Hypoxia Targeted Gene Therapy to Increase the Efficacy of Tirapazamine as an Adjuvant to Radiotherapy: Reversing Tumor Radioresistance and Effecting Cure

Rachel L. Cowen, Kaye J. Williams, Edwin C. Chinje, Mohammed Jaffar, Freda C. D. Sheppard, Brian A. Telfer, Natasha S. Wind, and Ian J. Stratford

School of Pharmacy and Pharmaceutical Sciences, University of Manchester, Manchester, United Kingdom

ABSTRACT

Solid tumors are characterized by regions of hypoxia that are inherently resistant to both radiotherapy and some chemotherapy. To target this resistant population, bioreductive drugs that are preferentially toxic to tumor cells in a hypoxic environment are being evaluated in clinical trials; the lead compound, tirapazamine (TPZ), is being used in combination with cisplatin and/or with radiotherapy. Crucially, tumor response to TPZ is also dependent on the cellular complement of reductases. In particular, NADPH:cytochrome P450 reductase (P450R) plays a major role in the metabolic activation of TPZ. In a gene-directed enzyme prodrug therapy (GDEPT) approach using adenoviral delivery, we have overexpressed human P450R specifically within hypoxic cells in tumors, with the aim of harnessing hypoxia as a trigger for both enzyme expression and drug metabolism. The adenovirus uses incorporates the hypoxia-responsive element (HRE) from the lactate dehydrogenase gene in a minimal SV40 promoter context upstream of the cDNA for P450R. In a human tumor model in which TPZ alone does not potentiate radiotherapeutic outcome (HT1080 fibrosarcoma), we witnessed complete tumor regression when tumors were virally transduced before treatment.

INTRODUCTION

Regions of acute/chronic hypoxia are present in the majority of solid human tumors (1, 2). The level of hypoxia in tumors has a profound influence on the outcome of cancer chemotherapy and radiotherapy and is a strong prognostic factor for disease progression and survival (3–5). To circumvent the therapeutic resistance induced by hypoxia, bioreductive drugs that are preferentially toxic to hypoxic cells are being developed for use as adjuvants with radiotherapy and chemotherapy regimens (6). The lead drug in this class is the hypoxia-selective cytotoxin tirapazamine [TPZ (7, 8)], which exhibits a high selectivity results from TPZ being a prodrug that is bioactivated under hypoxic conditions via one-electron reduction to a highly reactive free radical intermediate that generates 'OH to fragment DNA (11). The one-electron radical species is unstable in the presence of oxygen, undergoing rapid back oxidation to the prodrug with the concomitant production of the less toxic superoxide radical species that can be deactivated by cellular defense mechanisms.

Preclinical studies have demonstrated that TPZ can significantly enhance the antitumor effect of radiotherapy (12, 13) and chemotherapy, in particular, platinum-based drugs and taxanes, in bothmurine and human xenograft models (14–17). This encouraging data has initiated Phase I, II, and III trials with TPZ in combination with cisplatin for the treatment of solid tumors including non-small cell lung cancer, breast cancer, head and neck cancer, and melanoma (18–20) and as an adjuvant to radiotherapy in Phase II trials for head and neck cancer (21), cervical cancer (22), and glioblastoma multiforme (23). Phase III trials using cisplatin and TPZ for the treatment of advanced non-small cell lung cancer demonstrated a significant survival benefit for patients treated with the combined regimen showing a doubling in response rate compared with patients treated with cisplatin alone (24).

The response of tumors to bioreductive drugs such as TPZ not only depends on the tumor oxygenation but also on reductive enzymes within the tumor that are necessary to bioactivate the drug to a DNA-damaging species (25, 26). Therefore, tumor variability in bioreductive capacity, both in terms of hypoxic fraction and enzyme profile, will determine response and therapeutic outcome. Predictive screening to determine tumor reductase levels and/or oxygen status has been proposed to identify patients most sensitive to bioreductive drug treatment (27). An alternative to this is to use gene therapy to overexpress reductases specifically within the tumor to increase efficacy and uniformity of patient response.

