitro* and *in vivo*. Furthermore, we generated an adenoviral vector expressing P450R to investigate whether the data obtained from stable clones could be recapitulated when the enzyme is delivered in a clinically relevant format.

Materials and Methods

Vector Construction

The bicistronic expression vector pEF-IRES-P (F373) and the derivative that encodes DTD (pEF-DTD-IRES-P; F397) have been previously described (13, 14). The full-length cDNA for human P450R (2.3 kb) was isolated from pBabe/puro (15) following restriction with *Eco*RI/*Sal*I and subcloned into pCI^{neo} (Promega, Madison, WI) to create pCI-P450R^{neo}. The cDNA was then excised by *Eco*RI/*Not*I digestion and pEF-P450R-IRES-P was generated by cloning this fragment into F373. To generate an adenoviral vector encoding P450R, the full expression cassette was excised from pCI-P450R^{neo} by *Bgl*II/*Bam*HI digestion and inserted within the multiple cloning site of pShuttle (Stratagene, La Jolla, CA). Homologous recombination in *Escherichia coli* was then used to introduce the cassette into the whole adenovirus genome (16). pShuttle CMV P450R was linearized by *Pme*I digestion and co-transformed with pAd Easy (Stratagene) into BJ5183 cells using electroporation. pAd Easy contains the adenovirus genome subcloned in a backbone encoding for ampicillin resistance, whereas pShuttle CMV P450R confers kanamycin resistance. E1/E3 deleted transformants were selected for kanamycin resistance and small colonies were screened by restriction endonuclease digestion. A single positive colony was cultured and the DNA purified using standard techniques.

Propagation of the Cytochrome P450R Encoding Adenovirus

The plasmid encoding the adenovirus pAd CMV P450R was digested with *Pac*I to linearize the viral genome. The linearized DNA was then transfected into the permissive human embryonic kidney cell line 293 using Lipofectamine as described by the manufacturer (Invitrogen Ltd., Paisley, Scotland). These cells support adenoviral replication as they express the proteins encoded by the E1 and E3 regions that are deleted from the recombinant pAd CMV P450R plasmid. Cytopathic effect was evident in the monolayer typically 10–14 days after transfection. Once all the cells had rounded, they were harvested and lysed by three rounds of freezing and thawing. The cellular debris was removed by centrifugation and the supernatant used as the viral inoculum for infection of 293 cells on a large scale. Large-scale preparations of the recombinant adenovirus RAd CMV P450R were purified from infected cells using the BD Adeno-X chromatographic method (BD Biosciences, Clontech, Palo Alto, CA). The virus was then titred by plaque forming assay.

Cell Culture and Generation of Stable Cell Lines

The human breast carcinoma cell lines T47D, MDA468, and MDA231 were maintained in DMEM supplemented with 10% FCS, 2 mM glutamine, and 0.2 mM non-essential amino acids in a humidified atmosphere of 95% air:5% CO_2. To generate stable cell lines overexpressing either DTD or P450R, cells were transfected in mid-exponential growth phase using either standard electroporation techniques (17) or Lipofectamine following the manufacturer's recommended protocols. The pEF-P450R-P and pEF-DTD-P plasmids were linearized by restriction with *Nde*1 before use. Forty-eight to seventy-two hours after transfection, the cells were sub-cultured into medium containing 5 μg ml^{-1} puromycin. Puromycin-resistant colonies were evident 10–20 days later, depending on the cell line. These were isolated and expanded for evaluation of P450R and DTD enzyme activity. Stability of the clonal populations was ascertained by evaluating the enzyme activity following passage in the absence of puromycin selection.

Preparation of Cell Lysates

The method used for the preparation of lysates for use in enzyme assays was modified from that described by Hoban *et al.* (18). Briefly, exponential phase cells were harvested, washed with ice-cold PBS, and resuspended in ice-cold Nuclear Buffer A [10 mM HEPES/KOH (pH 7.9), 1.5 mM $MgCl_2$ 10 mM KCl, 0.5 mM DTT]. Cells were disrupted by three 5-s rounds of sonication, with chilling on ice in between. Cellular debris was pelleted by centrifugation at 9000 × *g* for 15 min at 4°C. Supernatants were stored at −80°C before analysis. For the preparation of lysates from tumor samples, tumor pieces were cross-chopped, rinsed with a small volume of PBS, and resuspended in Nuclear Buffer A. Samples were homogenized on ice before sonication and preparation as above.