Of the cellular complement of one-electron reductase enzymes, NADPH:cytochrome P450 reductase (P450R) has been shown to play a major role in TPZ activation. We have shown that cell lines expressing endogenously high P450R levels are more sensitive to hypoxic TPZ exposure in vitro (28) and have explored the potential use of the P450R/TPZ enzyme/prodrug combination in a gene therapy strategy. We have demonstrated that tumor cells stably engineered to overexpress P450R are sensitized to bioreductive drugs including TPZ (29), a finding that has been confirmed by others (30). However, overexpression of exogenous P450R, unlike high endogenous levels, potentiates the toxicity of TPZ not only under hypoxic conditions but also in normoxia, increasing the potential for toxicity in the normal tissue surrounding the tumor. Consequently, tumor-specific overexpression of P450R will be required.

We propose a refined enzyme prodrug gene therapy approach in which the expression of the reductase enzyme and drug toxicity are dependent on hypoxia, simultaneously increasing the therapeutic index of the bioreductive chemotherapy without increasing systemic toxicity. This is achieved by incorporation of a hypoxia-responsive promoter into the P450R expression cassette (31, 32). Hypoxia-responsive elements (HREs) within the promoter bind the transcription factor hypoxia-inducible factor 1 (33), resulting in the transcriptional activation of the reductase gene alongside the endogenous up-regulation of hypoxia-inducible factor 1 target genes under hypoxic conditions. The use of hypoxia-responsive promoters in gene therapy is well documented and holds great promise. A comparison of hypoxia-responsive promoter function within the tumor microenvironment and in normal healthy tissues has shown good tumor specificity and a considerable reduction in nonspecific expression in physiologically normal tissue compared with strong viral promoters routinely used in gene therapy (34). The use of hypoxia-responsive promoters circumvents the problems associated with tumor type-specific promoters that lack broad applicability and often lack strength.
of expression (35). Hypoxia-responsive promoters have been used to lend tumor specificity to cytokine deaminase/5-fluorocytosine (32), thymidine kinase/ganciclovir (34, 36), CYP2B6/cyclophosphamide (34), and nitroreductase/CB1954 (37) GDEPT approaches. To deliver a therapeutic cassette to the hypoxic tumor fraction, the innate ability of adenovirus (Ad) to infect dividing and quiescent human tumor cells can be harnessed (38). In this study, we have generated an adenoviral vector Ad LDH HRE P450R encoding for P450R with expression driven from a hypoxia-responsive promoter derived from a hypoxia responsive promoter derived from lactate dehydrogenase A (LDH). The aim of the study was to establish whether viral pretreatment could enhance the ability of TPZ to potentiate radiotherapeutic outcome. Hypoxia-targeted P450R expression was used to preclude the wasted bioactivation of TPZ in well-oxygenated regions of the tumor, concentrating the highest levels of P450R at the site of drug action within the radioresistant tumor subfraction. We have evaluated this viral-mediated gene therapy in the human HT1080 fibrosarcoma tumor model. This model was chosen because it would provide a stringent test of our hypothesis because HT1080 tumors have a low hypoxic fraction and low P450R level (39), which is likely to be the underlying reason(s) why previous studies showed that TPZ did not potentiate the antitumor effect of radiation in HT1080 xenografts (13).

MATERIALS AND METHODS

Viral Vector Construction and Propagation. Three E1/E3-deleted adenoviral vectors have been generated using the pAd Easy system according to the manufacturer’s protocol (Stratagene, La Jolla, CA). A trimer of the HRE sequence from the mouse LDH A gene (LDH, GCGACGTCGCGAGACCCACCGTG) in the reverse orientation or from human phosphoglycerate kinase (PGK) gene (PGK, TGTCACGTCCTGCACGACGCGAGTA) in the forward orientation was cloned upstream of a minimal SV40 promoter of a firefly luciferase (luc+) expression cassette (pGL3 promoter vector; Promega). The expression cassettes were excised with KpnI/Sall and cloned into the multiple cloning site of pShuttle. To generate Ad LDH HRE P450R, the LDH HRE and minimal SV40 promoter were excised from the pGL3 promoter vector by KpnI/HindIII digestion and inserted into the multiple cloning site of pShuttle. The full-length cDNA for human P450R (2.3 kb) and polyadenylation signal were isolated from pBabe/puro after restriction with HindIII and cloned downstream of the LDH HRE promoter. Large-scale preparations of the adenoviral vectors were purified using the BD Adeno-X chromatographic method (BD Biosciences, Chontec, Palo Alto, CA). Virus was titrated by plaque forming assay. Human tumor cell lines were infected at varying multiplicities of infection (MOIs) equivalent to plaque-forming units/Tumor cell.