DTD and Cytochrome P450R Activity Assays

Activities of both enzymes were measured at 37°C by spectrophotometric assay. Cytochrome ***c*** was used as the terminal electron acceptor. The rate of cytochrome *c* reduction was monitored at 550 nm. At this wavelength, reduced cytochrome ***c*** has a molar extinction coefficient of 21 mM^{-1} cm^{-1}. This was used to calculate enzyme activity in terms of nanomoles cytochrome ***c*** reduced min^{-1} mg^{-1} protein. DTD activity was measured in a reaction mixture containing menadione (20 μM) as a substrate, cytochrome ***c*** (70 μM), and NADH (500 μM) in 50 mM phosphate buffer containing 0.14% (w/v) BSA both with and without the addition of 100 μM Dicoumarol. Initial reduction rates were monitored and Dicoumarol-inhibited activity was

taken as that of DTD. For the analysis of P450R activity, lysates were added to 100 mM phosphate buffer (pH 7.4) containing cytochrome *c* (50 μM) and KCN (1 mM), with or without NADPH (200 μM). The difference in the initial rates of reaction obtained in the presence or absence of NADPH was used to calculate P450R activity.

Analysis of MMC Toxicity *in Vitro*

Cells were plated in a 150-μl volume into replicate wells of a 96-well plate. For anoxic MMC exposure, cell pellets were taken into an anoxic chamber (Bactron anaerobic chamber, Sheldon Manufacturing, Cornelius, OR), resuspended in pre-equilibrated medium, and seeded into primed 96-well plates. The cell numbers plated per well were 3000 for the MDA231 wt, DTD, and P450R clones; 6000 for the T47D wild-type and DTD clones; 8000 for the T47D P450R clone; and 10,000 for the MDA468 wt, DTD, and P450R clones. Cells were left to attach for 2–3 h and then 50 μl of serially diluted MMC (Sigma, Poole, United Kingdom), prepared at 4 times the required final concentration range (1 nM to 100 μM), were added to the wells in triplicate. Where the cells were exposed to drug under anoxic conditions, stock MMC was taken into the anoxic chamber and diluted using pre-equilibrated medium. Cells were exposed to MMC for 3 h. Drug-containing medium was then removed, replaced with fresh medium, and growth inhibition monitored 4 days later by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay as described previously (19).

Xenograft Studies

Cells for implantation were prepared at a concentration of 3×10^7 ml^{-1} in serum-free medium. To initiate tumor xenografts, 0.1 ml of the cell suspension was implanted i.d. on the midline of the back of cba *nu/nu* mice of age 9–12 weeks. Once the tumors had established, measurements were made every 2–3 days using callipers. For the MMC studies, tumor-bearing mice were randomly assigned to receive either two i.p. injections of saline or two i.p. injections of MMC (2 mg kg^{-1}) with a 7-day interval between each injection. The first treatment was administered when the tumors were well established (220–250 mm^3). Tumor size was monitored until a relative treatment volume 3 times that at the initiation of treatment (RTV_3) was achieved for the saline-treated tumors. These tumors were excised and snap frozen and/or formalin fixed for subsequent enzyme and/or immunohistochemical analysis. To evaluate the effect of adenoviral-mediated delivery of P450R on the response of MDA468 wild-type xenografts to MMC, RAd CMV P450R (5×10^8 pfu) was delivered by intra-tumoral injection (50 μl) to xenografts of approximately 180 mm^3 in volume. MMC or saline treatment was initiated 3 days later. Some tumors were excised and snap frozen at this point to evaluate the level of P450R activity achieved immediately before drug treatment. All procedures were carried out by approved protocols (Home Office Project License number 40-1770) in accordance with the Scientific Procedures Act 1986 and in line with the UKCCCR guidelines on the Welfare of Animals in Experimental Neoplasia (20).