Cell Culture. All cell lines were maintained in RPMI 1640 supplemented with 10% FCS and 2 mM glutamine in a humidified atmosphere of 95% air:5% CO2. All cell lines were maintained in RPMI 1640 supplemented with 10% FCS and 2 mM glutamine in a humidified atmosphere of 95% air:5% CO2.

Drugs and Chemicals. TPZ (3-amino-1,2,4-benzotriazine-1,4-dioxide; DRG2433) was synthesized in-house following the methodology of Fuchs et al. (40).

Adenoviral Infection of Monolayer Cultures. Cells were seeded at 106 cells/well in a 6-well plate and allowed to adhere. Cells were infected with increasing viral doses of Ad for 5 h. Cells were then exposed to either normoxia (95% air, 5% CO2) or hypoxia (21% oxygen; Bactron anaerobic chamber; Sheldon Manufacturing, Cornelius, OR) for 18 h, followed by either 3 h of reoxygenation or 24 h of reoxygenation. Where cells had been infected with luciferase-encoding viruses, they were lysed in passive lysis buffer, and activities were measured by standard firefly luciferase assay (Promega, Southampton, United Kingdom). For cells infected with Ad LDH HRE P450R, preparation of lysates and spectrophotometric detection of the reduction of cytochrome c by P450R to determine its activity have been described previously (41).

Metabolism of TPZ by Cell Lysates Prepared from Ad LDH HRE P450R-Infected Cells. HT1080 cells were infected with increasing viral doses of Ad LDH HRE P450R (MOI, 2–100). After 5 h, infected cells were exposed to hypoxia for 18 h, followed by 24 h of reoxygenation. Lysates were prepared and analyzed for both reductase activity and the ability to metabolize TPZ. The high-performance liquid chromatography methodology for quantification of the metabolite products of TPZ has been described previously (41).

Analysis of TPZ Toxicity in Vitro by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT) Proliferation Assay. HT1080 cells were infected with Ad LDH HRE P450R at MOI 0, 20, 50, or 100. Infected cells were subjected to 18 h hypoxic exposure followed by 24 h of reoxygenation. Both uninfected and infected cells taken into hypoxia as cell pellets, resuspended in pre-equilibrated medium, and seeded into primed 96-well plates at 2500 cells/100 ml/well. Cells were left to attach for 2–3 h. Stock TPZ was taken into the anoxic chamber and serially diluted using pre-equilibrated medium. TPZ was prepared at 2× the required final concentration range (0.1–1000 μM) and 100 μl added to the wells in triplicate. Cells were then exposed to TPZ for 3 h in normoxia, 1% O2, or hypoxia. All plates were returned to standard culture conditions and drug-containing medium was then removed and replaced with fresh medium and growth inhibition was monitored 3 days later by MTT assay (42).

Xenograft Studies. Cells for implantation were prepared at a concentration of 5×106 ml−1 in serum-free medium. To initiate tumor xenografts, 0.1 ml of the cell suspension was implanted by intradermal injection on the midline of the back of female cba nude mice of 9–12 weeks of age. Once the tumors were established, measurements were made every 2–3 days using calipers. When xenografts reached approximately 100 mm3, Ad LDH HRE P450R (106 plaque-forming units) was delivered by a single intratumoral injection (50 μl) into the center of the tumor. Treatments were initiated 2 days later, when tumors had reached approximately 200 mm3. Sample tumors were excised and snap-frozen at this point to evaluate the level of P450R activity achieved immediately before drug and/or radiation treatment. In addition, the bioreductive hypoxia marker, pimonidazole (Hypoxyprobe-1; Chemicon International Inc.) was administered at a dose of 60 mg kg−1 in 0.9% (w/v) NaCl solution by i.p. injection, 2 h before sacrifice to identify the fraction of hypoxic cells in the 200 mm3 HT1080 tumors. Mice receiving radiation treatment were restrained while localized radiotherapy (X-rays) was delivered at a dose rate of 2 Gy min−1 for 5 min (10 Gy). TPZ was prepared in 0.9% (w/v) NaCl solution and administered either alone or immediately after radiotherapy at a dose of 50 mg kg−1 by i.p. injection. Tumor size was monitored daily until a relative tumor volume 4× that at the initiation of radiation/drug treatment (RTV4) was achieved. Mice were sacrificed at either RTV1, or, if RTV4 had not been reached, 90 days after treatment. All procedures were carried out by approved protocols (Home Office Project License 40-1770) in accordance with the Scientific Procedures Act 1986 and in line with the United Kingdom Coordinating Committee on Cancer Research guidelines on the Welfare of Animals in Experimental Neoplasia (43). The difference in growth delay effects between the 0.9% NaCl solution control group or 10 Gy radiation group and each of the other treatment groups was statistically examined by the nonparametric log-rank P test. Ps of ≤0.05 were considered significant.