Analysis of Protein Expression *in Vivo* by Immunohistochemistry

Sections of 3–4 μm in thickness were prepared from formalin-fixed, paraffin-embedded tumor pieces. Both P450R and DTD proteins were detected using rabbit polyclonal antibodies at a 1/1000 dilution (kindly provided by Professors Roland Wolf and Paul Workman, respectively). Control slides were treated with similarly diluted or neat pre-immune rabbit serum, and the subsequent visualization of primary antibody binding achieved using reagents supplied in the Envision anti-rabbit kit (DAKO, Glostrup, Denmark).

Results

Generation of Breast Cancer Cell Lines Overexpressing DTD and P450R

The vector used to express both the DTD and P450R cDNAs (pEF-IRES-P) has been previously shown to give robust constitutive expression in mammalian cells, afforded by the elongation factor 1α (EF-1α) promoter (13). Both the gene of interest and that encoding for puromycin resistance (*pac*) are encoded by a single transcript through the incorporation of an internal ribosomal entry site (IRES). This effectively eliminates the potential for clones selected on the basis of antibiotic resistance to lack expression of the recombinant protein. Following transfection of T47D, MDA231, and MDA468 cells with the expression vectors detailed in Fig. 1, stable clones were isolated that had DTD and P450R activity levels that were 250- to 620- and 20- to 60-fold higher than wild-type cells for the two enzymes, respectively (Table 1).

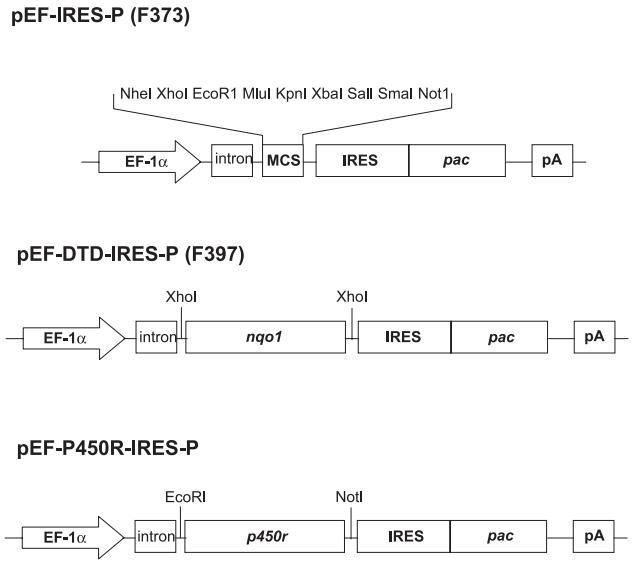

Figure 1. Schematic representation of the bicistronic vectors used in the generation of the P450R and DTD-overexpressing clones. The cDNAs for DTD (*nqo1*) and P450R were inserted into the multiple cloning site of the pEF-IRES-P (F373) vector to generate pEF-DTD-IRES-P and pEF-P450R-IRES-P.

Table 1. Enzyme activity and MMC toxicity in the panel of breast carcinoma wild-type and transfected cell lines

Cell Line	Clone	Enzyme Activity[a]		IC_{50} (μM)[b]		HCR[c]
		DTD	P450R	Air	Anoxia	
T47D	Wild type	31 ± 0.4 (3)	13 ± 2.9 (5)	2.3 ± 0.9 (5)	0.75 ± 0.13 (3)	3.1
	DTD	9836 ± 304 (4)	11 ± 2.4 (4)	2.7 ± 0.7 (4)	1.01 ± 0.18 (4)	2.7
	P450R	15 ± 1.7 (3)	441 ± 22 (3)	0.13 ± 0.03 (4)	0.13 ± 0.06 (4)	1
MDA468	Wild type	27 ± 8 (3)	11.2 ± 3.5 (4)	2.6 ± 0.73 (5)	0.6 ± 0.1 (5)	4.4
	DTD	7027 ± 254 (5)	8 ± 0.6 (5)	0.095 ± 0.03 (5)	0.32 ± 0.06 (5)	0.3
	P450R	14 ± 4 (3)	248 ± 67 (4)	0.46 ± 0.11 (5)	0.32 ± 0.09 (4)	1.4
MDA231	Wild type	17 ± 5.4 (4)	4 ± 0.6 (4)	12.7 ± 1.5 (5)	1.8 ± 0.15 (4)	7.1
	DTD	8760 ± 1745 (4)	4 ± 0.3 (3)	2.1 ± 0.4 (4)	2.5 ± 0.61 (5)	0.8
	P450R	8 ± 1.9 (3)	238 ± 31 (4)	2.7 ± 1.1 (4)	3.0 ± 0.24 (3)	0.9

[a]Mean values ± SE. Units are nanomoles cytochrome *c* reduced min^{-1} mg $protein^{-1}$. The number of samples from which the data were obtained is given in parentheses.
[b]IC_{50} values were determined 96 h after a 3-h drug exposure. Data given are mean values ± SE obtained from the number of independent experiments given in parentheses.
[c]Hypoxic-cytotoxicity ratio.

DTD and P450R Overexpression Enhances the Cytotoxicity of MMC Particularly under Aerobic Conditions

The panel of transfected and wild-type breast carcinoma cells was exposed to MMC for 3 h either in air or under conditions of catalyst-induced anoxia. Proliferation relative to that of untreated control cells was addressed 4 days later by MTT assay. The concentration required to reduce proliferation to 50% of control levels (IC_{50}) was calculated from the dose-response curves obtained. Representative dose response data for the MDA468 cell lines used are given in Fig. 2.

In all three of the cell lines, P450R overexpression reduced the IC_{50} for MMC in air, whereas sensitization was only apparent in the T47D and MDA468 P450R cells when the drug exposure was carried out under anoxic conditions (Table 1). DTD overexpression yielded aerobic MMC sensitization in the MDA468 and MDA231 cell lines. The effect was most pronounced in the MDA468 cells, where DTD overexpression resulted in a 27-fold reduction in IC_{50} in air compared to wild-type cells. Anoxic sensitization was also apparent in these cells, whereas DTD overexpression did not influence the anoxic IC_{50} for MMC in either the T47D or MDA231 DTD clones. The enhanced level of sensitization in air *versus* anoxia resulted in the hypoxic-cytotoxicity ratio (HCR; the ratio of the aerobic IC_{50} dose to that obtained when the cells were exposed to drug under anoxic conditions) being reduced in all transfectants compared with that seen in wild-type cells (Table 1).

Validation of the Potential Use of the Clonal Populations *in Vivo*

Of the three parental breast cancer cell lines used in this study, the MDA231 and MDA468 cells readily form tumors when implanted into nude mice. Initial evaluation of the transfected clones revealed that the growth rates of these cells as tumor xenografts varied from those derived from wild-type cells (Table 2). In addition, preliminary experiments suggested that the growth rate of the MDA468 clonal populations slowed after a size of around 700 mm^3 was attained which was not apparent in the wild-type tumors (data not shown). Immunohistochemical analysis revealed that the tumors derived from the transfected clones maintained the respective protein overexpression (Fig. 3).

The enzyme activities measured in lysates prepared from xenografts of transfected cells were higher than those in tumors derived from the wild-type cells. However, the levels did not achieve those seen *in vitro* (Tables 1 and 2). In only the MDA468 DTD clone did the relative fold overexpression of DTD achieve that seen when the cells were

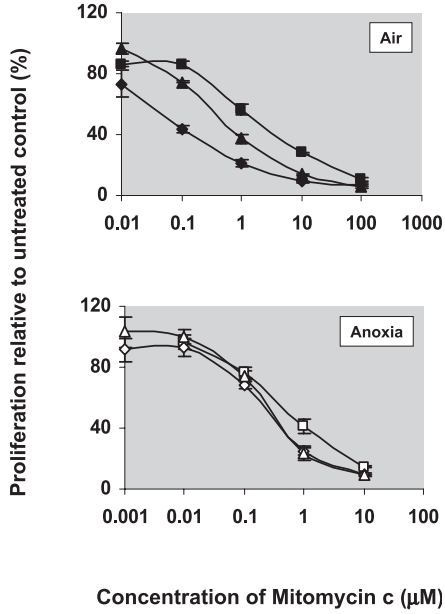

Figure 2. Representative dose-response curves for MDA468 wild-type (*squares*), DTD (*diamonds*), and P450R (*triangles*) cell lines to MMC. Proliferation relative to control cells was determined by MMT assay 96 h after a 3-h exposure to MMC either in air (*closed symbols, top panel*) or under conditions of catalyst-induced anoxia (*open symbols, bottom panel*).