Analysis of Protein Expression by Immunohistochemistry. Cells in monolayer culture were grown on glass coverslips and fixed in 10% formalin for 10 min at room temperature. They were permeabilized by incubation in PBS containing 1% FCS and 0.1% Triton X-100 for 10 min at 4°C. P450R protein was detected by incubation with a rabbit polyclonal anti-P450R antibody (1:10,000 dilution in PBS containing 1% FCS) for 1 h at room temperature followed by a Texas red-conjugated goat antirabbit antibody (1:100 dilution in PBS containing 1% FCS) for 30 min at room temperature. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI), and cells were mounted in DAKO fluorescent mounting medium (DAKO Corp.) and analyzed by fluorescence microscopy. Tumor sections of 10-μm thickness were prepared by cryostat sectioning of snap-frozen tumor pieces. P450R protein and pimonidazole adducts were detected simultaneously by incubating sections overnight at room temperature with a rabbit polyclonal anti-P450R antibody (1:1000 dilution; kindly provided by Prof. Roland Wolf) and a mouse monoclonal anti-pimonidazole antibody (1:50 dilution: Hypoxyprobe-1 Mab1; Chemicon International Inc.). Antibodies were diluted in PBS containing 1% BSA and control slides were treated with dilution buffer only. After rinsing, the sections were incubated for 1 h at room temperature with FITC-conjugated goat antirabbit antibody (1:100 dilution; Oncogene Research Products) and Texas red-conjugated goat antimouse antibody (1:100 dilution). The nuclei were stained for 10 min with DAPI (diluted 1:500 in water) in the dark. Sections were mounted in DAKO fluorescent mounting medium (DAKO Corp.) and analyzed by fluorescence microscopy.
RESULTS

Generation of an Adenoviral Vector Encoding for Hypoxia-Dependent P450R Expression. Initial studies were undertaken to select a promoter that achieved tight hypoxic regulation without compromising the absolute level of hypoxia-stimulated expression. A comparative analysis using Ads encoding for the reporter gene luciferase, driven by either the LDH HRE or the commonly used PGK HRE (32, 34), demonstrated that the LDH HRE promoter consistently afforded greater fold increases in hypoxia-induced gene expression in HT1080 cells [6.5-fold (LDH) versus 4-fold (PGK)] and in other cell lines [HCT116 (colon carcinoma), 12-fold versus 10-fold; T47D (breast carcinoma), 11-fold versus 5-fold]. Consequently, the LDH HRE was selected to drive expression of P450R and the Ad LDH HRE P450R was generated.

Tumor Cells Infected with Ad LDH HRE P450R Express High Levels of P450R after Hypoxic Exposure in Vitro. HT1080 tumor cells were infected under aerobic conditions with Ad LDH HRE P450R at increasing viral dose (MOI 2, 10, 20, 50, or 100). After allowing for viral infection, cells were exposed to normoxia or hypoxia for 18 h followed by 3 h of reoxygenation. Immunocytochemical analysis of the infected cells clearly showed high levels of P450R protein within the endoplasmic reticulum (Fig. 1A). Analysis of the cell lysates for P450R activity showed a viral dose-dependent increase in P450R levels (Fig. 1B). Under hypoxic conditions, there was a highly significant viral dose-dependent increase in P450R reaching 28-fold above the basal level at the highest viral dose (322.4 ± 90.4 nmol cytochrome c reduced min⁻¹ mg⁻¹). Although there was a viral dose-dependent increase in normoxia, this reached a plateau at MOI 50 (48.6 ± 17.8 nmol cytochrome c reduced min⁻¹ mg⁻¹). Importantly, the differential between normoxic and hypoxic P450R expression levels increased with increasing MOI. Furthermore viral doses as low as MOI 2 gave a 2-fold enhancement in hypoxic P450R levels relative to those seen in normoxia. To ensure that the levels of virally delivered P450R were sufficiently high to enhance the metabolism of TPZ, the formation of the two-electron reduction product SR4317 by the cell lysates was monitored by high-performance liquid chromatography (41). A clear correlation between lysate P450R levels and the rate at which TPZ is converted to SR4317 was observed, with higher P450R levels resulting in a greater rate of metabolism of TPZ (Fig. 2).