Table 2. Summary of the *in vivo* evaluation of the transfected clones and response of the MDA468 xenografts to MMC

Xenograft	Clone	Enzyme Activity[a]		TD[b]	Size[c] (Mean [Range])	
		DTD	P450R		Saline	MMC
MDA468	Wild type	5 ± 1.7 (3)	2 ± 0.4 (5)	19 ± 3 (9)	585 [315–726]	599 [550–648]
	DTD	705 ± 229 (5)	–	21 ± 2 (6)	600 [480–660]	102 [60–180]*
	P450R	–	15 ± 2 (8)	36 ± 5 (6)	459 [224–700]	236 [108–504]**
MDA231	Wild type	8 ± 4 (6)	3 ± 0.5 (4)	7 ± 0.7 (12)	–	–
	DTD	1729 ± 458 (4)	–	5 ± 0.3 (4)	–	–
	P450R	–	71 ± 9 (5)	5 ± 0.4 (5)	–	–

[a]Mean values ± SE obtained from the number of tumor samples given in parentheses. Units are nanomoles cytochrome *c* reduced min^{-1} mg $protein^{-1}$.
[b]Tumor volume doubling time (days). Mean values ± SE were determined from the number of tumors given in parentheses.
[c]Tumor volume (mm^3) at the designated experimental end point when a relative tumor volume three times that at the start of treatment was achieved in the first saline-treated tumor.
*$P < 0.001$ for MMC *versus* saline-treated tumors.
**$P = 0.04$ for MMC *versus* saline-treated tumors.

grown *in vitro*. Within this tumor group, DTD activity was significantly reduced as tumor size increased. In tumors excised at the designated experimental treatment size (220–250 mm^3), DTD activity was greater than 1000 nmol cytochrome *c* reduced min^{-1} mg $protein^{-1}$. At a relative tumor volume three times this (RTV_3), the DTD activities obtained were 400 nmol cytochrome *c* reduced min^{-1} mg $protein^{-1}$ and below. The enzyme activities attained in the other tumor types appeared to be irrespective of tumor excision size, although an exhaustive evaluation was not undertaken.

MMC Sensitization Is Maintained in the MDA468 Clones When Grown as Xenografts in Nude Mice

The MDA468 clones had shown the most marked MMC sensitization *in vitro,* hence the response of tumors derived from these clones to MMC treatment was evaluated *in vivo*. Both DTD and P450R overexpression significantly sensitized the derivative tumors to MMC (Fig. 4; Table 2). Xenografts derived from wild-type MDA468 cells failed to respond to MMC and the size of the treated tumors as a percentage of the control at the point of excision (T/C) was 102%. For the P450R tumors, the T/C value was reduced to 51%. However, in keeping with the *in vitro* findings, the DTD-overexpressing tumors exhibited the most pronounced MMC response, and the T/C obtained was 17%.

Adenoviral Delivery of P450R Sensitizes Established Wild-Type MDA468 Tumors to MMC Treatment

The growth rate and treatment response of MDA468

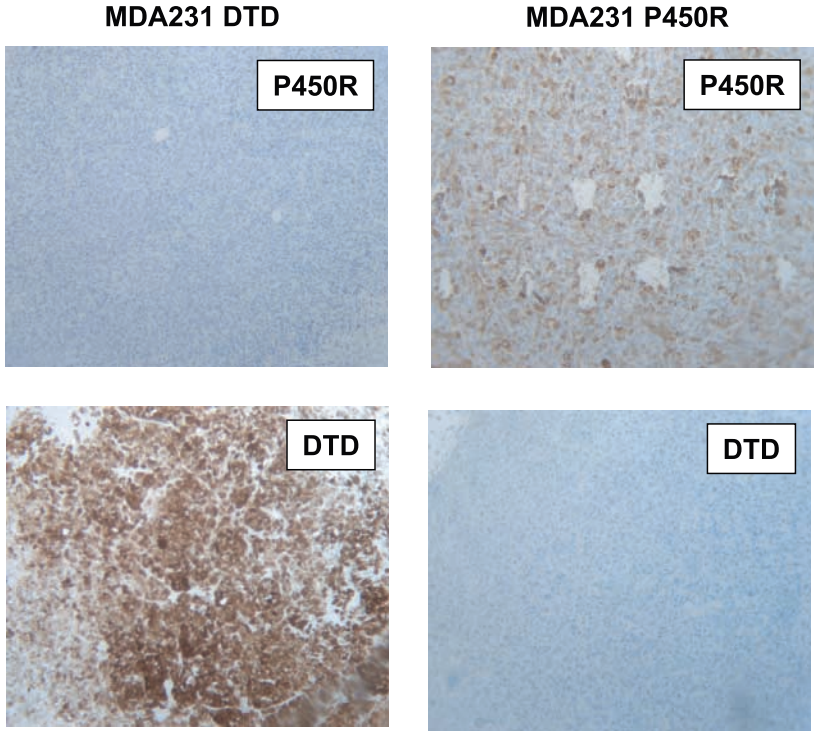

Figure 3. Immunohistochemical analysis of DTD and P450R expression in xenografts derived from the MDA231 overexpressing clones. Endogenous expression of the non-transfected protein is minimal in both wild-type xenograft types.

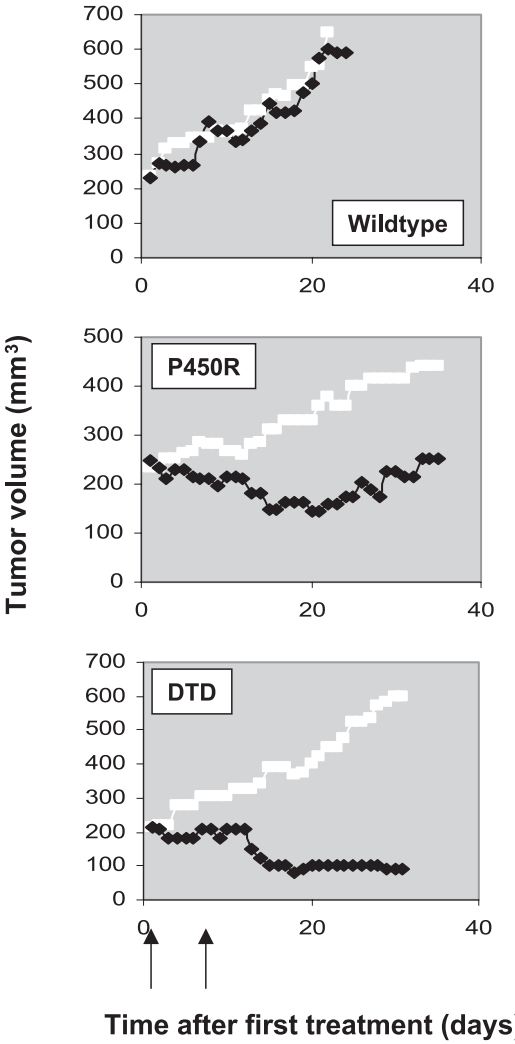

Figure 4. Response of the MDA468 wild-type, P450R, and DTD xenografts to MMC treatment *in vivo*. MMC (2 mg kg^{-1}, IP; *closed symbols*) or saline (IP; *open symbols*) was administered on days 1 and 8 (indicated by *arrows*) and median values are plotted. Four mice per group were treated for the wild-type tumors, and six per group for the two clones. One mouse was removed from the DTD MMC group 18 days after treatment due to poor condition. The tumor mass at this point measured 50 mm^3. The data presented for this group were obtained from the remaining five mice.

P450R tumors were somewhat variable, suggesting that this clonal population may not be as robust as the DTD variant for making *in vitro/in vivo* correlations. To validate the data obtained with the clonal population, an adenoviral vector encoding P450R was generated (schematic, Fig. 5A). RAd CMV P450R was administered to MDA468 wild-type tumors 3 days before the first dose of MMC. The P450R activity achieved in the virus-treated tumors at the point of administering the first MMC dose was 10 ± 4 nmol cytochrome *c* reduced min^{-1} mg $protein^{-1}$ (n = 3), which compared favourably with that of the tumors derived from the clonal population (Table 2). RAd CMV P450R significantly enhanced the response of wild-type tumors to MMC treatment (Fig. 5B). Given that the growth rate of MDA468 wild-type xenografts remained linear over a greater period of time than the clonal populations, the end point for this set of experiments was taken as the maximum tumor burden allowed for ethical reasons [approximately 4% body weight, 1 g tumor burden (20)]. The mean size of the virally transduced, saline-treated tumors at this point was 940 mm^3 (range 720–1100) and for the virally transduced, MMC-treated tumors 431 mm^3 (range 280–550; P = 0.008 *versus* saline-treated virally transduced tumors). The T/C was similar to that of the xenografts derived from the P450R-transfected MDA468 cells at 46%.