Ad LDH HRE P450R Enhances the Sensitivity of HT1080 Tumor Cells to TPZ in Vitro. Analysis by standard MTT assay demonstrated the hypoxia selectivity of TPZ. Nontransduced HT1080 cells have an IC₅₀ of 559 ± 160 µM when seeded and exposed to TPZ for 3 h in normoxia. Toxicity increases 15-fold (IC₅₀ 36.5 ± 14 µM) and 50-fold (IC₅₀ 11 ± 2.8 µM) when cells are exposed to TPZ in 1% O₂ and hypoxia, respectively (Table 1). The effect of viral infection and hypoxia-induced P450R overexpression on TPZ toxicity was assessed by preinfection with increasing viral doses of Ad LDH HRE P450R (MOI 20, 50, and 100). P450R expression was stimulated by hypoxic exposure (18 h). Cells were allowed to recover overnight in normoxia, resulting in a 24-h reoxygenation before a 3-h drug exposure in normoxia, 1% O₂, or hypoxia. Table 1 shows the levels of...
P450R within infected cells measured at the point of drug dosing, which were slightly higher than those after 18 h of hypoxia followed by 3 h of reoxygenation, reaching a maximum of 425 ± 111 nmol cytochrome c reduced min⁻¹ mg⁻¹ with MOI 100. Viral-mediated toxicity was not observed at any of the viral doses used.

As expected from a bioreductive drug, the toxicity of TPZ on uninfected HT1080 cells increases with reducing oxygen tension. This resulted in hypoxic cytotoxicity ratios (hypoxic cytotoxicity ratio = IC₅₀ air/IC₅₀ hypoxia) of 15.3 and 50.8 for 1% O₂ and hypoxia, respectively. This toxicity was greatly enhanced by preinfection with Ad LDH HRE P450R in all three conditions, with a maximal enhancement in IC₅₀ of 8-fold in normoxia, 80-fold in 1% O₂, and 215-fold in hypoxia (Fig. 3A). In normoxia, 1% O₂, and hypoxia, there was a direct correlation between viral dose and reduction in IC₅₀.

Although radical metabolites of TPZ would be predicted to be short-lived, causing damage close to the site of their formation, it has been suggested that at intermediate oxygen levels, oxygen radical-dependent bystander cell killing may occur (30). Using a reporter Ad encoding for the constitutive expression of β-galactosidase, we assessed the percentage of HT1080 cells infected at MOI 20, 50, and 100. HT1080 cells are readily infected with Ad type 5, and when they were infected at MOI 20, ~50% of cells turned blue on 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside staining. This increased to ~80% with infection at MOI 50 and ~100% with a viral dose of MOI 100 (Fig. 3B). Our data suggest an absence of bystander cell killing because we did not witness any enhanced increase in TPZ toxicity in 1% O₂ compared with what was apparent in normoxia and hypoxia. In particular, when only 50% of cells overexpressed P450R (MOI 20), we would have expected a greater decrease in IC₅₀ in 1% O₂ compared with hypoxia as a result of bystander-mediated cell killing.

**Intratumoral Injection of HT1080 Tumors with Ad LDH HRE P450R Results in Elevated P450R within Hypoxic Tumor Regions.** Ad LDH HRE P450R was administered by single intratumoral injection (50-µl volume) at a dose of 10⁸ plaque-forming units. HT1080 tumors were virally transduced above 100 mm³ and 48 h later, transduced tumors were excised, having reached a volume of 200 mm³. The hypoxic marker pimonidazole was administered 2 h before excision. Immunohistochemical analysis of transduced tumors (Fig. 4) shows focal overexpression of P450R colocalizing with pimonidazole. In the three tumors analyzed, three of four areas staining for pimonidazole stained for P450R. P450R staining was not seen in the absence of pimonidazole staining. Pimonidazole forms adducts within hypoxic cells (pO₂ < 10 mmHg), which, unlike TPZ, is independent of P450R activity. The focal overexpression of P450R translated to a 2-fold increase in total tumor reductase activity that rose from 2.7 ± 0.22 nmol cytochrome c reduced min⁻¹ mg⁻¹ (untreated tumors) to 5.2 ± 1.3 nmol cytochrome c reduced min⁻¹ mg⁻¹ for virally injected tumors (n = 3). This is consistent with the low hypoxic fraction (mean of 6%) that we have reported previously for the HT1080 model (39).

**Tumors Presensitized by Injection with Ad LDH P450R Show Complete Regression after Combined TPZ Chemotherapy and Radiotherapy.** Mice bearing palpable HT1080 tumors of approximately 200 mm³ were treated with a single i.p. injection of TPZ (50 mg kg⁻¹) or 0.9% NaCl solution. Tumors in both treatment groups reached RTV⁴ by day 9 after treatment. Intratumoral injection of Ad LDH HRE P450R (50 µl volume/10⁶ plaque-forming units) 2 days before TPZ treatment did not enhance tumor response (Fig. 5). A single dose of 10 Gy of radiation increased the time for HT1080 tumors to reach RTV⁴ from 9 ± 4 days to 32 ± 4 days (Fig. 6).

Administration of TPZ immediately after 10 Gy of radiation did not significantly enhance this response, and the observed RTV⁴ was 36 ± 6 days (P = 0.1916, nonsignificant versus 10 Gy alone), which confirmed previous observations (13). However, when Ad LDH HRE P450R was administered 2 days before the combined TPZ and 10 Gy radiation treatment, 85% of tumors completely regressed, and mice were tumor free 90 days after therapy. This was not a consequence of radiosensitization by viral pretreatment alone because this did not significantly alter regrowth time compared with 10 Gy alone (RTV⁴ at 40 ± 9 days; P = 0.2172). The success of this gene therapy appeared to be independent of tumor size over the range tested. Palpable tumors ranging in size from 115 to 280 mm³ (median, 239 mm³) at the time of drug/radiation dosing were successfully eradicated. The single nonresponsive tumor was treated at 256 mm³.

**DISCUSSION**

Strong preclinical data have endorsed the use of TPZ as an adjuvant to improve the therapeutic outcome of radiotherapy and cisplatin-based chemotherapy, leading to the initiation of clinical trials for a variety of solid neoplasms. However, the reproducibility of patient response to TPZ-inclusive regimens will be intimately linked with tumor hypoxic fraction and reductase enzyme complement. TPZ has been shown to potentiate radiotherapy in all of the murine and human tumor models in which it has been tested, with the exception of the human HT1080 fibrosarcoma tumor model (12, 13). The reductive capacity and TPZ-induced toxicity profile of the HT1080 cell line in vitro were shown to be similar to other human tumor cell lines that responded to TPZ/radiotherapy treatment in vivo. Therefore, it was hypothesized that the lack of efficacy of TPZ toward HT1080 tumors in vivo may be the result of factors such as vascularization, drug delivery, kinetics of reoxygenation, and rehypoxiation (13).

**Table 1. In vitro TPZ toxicity**

The table shows the response to TPZ of HT1080 cells preinfected with varying doses of Ad LDH HRE P450R (MOI 0–100) and stimulated to overexpress P450R by 18 h of hypoxia and recovery in normoxia. Cells were seeded in normoxia, drug was added in normoxia and replaced with fresh media 3 h later, or cells were seeded in hypoxia, drug was added in hypoxia, and plates were then immediately removed to 1% O₂ or hypoxia for the 3-h drug exposure. The viral-mediated sensitization to TPZ exposure in normoxia, 1% O₂, and hypoxia is compared and related to the level of P450R at the time of drug exposure.