Discussion

The extent of tumor hypoxia has been identified as a significant prognostic indicator for radiotherapy outcome in breast, head and neck, squamous cell, and cervical cancer (21–24). One way to enhance the efficacy of radiotherapy is to use a combined treatment approach whereby the hypoxic tumor cells are selectively targeted through the use of a bioreductive cytotoxin.

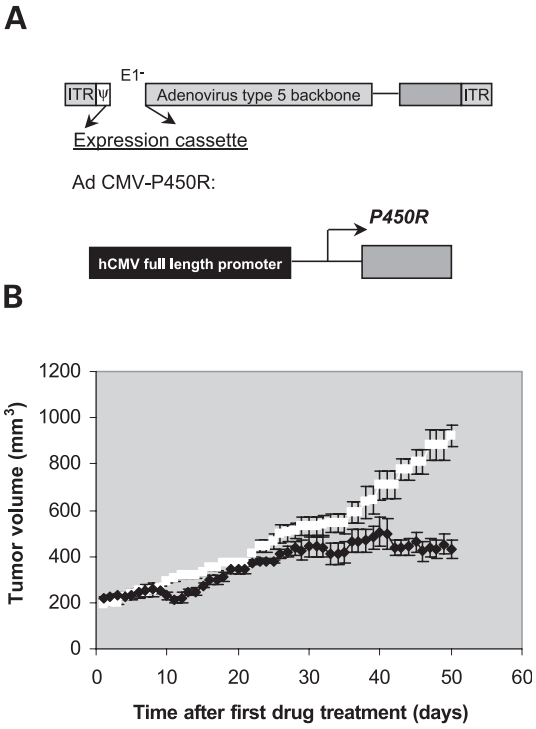

Figure 5. Adenoviral mediated delivery of P450R sensitizes wild-type MDA468 tumors to MMC treatment *in vivo*. RAd CMV P450R was generated according to the schematic given in **A**, and administered to MDA468 wild-type xenografts (5×10^8 pfu in a 50-μl volume, IT) 72 h before treatment with the first dose of MMC (2 mg kg^{-1}, IP) or saline (IP). MMC or saline treatment was repeated 7 days later. Data plotted are median values, n = 5 per group.

Three classes of bioreductive agents have been identified; quinones, nitro compounds, and benzotriazine di-*N*-oxides. We have a particular interest in the development of indolequinones that are structurally related to the prototype quinone bioreductive alkylating agent MMC. Reductive activation can be enhanced both by hypoxia and the bioreductive enzyme complement of tumor cells (25). Assessing the relative contribution of key enzymes to drug cytotoxicity in both air and anoxia is therefore an important step in the rational design of these hypoxia-selective agents. We have created a panel of breast cancer cell lines that overexpress either DTD or P450R for this purpose and used MMC as a model drug with the aim of establishing *in vitro/in vivo* toxicity profiles. Although MMC is generally regarded as a poorly hypoxia-selective drug (26), clinical utility supports the use of MMC as a benchmark against which novel agents can be tested.

Numerous *in vitro* studies have suggested that DTD is important in the aerobic metabolism of MMC (2) and cell line data have supported a potential predictive relationship between DTD activity levels and MMC response (27, 28). There are examples where this has been established both in xenografts derived from certain tumor types (*e.g.*, non-small cell lung cancer) (4) and clinical material (8, 10). However, such a ''global'' relationship is contended (29) when analyzing xenografts established from a broad range of human tumors, where DTD activity in isolation is of little predictive value to MMC response (12). Contributing factors to this lack of *in vivo* correlation could be extracellular pH, because MMC has been established as a good substrate for DTD under acidic, but not neutral conditions (30–32). In addition, the inherent sensitivity of the chosen model to DNA alkylation will influence response. This latter point is illustrated in the present study, whereby similar levels of DTD activity in the overexpressing transfected cell lines gave rise to the most marked aerobic MMC sensitization in the cell line that exhibits the greatest sensitivity to DNA-alkylating agents (MDA468).