<table>
<thead>
<tr>
<th>MOI</th>
<th>P450R Level (nmol cyt.c min⁻¹ mg⁻¹)</th>
<th>IC₅₀ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normoxia</td>
<td>1% O₂</td>
</tr>
<tr>
<td>0</td>
<td>21 ± 9.8</td>
<td>559 ± 160</td>
</tr>
<tr>
<td>20</td>
<td>140 ± 35 (6.6-fold ↑)</td>
<td>178 ± 17</td>
</tr>
<tr>
<td>50</td>
<td>287 ± 67 (13.6-fold ↑)</td>
<td>117 ± 12</td>
</tr>
<tr>
<td>100</td>
<td>425 ± 111 (20-fold ↑)</td>
<td>69 ± 21</td>
</tr>
</tbody>
</table>

*TPZ, tirapazamine; MOI, multiplicity of infection; P450R, NADPH:cytochrome P450 reductase; cyt., cytochrome; Ad, adenovirus; LDH, lactate dehydrogenase; HRE, hypoxia-responsive element.*
In this study, we demonstrate the importance of P450R levels within the hypoxic tumor fraction that can be elevated by adenoviral-mediated, hypoxia-targeted gene therapy to reverse the chemoresistant and radioresistant phenotype of the HT1080 tumor, resulting in cure. Using the vector Ad LDH HRE P450R, we were able to transfect HT1080 cells \textit{in vitro} to confer increased P450R activity after a hypoxic stimulus. Without inducing viral toxicity, P450R levels could be increased 40-fold in hypoxic tumor cells. The rise in reductase level in HT1080 cells directly correlated with an increase in sensitivity to TPZ. At the highest viral dose of Ad LDH HRE P450R (MOI 100), the hypoxic toxicity of TPZ was 215-fold higher compared with untransduced HT1080 tumor cells in normoxia. Furthermore, a single direct intratumoral injection of the virus into HT1080 xenografts elevated P450R specifically within hypoxic tumor regions, increasing the potency of TPZ as an adjunct to radiotherapy such that 85% of tumors regressed without regrowth.

This work validates the therapeutic utility of the LDH HRE promoter, which proved to be a powerful hypoxia-responsive promoter. In comparison with previous studies that have used constitutive viral promoters to drive expression of P450R in stable tumor cell lines (29, 30), we witnessed a greater increase in P450R levels on hypoxic stimulation of the LDH HRE promoter (maximum of 425 nmol cytochrome \(c\) reduced min \(^{-1}\) mg \(^{-1}\)) in infected HT1080 cells. Constitutive overexpression of P450R has been shown to result in an equal sensitization to TPZ in normoxia and hypoxia. For example, Jouaidi \textit{et al.} (30) demonstrated a 1.5- to 2-fold decrease in IC\(_{50}\) in response to TPZ toward 9L glioma cells with elevated P450R (maximum, 100 nmol cytochrome \(c\) reduced min \(^{-1}\) mg \(^{-1}\)) in both normoxia and hypoxia. Constitutive overexpression of P450R in stable human breast MDA 231 cell lines also resulted in a similar sensitization to TPZ in both normoxia and hypoxia (~7-fold in each condition; Ref. 29). In contrast, in this present study, as P450R levels are elevated to a markedly greater extent in hypoxia than in normoxia, the sensitization observed increases with decreasing oxygen level, reaching a maximum of 80-fold and 215-fold in 1% O\(_2\) and hypoxia compared with only 8-fold in normoxia (MOI 100).

Previous studies have highlighted the importance of nuclear-localized reductase enzyme activity on hypoxic TPZ drug metabolism and
consequential cytotoxicity. Drug metabolism by cytoplasmic reductases was suggested to be unrelated to the hypoxic cytotoxicity of TPZ (44). Although the lifetime of the toxic TPZ free radical species in the cell is unknown, it would be predicted to be short-lived, adding weight to the suggestion that only the activation of TPZ by nuclear reductases would contribute to DNA damage (45). Endogenous P450R is absent from the nuclear fraction because it resides exclusively within the endoplasmic reticulum. Therefore, the data imply that to overexpress P450R in a gene therapy approach, it may be prudent to target this enzyme to the nucleus by the introduction of a nuclear signal sequence. However, we have clearly shown that exogenous overexpression of P450R in its natural setting in the endoplasmic reticulum potentiates TPZ toxicity in vitro and TPZ/radiotherapy response in vivo.