The development of cell lines overexpressing DTD as tools for the evaluation of bioreductive drugs is not novel. In a previous study, Sharp *et al.* (14) transfected DTD into the BE cell line which has a null DTD phenotype, as a result of a homozygous point mutation in the *NQO1* gene (33). *In vitro* studies established that these cells were sensitive to MMC and related indolequinone analogues such as E09 following aerobic exposure (14, 28). However, enhanced responsiveness to MMC was not maintained when these cells were established as xenografts in nude mice. This is in contrast with the current findings, where we were able to correlate *in vitro* sensitivity with enhanced *in vivo* response for the MDA468 DTD-transfected cells. This may relate to differences in the relative enzyme activity observed *in vitro* and *in vivo*. In the present study, we found that DTD activity in MDA468 tumor extracts was approximately 10-fold lower than that seen when the cells were grown *in vitro*. Similarly, MDA231 DTD transfectants showed a 5-fold reduction in activity when *in vivo/in vitro* comparisons were made. In spite of this, the actual activities *in vivo* were 705 ± 229 and 1729 ± 458 nmol min^{-1} mg^{-1} protein for the MDA468 and MDA231 DTD-transfected xenografts, respectively. These values are markedly higher than that reported for the previously described BE-derived DTD transfectant [6.1 ± 0.4 nmol min^{-1} mg^{-1} *in vivo* compared with 1400 ± 500 *in vitro*; >200-fold reduction (14)]. Interestingly, DTD overexpression in the BE cell line yielded a similar enhancement in MMC toxicity *in vitro* to that obtained with the MDA468 P450R clone used here and shown to be sufficient to yield an enhanced *in vivo* response in the present study.

The relationship between endogenous P450R levels and MMC is even more tenuous than that of DTD. Comparative analysis of P450R activity and MMC response across the National Cancer Institute tumor cell line panel revealed no correlation between the two (27). In spite of this, P450R activity has been shown to correlate with MMC toxicity in human bladder cancers (8). In the present study, we found that overexpression of P450R sensitized all of the cell lines used to MMC treatment in air. In contrast with the DTD clones, the level of sensitization attributable to P450R overexpression did appear to go hand in hand with the level of P450R activity achieved in the clonal lines. An 18-fold sensitization was apparent in the T47D cell line, which exhibited enzyme levels approximately twice that seen in the MDA468 and MDA231 clones where a 5- to 6-fold sensitization was seen. These findings are in keeping with those reported recently, showing the potential for reversing MMC resistance through P450R overexpression in Chinese hamster ovary cells (34). However, the fact that relative sensitization in all cell lines in air was greater than that seen in anoxia perhaps conflicts with the commonly held belief that P450R plays a greater role in the metabolism of MMC under hypoxic rather than aerobic conditions (2).

In our drug development programme, we are interested in designing indolequinone agents that show preferential toxicity in P450R-overexpressing cells particularly under hypoxic conditions. The cell lines we have developed here have already allowed us to identify lead P450R-targeted compounds that exhibit far superior hypoxic-cytotoxicity ratios than those seen with MMC (35). The aim is to use these novel agents in a gene-directed enzyme pro-drug cancer therapy approach in combination with a standard treatment regimen (*e.g.*, radiation) to control oxic cells. Although clonal cell lines are useful in proof of principle experiments (19), a substantial advancement is afforded through the demonstration of *in vivo* efficacy following gene delivery in a clinically viable format. One previous study has demonstrated that adenoviral delivery of DTD can sensitize BE cells to MMC *in vitro* (36). Here, we have shown that the response of MDA468 wild-type tumors can be engineered to mimic that of xenografts derived from the MDA468-transfected P450R clone following delivery of P450R in an adenoviral context by intratumoral injection of the vector. As we would not envisage that the viral titre used (5×10^8 pfu) was sufficient to transduce the whole

tumor, this suggests that MMC may have a bystander effect, which is consistent with the *in vitro* findings of Misra *et al.* (36). To our knowledge, the present study is the first to demonstrate *in vivo* enhancement of bioreductive drug response through the administration of adenovirally encoded P450R and we aim now to evaluate the efficacy of our novel indolequinone drugs using this system.

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