The hypoxic tumor microenvironment can lead to an inhibition of cell proliferation. Therefore, to specifically target this region using gene therapy, it is essential to select a vector that is able to transduce both viable quiescent tumor cells and tumor cells progressing rapidly through the cell cycle (38). Adenoviral vectors have clinical application, are readily taken up into the majority of human tumor cell types, and are not restricted to dividing cells. A precedent also exists for using Ad to deliver a hypoxia-regulated therapeutic cassette (34, 46). In this study, we demonstrate that a single Ad injection, directly into the tumor, is sufficient to achieve viral dissemination into hypoxic regions, despite the chaotic tumor vasculature and significant perfusion heterogeneity existing within the tumor microenvironment that may obstruct viral dissemination. Ad injection results in focally high levels of P450R that colocalize with the hypoxic marker pimonidazole, leading to a dramatic increase in treatment response to TPZ/radiotherapy. This response was not mediated by increased tumor immunogenicity as a consequence of adenoviral injection because studies were carried out in athymic mice. In addition, viral injection alone did not potentiate TPZ chemotherapy or radiotherapy when given as single agents.

We predicted that HT1080 tumor xenografts would be completely unresponsive to Ad LDH HRE P450R/TPZ treatment without radiotherapy. This enzyme/prodrug combination specifically targets the hypoxic tumor fraction, which in HT1080 tumors ranges from 1% to 11% of the tumor mass (39). Therefore, without a substantial bystander effect, a significant impact on tumor growth would not be predicted. A hypoxia-targeted enzyme prodrug combination with a bystander component has been tested previously in the HT1080 model without success. HT1080 tumor cells have been stably engineered to express nitroreductase under hypoxic conditions. A small growth delay was recorded after systemic administration of CB1954, which only reached statistical significance when tumor-bearing mice breathed 10% oxygen before drug dosing (37). This work emphasizes the potential problems and variability in response that may be encountered as a result of using hypoxia-responsive promoters to confer tumor selectivity on a classical GDEPT strategy. If the hypoxia-driven gene therapy is used as a stand-alone treatment, the outcome will be highly dependent on the hypoxic fraction. In addition, hypoxia-regulated expression of activating enzymes such as thymidine kinase would result in prodrug activation within cells against which the active metabolite is the least potent (quiescent cells). Conversely, to activate TPZ, the use of a constitutive or tumor-specific promoter driving expression of P450R throughout the tumor mass would result in futile cycling of the bioreductive drug.

Having demonstrated the suitability of our hypoxia-mediated GDEPT approach with radiotherapy, we would predict that it would also enhance the response to cisplatin and other platinum-based chemotherapies. Delivery of this gene therapy strategy using a first-generation adenoviral vector restricts its application to the local control of solid human tumors. However, with increasing vector sophistication, it could be given systemically for the treatment of disseminated disease in combination with chemotherapy. This is supported by recent research using an Ad encoding for the reporter gene lacZ with expression driven by the hypoxia-responsive promoter derived from PGK. Systemic administration of this vector resulted in a dramatic reduction in potentially toxic transgene expression in the liver, which has high concentrations of the Ad type 5 receptor coxsackie adenovirus receptor (CAR) (~1000-fold reduction in β-galactosidase expression from the HRE promoter compared with levels obtained from the cytomegalovirus promoter; Ref. 34).

In conclusion, we have developed a hypoxia-mediated gene therapy approach that can potentiate TPZ as an adjuvant to radiotherapy, resulting in a dramatic increase in treatment response to hypoxia-directed gene therapy and radiotherapy in vivo.
in cancers in a previously radioresistant tumor model. Furthermore, we have engineered in two levels of control, with tumor hypoxia being a requirement for both enzyme expression and prodruk activation, making this an attractive strategy for future clinical development.

ACKNOWLEDGMENTS

We thank Prof. Roland Wolf for the cDNA encoding human cytochrome P450R and the P450R antibody. We thank David Garvey for excellent technical assistance.

REFERENCES

Hypoxia Targeted Gene Therapy to Increase the Efficacy of Tirapazamine as an Adjuvant to Radiotherapy: Reversing Tumor Radioresistance and Effecting Cure


Cancer Res 2004;64:1396-1402.

Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/64/4/1396

Cited articles
This article cites 46 articles, 16 of which you can access for free at:
http://cancerres.aacrjournals.org/content/64/4/1396.full.html#ref-list-1

Citing articles
This article has been cited by 10 HighWire-hosted articles. Access the articles at:
/content/64/4/1396.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.